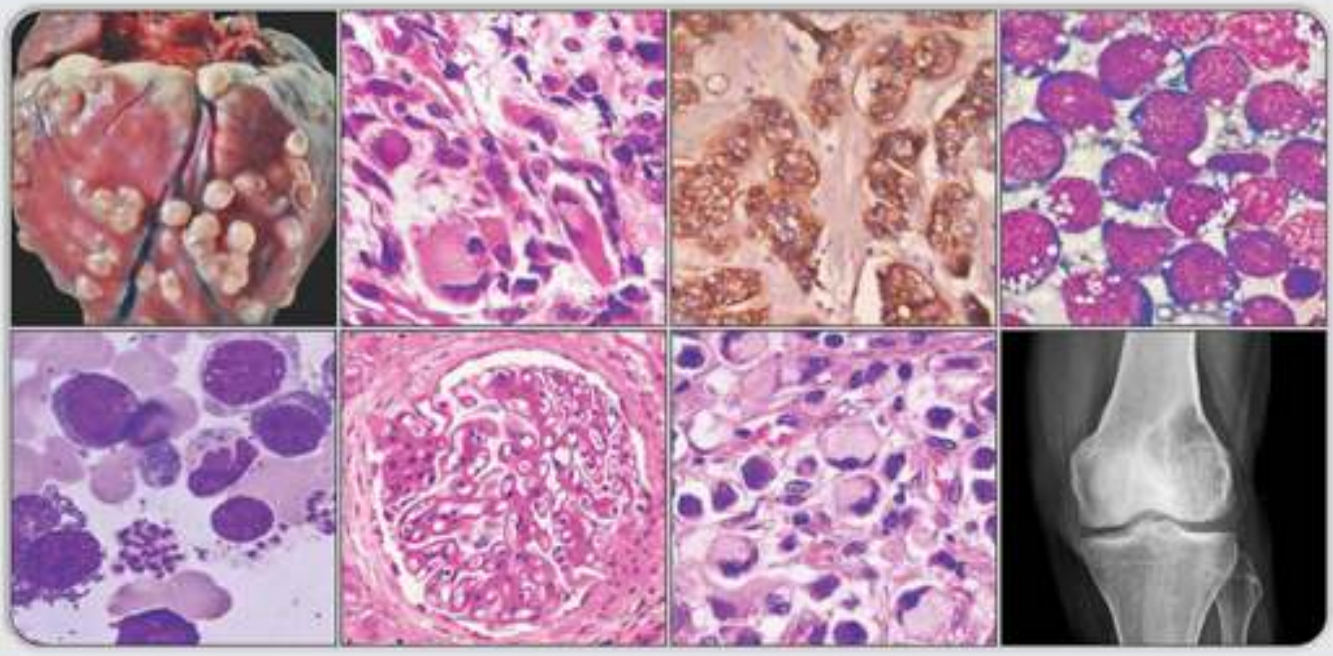
2nd Edition

Textbook of Pathology

General Pathology & Hematology

Vinay Kamal

Volume I



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Textbook of

Pathology

Second Edition

VOLUME I: General Pathology and Hematology

Textbook of
Pathology

Second Edition

VOLUME I: General Pathology and Hematology

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Volume I: General Pathology and Hematology

Volume II: Systemic Pathology and Molecular Diagnostics

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Dedication

Dedication of this small work on Second Edition of Textbook of Pathology to the great scientists Juan Rosai (Rosai and Ackerman's Surgical Pathology), and Christopher DM Fletcher (Diagnostic Histopathology of Tumors), whose profound contribution to the field of Pathology has touched countless lives. Their legacy will continue to inspire and educate the generation of pathologists.

Dedication to our worthy teachers for teaching basics of pathology and excellence in medicine, Esteemed Faculty Members, and promising undergraduate and postgraduate Medical Students for enduring inspiration and encouragement, while working on Second Edition of Textbook of Pathology.

Dr Vinay Kamal
Dr Anubhav
Dr Vigyat

Dedication

Second Edition of Textbook of Pathology is dedicated to our family, who inspired, supported, encouraged, and patiently endured long hours of inattention and hearing from me during the creation of this final project to achieve an academic career. But for their understanding, it would be difficult to get away with the disproportionate amount of time that such projects consume.

Dr Mrs Manita Kamal

Siya Kamal

Dr Anubhav

Dr Vigyat

Dr Spriha Arun

and

Dr Mansi Siddharth Dhende

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Preface to the Second Edition

Rudolph Virchow (1821–1902) is the father of 'Cellular Pathology' in terms of the dysfunction of cells. Many pathologic observations are being published in the Textbooks and Journals by great authors across world. Their outstanding contribution in the field of medical practice and education has been a great source of inspiration.

Second Edition of *Textbook of Pathology*, extensively revised, updated with recent developments as needed and amended as per the 'Competency-Based Medical Education Curriculum' is intended to provide undergraduate and postgraduate medical students with clear, and concise presentation of the pathologic basis of the human disorders.

The field of pathology emerged from the application of the scientific method to study of nature, etiology, molecular mechanism, and interrelated anatomical, functional, clinical manifestations and consequences of human diseases through examination of blood and tissue specimens.

The description of the human disorders evolved over time from gross morphology observation to the histologic examination of the diseased tissues based on the specific immunohistochemical stains, immunofluorescence microscopy, immunophenotyping, fluorescence *in situ* hybridization, molecular diagnostics and more recently to ultrastructural analysis of the disease with the advent of electron microscope. Thus, molecular pathology as a discipline represents the complementary intersection of medicine and basic science discoveries and the basis for the development of new strategies for disease prevention.

Textbook of Pathology has three sections: (i) General Pathology, (ii) Hematology including chapter on 'Blood Banking and Transfusion Practices', and (iii) Systemic Pathology and Molecular Diagnostics. This textbook contains approximately 1901 high-resolution colored figures and 1719 tables, which would complement quick revision of the chapters. It provides comprehensive coverage of etiology, molecular pathogenesis of human disorders, clinical manifestations, clinicopathological correlation and diagnostic approach.

Pathology is the fundamental bridging discipline linking the basic biomedical sciences to clinical medicine. Therefore, our basic aim is for our undergraduate as well as postgraduate medical students to use their knowledge of pathology to become scientifically grounded effective clinicians and healthcare professionals.

We hope that *Textbook of Pathology* will accomplish its purpose of providing medical students and researchers with in-depth coverage of molecular basis of a wide spectrum of human disorders in the context of understanding the molecular mechanisms of the disease and advancing the theory and practice of molecular medicine.

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Anubhav
Vigyat

Preface to the First Edition

The study of pathology is essential for safe clinical practice. In modern era, tremendous growth is in progress in diagnostic pathology with recent advances in molecular methods, cytogenetics and immunohistochemistry. Rapidly changing hematology needs integration of guidelines and diagnostic criteria of hematological disorders.

I joined India's premier institution in Department of Pathology, Maulana Azad Medical College, New Delhi. I puzzled over the fact that students admitted of very high caliber still had problems in grasping the teaching material. I interacted with students and eminent faculty members across country. I discussed problems faced by the students in reproducing text. I analyzed their inputs and then started working on Textbook of Pathology in 2007. The primary aim of this book is to discuss complex topics in a 'straightforward', clear and organized manner.

All the chapters have been shared with the eminent teachers and young budding students across country. Their valuable opinion on various aspects has been taken positively during drafting of the chapters to meet the need of the young generation of pathologists. Each chapter focuses on the essentials necessary to build a broad fundamentals backed up with numerous colored figures, gross morphology, and light microscopy photographs, tables and flow diagrams.

I trust, you will enjoy reading this book. I have a great deal of time and energy ensuring easy grasp and retaining the topics. I am inviting your comments, valuable suggestions and criticism.

Vinay Kamal
Email: vinaykamal@hotmail.com
Mobile: 09818001202

Foreword

It gives me great pleasure to write the Foreword for this wonderful Second Edition of *Textbook of Pathology* written by Dr Vinay Kamal, Ex-Director Professor of Pathology, Maulana Azad Medical College, New Delhi. His vast teaching experience for undergraduate and postgraduate medical students is well-known in the corridors in the premier institution, where medical students have interacted to seek the advice.

I have gone through many chapters of this *Textbook of Pathology* and find his unique teaching experience that has been successfully transformed into wonderful method of writing and communication. Textbook abounds with revised WHO classification of neoplasms, approximately 1901 high-resolution colored figures and 1719 tables, and molecular diagnostics that explain everything in simple language—always the best way to teach.

I have no doubt that this *Textbook of Pathology* will help undergraduate and postgraduate medical students to achieve a better understanding and create strong foundation for medical education.

Dr Venkateswaran K Iyer
Senior Professor and Head
Department of Pathology
All India Institute of Medical Sciences
New Delhi

Foreword

It is with great enthusiasm, I look forward to Second Edition of *Textbook of Pathology* written by Dr Vinay Kamal, Ex-Director Professor, Maulana Azad Medical College, New Delhi. The first edition of Textbook by him received a brilliant response from the undergraduate as well as postgraduate medical students throughout the country.

Bringing out the Second Edition of *Textbook of Pathology* within a short span of time, speaks volumes about the passion of the author to constantly update the information and share it in a presentable format to his medical students.

All the chapters of textbook have been updated with incorporation of revised WHO classification of neoplasms, new information on immunohistochemistry and molecular alterations. *Textbook of Pathology* contains around 1901 high-resolution colored figures and 1719 tables.

Enormous effort has been put in recreating the tables with additional content, making the flowcharts and illustrations as well as adding new and clear microphotographs. All these are the unique feature of this textbook to enable the medical students to learn pathology in context of etiology and pathogenesis making it a valuable source for gaining knowledge to build a solid foundation in pathology for the future doctors.

I hope the undergraduate and postgraduate medical students and teachers will benefit from the Second Edition of *Textbook of Pathology*.

Dr Nita Khurana
Director Professor and Head
Department of Pathology
Maulana Azad Medical College
New Delhi

Foreword

It is my privilege to write the Foreword to Second Edition of *Textbook of Pathology* written by Dr Vinay Kamal, Ex-Director Professor of Pathology, Maulana Azad Medical College, New Delhi, releasing in 2025. The author of this *Textbook of Pathology* has more than three-decade long teaching experience at one of the country's finest and reputed medical institutes.

I had an opportunity to read the preprint of few topics, and I am immensely pleased to express that the latest Second Edition of *Textbook of Pathology* is in accordance with the 'Competency Based Medical Education Curriculum' recommended by the National Medical Commission.

Textbook of Pathology contains around 1901 high-resolution colored figures and 1719 tables, which makes the understanding of concepts very lucid for the undergraduate and postgraduate medical students.

Textbook of Pathology includes important topics like tissue fixatives, microscopy, frozen/cryostat section techniques, and histochemical stains which covers the much-needed practical aspects of the subject. Topics like recent updates, molecular pathogenesis, revised WHO classification of neoplasms, immunohistochemistry, hematology, flow cytometry, cytogenetic study, fluorescence *in situ* hybridization (FISH), enzyme-linked immunosorbent assay (ELISA), DNA sequencing, tissue microarray (TMA), high-performance liquid chromatography (HPLC), and electrophoresis have been presented in such a way that it would help the postgraduate medical students, teaching faculty and fellow practitioners also. Updated content on 'Blood Transfusion' being included, makes the *Textbook of Pathology* a very comprehensive read.

I wish Dr Vinay Kamal for all the success and hope that this latest Second Edition of *Textbook of Pathology* will receive wide acceptance by the undergraduate and postgraduate medical students and faculty members.

Dr Srivani N
Senior Professor and Head
Department of Pathology
Government Medical College
Nalgonda, Telangana

Foreword

I have known Dr Vinay Kamal, Ex-Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi for more than two decades, and have been interacting with him on various issues of pathology. The importance of pathology can be summarized in a quote by Great Sir Boyd "*As good is your pathology, so good will be your clinical practice*."

The undaunted association with teaching undergraduate and postgraduate medical students for more than three decades as faculty at Maulana Azad Medical College, New Delhi, distinguishes him from many other academicians by way of his integrated innovative approach to make the subject of pathology simple, and easy with clinico-pathological correlation of various diseases for medical students.

It is my proud privilege to write the Foreword to Second Edition of *Textbook of Pathology* written by Dr Vinay Kamal, which consists of the essence of his teaching experience in academics. This textbook contains revised WHO classification of neoplasms, molecular pathogenesis, and chapters on Blood Banking and Transfusion Practices and Cellular–Molecular Diagnostic Techniques in Clinical Practice. This textbook contains numerous high-resolution colored figures, tabulated data analysis, and methodology for diagnosis of various diseases accurately.

I am confident that this *Textbook of Pathology* by Dr Vinay Kamal will open a whole new avenue for all students in world of pathology, which will go long away in making undergraduate as well as postgraduate medical students to understand pathology and its relevance, and significance in medicine. I wish great success of new release Second Edition of *Textbook of Pathology*.

Dr Hansa Goswami
Senior Professor and Head
Department of Pathology
BJ Medical College
Ahmedabad, Gujarat

Foreword

I joined MAMC as a postgraduate student. He connects with the students and takes keen interest in their learning and tries to help them out in all their personal and academic problems. A similar helpful attitude towards staff with all help extended to them has been his nature at all times.

It is my distinct pleasure and honour to endorse the Second Edition of *Textbook of Pathology* written by Dr Vinay Kamal, Ex-Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi, whom I have known for some years and interacted on various issues of pathology.

Second Edition of *Textbook of Pathology* written by Dr Vinay Kamal covers various aspects of the subject in a systematic manner. The content in this textbook is in simple language, which makes it reader-friendly and is well illustrated by numerous gross and microscopic pictures.

The beauty of the *Textbook of Pathology* is that it highlights the clinicopathological correlation, diagnostic approach and includes revised WHO classification of neoplasms. Various illustrations in the form of figures, tables, flow diagrams and incorporation of basics of immunohistochemistry and molecular biology in this textbook provide a comprehensive understanding to medical students in pathology. The key highlights in every section of textbook also helps in quick revision of the topics.

I congratulate Dr Vinay Kamal for his dedication and immense passion of teaching medical students, who will enjoy reading this textbook and understand the basic concepts along with updated knowledge in the subject.

Dr Ranjana Solanki
Senior Professor
Department of Pathology
SMS Medical College
Jaipur, Rajasthan

Foreword

A seasonal writer—is the most appropriate word for Dr Vinay Kamal, Ex-Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi, which has more than three decades of teaching experience in the premier institution.

It gives me an immense pleasure to write the Foreword to Second Edition of *Textbook of Pathology* with recent updates. I have gone through many chapters penned down by him in this textbook. Nothing can be more informative and more simplified for undergraduate as well as postgraduate medical students than this version.

Beautifully integrating numerous high-resolution colored illustrations, images, flowcharts, tables and providing relevant knowledge in a well-structured and designed text is an art and his textbook is a perfect example of these qualities. The contents not only include the knowledge of basic *Textbook of Pathology* for undergraduate students but also integrates trivial topics of recent diagnostic techniques and advancements, and molecular and genetic pathology for postgraduate students as well.

I congratulate Dr Vinay Kamal for having the insight to produce a novel *Textbook of Pathology* that allows the undergraduate as well as postgraduate medical students of all levels to understand pathology with clinicopathological correlation and relish it while reading it cover to cover.

I wish and hope that this splendid contribution of Dr Vinay Kamal gets the well-deserved acclamation and applause.

Dr Reeni Malik
Senior Professor and Head
Department of Pathology
Gandhi Medical College
Bhopal, Madhya Pradesh

Foreword

I have this proud privilege to write the Foreword to Second Edition of *Textbook of Pathology* written by Dr Vinay Kamal, Ex-Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi. He is a renowned Pathologist, who enjoys teaching undergraduate and postgraduate medical students and has put in dedicated efforts to facilitate teaching–learning methods.

Textbook of Pathology is in two volumes. The first volume deals with General Pathology and Hematology including chapter on Blood Banking and Transfusion Practices. The second volume deals with Systemic Pathology including a chapter on Cellular–Molecular Diagnostic Techniques in Clinical Practice.

This Second Edition of *Textbook of Pathology* is an integration of molecular pathology and routine histopathology and thorough in all aspects. The upcoming scientific discipline of molecular basis of disease development has been described in a simplified yet extensively detailed manner in this textbook. Numerous high-resolution colored illustrations are eye-catching and self-explanatory. All topics are covered comprehensively making it extremely student friendly for undergraduate and postgraduate medical students.

I congratulate Dr Vinay Kamal for his dedication and immense passion of teaching undergraduate and postgraduate medical students, I am confident that this splendid contribution of the author gets the well-deserved acclamation and applause.

Dr Roma Issacs
Senior Professor
Department of Pathology
Christian Medical College and Hospital
Ludhiana, Punjab

Foreword

It gives me an immense pleasure to write the Foreword to Second Edition of *Textbook of Pathology* written by Dr Vinay Kamal, Ex-Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi, who brings out new concepts defining various learning domains for the undergraduate as well as postgraduate medical students of pathology.

As all of us know that pathology is a very vast subject but it forms the basis for understanding the disease process. This textbook covers all the aspects of pathology in a systemic manner. Every effort has been made to make the subject interesting and easy to understand by using numerous well illustrated diagrams, flowcharts, tables as well as key points placed separately in the boxes for quick revision.

An attempt has also been made to include revised WHO classification of neoplasms, recent advances in pathology, molecular and immunohistochemical correlations in various aspects of tumor diagnosis, which are aimed at individualized treatment.

I congratulate Dr Vinay Kamal for this new venture and wish him all the success in his future endeavors.

Dr Sunita Singh
Senior Professor and Head
Department of Pathology
Pandit Bhagwat Dayal Sharma Post Graduate
Institute of Medical Sciences
Rohtak, Haryana

Foreword

The first edition of *Textbook of Pathology* authored by Dr Vinay Kamal released in the year 2017, was well appreciated by undergraduate as well as postgraduate medical scholars, and eminent faculty all over India. Textbook of Pathology was the result of years of hard work and commitment by a professional teacher and experienced pathologist.

I congratulate Dr Vinay Kamal, Ex-Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi for bringing out the Second Edition of *Textbook of Pathology*. Keeping in line with the recent advances in the field of pathology, the focus in this new venture is on the molecular pathogenesis of various disorders and clinicopathological correlation. The revised WHO classification of neoplasms has been incorporated in the discussions on various entities. Updated information on immunohistochemical markers has also been provided.

I am sure that Second Edition of *Textbook of Pathology* will be of immense help to the budding undergraduate as well as postgraduate medical students, and pathologists in reproducing their knowledge.

I wish great success to the latest endeavor by Dr Vinay Kamal.

Dr Syed Besina Yasin
Senior Professor and Head
Department of Pathology
Sher-I-Kashmir Institute of Medical Sciences
Srinagar, Jammu and Kashmir

Foreword

Dr Vinay Kamal, Ex-Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi, is well known name in the field of pathology, who needs no introduction. He has more than three decades of teaching experience in nation's most prestigious institution at Maulana Azad Medical College, New Delhi. I am delighted to know about the new release of his Second Edition of *Textbook of Pathology* with recent updates.

This is a very well written textbook with numerous high-resolution figures and tables which make it a handy tool to understand the fine nuances of the subject. This textbook would make the subject interesting for both the undergraduate as well as postgraduate medical students. Seldom we come across a thorough professional who is also a person with a kind heart, always keen to go extra mile to help others.

I congratulate Dr Vinay Kamal all the best for new release of *Textbook of Pathology*. I am sure this textbook is going to be a milestone for all the medical fraternity in the times to come.

Dr Geeta Pachori
Senior Professor and Head
Department of Pathology
Jawahar Lal Nehru Medical College
Ajmer, Rajasthan

Foreword

Second Edition of *Textbook of Pathology* written by Dr Vinay Kamal, Ex-Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi, is in your hands.

Dr Vinay Kamal has worked very hard and has rewritten all the chapters of textbook incorporating most of the contemporary knowledge and developments in the field as relevant within the scope of the book.

Many new tables, flow diagrams and illustrations have been added for easy remembrance. He has been constantly updating the content and has made tremendous efforts to present the content in a comprehensive yet concise style.

Revised WHO classification of neoplasms, concepts in the molecular pathogenesis, current Cellular–Molecular Diagnostic Techniques in Clinicale Practice of pathology and diagnostic approach have been incorporated.

I am sure the textbook is going to be useful not only to undergraduate but also to postgraduate medical students, and teachers of pathology.

Dr Rajeev Sen
Senior Professor and Head
Department of Pathology
Faculty of Medicine and Health Sciences
SGT University
Gurugram, Haryana

Foreword

Ever since the first edition of *Textbook of Pathology* by Dr Vinay Kamal was released in the year 2017, the author started working on its new edition as the book became so popular in just a couple of months that most of the undergraduate and postgraduate medical students, as well as faculty in pathology across the country procured this book for personal use and for the library of their institutions. This was due to numerous qualities of the textbook such as unique presentation, topics discussed in a simple, clear, and well-organized manner backed up with numerous colored figures, photographs of gross and microscopic lesions, tables, and diagrams along with the recent updates.

Second Edition of *Textbook of Pathology* by Dr Vinay Kamal, Ex-Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi, is releasing in 2025. In this textbook, all the chapters have been updated with incorporation of revised WHO classification of neoplasms, etiopathogenesis of the disease, clinical manifestations, clinicopathological correlation, and molecular diagnostics.

Textbook of Pathology contains around 1901 high-resolution colored figures, 1719 tables, chapters on Blood Banking and Transfusion Practices and Cellular–Molecular Diagnostic Techniques in Clinical Practice. All the chapters have been shared with eminent teachers and young budding residents across the country and their opinions have been given due weightage during drafting of the chapters.

While going through the chapters, the Author's honest, hard, and intelligent work is clearly felt by the reader. The book is designed to provide accurate, authoritative, and most current information and thus meets the need of the young generation of pathologists.

Dr Vinay Kamal's heart always beats for every medical student. I congratulate Dr Vinay Kamal on the Second Edition of *Textbook of Pathology* and wish good luck in his future endeavors.

Dr Kuldeep Kumar Kaul
Medical Advisor
J and K Thalassaemia Welfare Society
Former Professor and Head
Department of Pathology
Government Medical College
Jammu, Jammu and Kashmir

Foreword

It is indeed a pleasure and honour to write the Foreword to Second Edition of *Textbook of Pathology* written by Dr Vinay Kamal, Ex-Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi. The book is an excellent compilation of knowledge of pathology in a very organized manner.

I have been associated with Dr Vinay Kamal for the last fifteen years and had the opportunity to review many of the chapters in this textbook. All the chapters in this new release *Textbook of Pathology*, have been updated with recent advances with strong emphasis on molecular pathogenesis, immunohistochemistry, flow cytometry and other molecular techniques. The book is easy to read and comprehend and shall prove student-friendly for undergraduate as well as postgraduate medical students.

I wish all the best to Dr Vinay Kamal for success of Second Edition of *Textbook of Pathology* and sincerely hope that he will keep on contributing more to the knowledge of medical students.

Dr Subhash Chander Bhardwaj
Senior Professor and Head
Department of Pathology
Government Medical College
Jammu, Jammu and Kashmir

Foreword

Books are the best to windows of the world as they are best source to gain knowledge and discipline. Pathology is the scientific study of the diseases. I have known Dr Vinay Kamal, Ex-Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi for many years by reading an excellent *Textbook of Pathology* authored by him in the year 2017.

It is my pleasure, and an honour to write the Foreword for the Second Edition of *Textbook of Pathology* by Dr Vinay Kamal releasing in 2025. The only thing that surpasses his knowledge is his humility about capabilities.

Textbook of Pathology contains around 1901 high-resolution colored figures and 1719 tables with recent updates including revised WHO classifications of neoplasms, molecular pathogenesis, immunohistochemistry, flow cytometry and molecular diagnostic techniques. This textbook contains two chapters on Cellular–Molecular Diagnostic Techniques in Clinical Practice and Blood Banking and Transfusion Practices.

Undergraduate and postgraduate medical students seeking a resource should consider Second Edition of *Textbook of Pathology* written by Dr Vinay Kamal. I congratulate Dr Vinay Kamal for grand success of the textbook releasing in 2025.

Dr Soumitra Biswas
Senior Professor and Head
Department of Pathology
Calcutta National Medical College
Kolkata, West Bengal

Foreword

The influence of a good teacher can never be erased. Dr Vinay Kamal, Ex-Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi, besides being a dedicated teacher and pathologist par excellence, is an awakener.

It is my proud privilege and I feel honored to be given an opportunity to write the Foreword to Second Edition of *Textbook of Pathology*. All the chapters of the book have recent updates including molecular pathogenesis. Many features such as revised WHO classification of neoplasms, immunohistochemistry, microscopy, histochemical stains, flow cytometric analysis, cytogenetic study, fluorescence *in situ* hybridization, enzyme-linked immunosorbent assay, DNA sequencing and tissue microarray.

This textbook contains around 1901 high-resolution colored figures and 1719 tables that will help undergraduate and postgraduate medical students to understand pathology of diseases and their clinicopathological correlation.

I convey my heartfelt appreciation for the untiring efforts and the hard work done by Dr Vinay Kamal for conceptualizing and executing his work. This new release Textbook of Pathology is a great boon to the undergraduate and postgraduate medical students, and will enjoy reading this book.

I wish Dr Vinay Kamal a grand success in his endeavor and happy to release this Second Edition of *Textbook of Pathology*.

Dr Pranita Medhi
Senior Professor and Head
Department of Pathology
Assam Medical College and Hospital
Dibrugarh, Assam

Foreword

I am privileged to write the Foreword to Second Edition of *Textbook of Pathology* written by a well experienced and excellent talented teacher Dr Vinay Kamal, Ex-Director Professor, Maulana Azad Medical College, New Delhi.

Textbook of Pathology contains 1901 high-resolution colored images, 1719 tables, chapters on 'Cellular–Molecular Diagnostic Techniques in Clinical Practice' which makes the understanding of concepts very lucid for the undergraduate and postgraduate medical students, which make the book unique.

The author has meticulously considered valuable suggestions and criticism after thorough discussions with many subject experts in upgradation of this Second Edition of *Textbook of Pathology*. Inclusion of recent advances in diagnostic pathology, cytogenetics, molecular pathology, and immunohistochemistry are the added advantages of the textbook. Recently revised WHO classification of neoplasms has also been included in the book. All the chapters of textbook are written, and edited with recent updates.

I am sure this Textbook of Pathology will prove to be an asset for undergraduate and postgraduate medical students in view of the 'Competency Based Medical Education' implemented by National Medical Commission. Salient features given in the boxes will help the medical students in quick revision of the course prior to their formative and summative assessments.

I wish Dr Vinay Kamal all the best in his endeavor.

Dr Naval Kishore Bajaj
Senior Professor and Head
Department of Pathology
Osmania Medical College
Hyderabad, Telangana

Acknowledgments

Our motivation in writing Second Edition of *Textbook of Pathology* continues to share recent scientific concepts, clinical applications, and molecular diagnostics with colleagues and undergraduate and postgraduate medical students. We have strived to provide explanatory text, 1901 photographs and 1719 tables to enhance pictorial insights into human diseases.

We acknowledge the support and encouragement of colleagues and undergraduate and postgraduate medical students, who have critically revied the draft chapters and provided photographs related to surgical pathology and hematology section. Their constructive invaluable suggestions have served to significantly improve the work.

We gratefully recognize the continual invaluable support of Dr Naval Kishore Bajaj (Senior Professor and Head, Department of Pathology, Osmania Medical College Hyderabad, Telangana), Dr Nita Khurana (Director Professor and Head, Department of Pathology, Maulana Azad Medical College, New Delhi), Dr Jyoti Priyadarshini (Professor, Department of Pathology, GR Medical College Gwalior, Madhya Pradesh), Dr Tushar Kalonia (Assistant Professor, Department of Pathology), Dr Neha Kumari (Assistant Professor, Department of Pathology), Rajiv Aggarwal, Sh PL Jayant, Smt Shanti Devi, Dr Satya Pal Jayant, Dr Vijaya Jayant, Mr Arjun Jayant, Dr Rohan Jayant, Dr Vimal Singh, Dinesh Kumar, and Dr Divyark Singh.

Words are less to express our gratitude to eminent faculty colleagues for showering their blessings by endorsing Second Edition of *Textbook of Pathology*. Comments from eminent faculty and undergraduate and postgraduate medical students are always welcome.

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We express our gratitude to Mr Satish Kumar Jain CHAIRMAN AND MANAGING DIRECTOR, Mr Varun Jain MANAGING DIRECTOR and Mr Sunil Dutt MANAGER PROMOTION AND DEVELOPMENT of CBS Publishers and Distributors Private Limited for extending their whole hearted support at every step in completion of this book.

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Noida, Uttar Pradesh

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• Dr SD Ganokar • Dr Amrin Fatima • Dr Pramod Sarwade

MGM Institute of Health Sciences Aurangabad, Maharashtra
Senior Professor and Head of Pathology
• Dr CP Bhale

Professors of Pathology
• Dr SS Kale • Dr AA Vare • Dr Suparna Bindu

Associate Professors of Pathology
• Dr Reeta Taksali • Dr Archana Bidari • Dr Meera Mahajan
• Dr Ganesh Kadam • Dr Anuradha Patil • Dr Neha Borde

Assistant Professors of Pathology
• Dr Rohini Shewale • Dr Praveen Kumar R • Dr Pranita Sontakke

Senior Residents of Pathology
• Dr Snehal Patil • Dr Kalyani Deshmukh

Tutor of Pathology
• Dr Sangeeta Kulkarni

Indira Gandhi Government Medical College and Hospital Nagpur, Maharashtra
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• Dr Balwant D Kowe

Associate Professors of Pathology
• Dr Nitin Y. Shende • Dr Rekha Patil • Dr Saroj Gaikwad Meshram
• Dr Jayshree Joshi Sathe • Dr Amit Agrawal

Assistant Professors of Pathology
• Dr Anjali Bode • Dr Sonali Datar • Dr Amrapali Gaiwad
• Dr Pradnya Bhadarje • Dr Sayyed Wasim

Government Medical College and Hospital Nagpur, Maharashtra
Head of Pathology
• Dr Satish Halwatkar

Associate Professors of Pathology
• Dr Poornima Kodake • Dr Tulsi Chadi • Dr Pradeep Butle
• Dr Prajakta Gaddewar • Dr Supreeta Nayak • Dr Rajna Pohekar
• Dr Anjali Bode

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• Dr Shubhangi Jibkate • Dr Shubhangi Khamankar • Dr Archana Khade
• Dr Manjiri Makde • Dr Fatema Kamal • Dr Renuka Badnale
• Dr Nikhil Charmode • Dr Pravin Meshram • Dr Aniket Meshram
• Dr Anushree Wagh • Dr Sonal Meshram • Dr Shalakha Khade

NKP Salve Institute of Medical Sciences Nagpur, Maharashtra
Associate Professors of Pathology
• Dr Anne R Winkinson

Mahatma Institute of Medical Sciences Sewagram, Maharashtra
Director Professor and Head of Pathology
• Dr Anupama Gupta

Director Professor of Pathology
• Dr VB Shiv Kumar

Professor of Pathology
• Dr Anshu

Associate Professors of Pathology
• Dr Mansi Bhalavi • Dr Bharat Patil • Dr Abhay Deshmukh

Assistant Professors of Pathology
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• Dr Shikha Singh

Jawaharlal Nehru Medical College Sawangi, Maharashtra
Senior Professor and Head of Pathology
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Professor of Pathology
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Vasantrao Pawar Medical College Nashik, Maharashtra
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• Dr Preeti Bajaj

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• Dr Sheetal Sameer Dharrao

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• Dr Kale Vidya Gaurav • Dr Purva More

SMBT Institute of Medical Sciences and Research Center Nashik, Maharashtra
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• Dr Dnyaneshwar Shivajirao Jadhav

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• Dr SA Bora • Dr BM Patil

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Assistant Professors of Pathology
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• Dr Ubhale Sarika B • Dr Agrawal Sakshi S • Dr Jain Sapna P

Senior Residents of Pathology
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• Dr Desale Shruti S • Dr Newadkar Neha D • Dr Chaudhari Shubhangi R

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• Dr Shende Ketaki R • Dr Saoji Shreeya M

Tutors of Pathology
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• Dr Rajharsh Dattatray Hanmate • Dr Smita Shashikant Pudale
• Dr Rahul Suryakant Rao Abhange • Dr Kacharu Tukaram Dalve

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• Dr Khule Subhangi Kamlakar

Maharashtra Institute of Medical Sciences and Research (MIMSR) Latur, Maharashtra
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ODISHA

MKCG Medical College Berhampur, Odisha
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• Dr Sulata Choudhary

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• Dr Lity Mohanty

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• Dr Jawahar

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Government Medical College Amritsar, Punjab
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Professor of Pathology
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Assistant Professors of Pathology
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Guru Govind Singh Medical College Faridkot, Punjab
Senior Professor and Head of Pathology
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• Dr Sumit Grover

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RAJASTHAN

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• Dr Karuna Gupta • Dr Sangita Sehgal • Dr Dilip Ramrakhiani
• Dr Dipika Mishra • Dr Anita Harsh

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• Dr Dipika Hemrajani • Dr Saroj Pachori • Dr Kalpana Mangal
• Dr Priti Saini • Dr Neeraj Verma • Dr Ashish Bagadiya
• Dr Mukesh Khinchi • Dr Subhash Sharma • Dr Shruti Bhargav

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• Dr Alpana Jain • Dr Namrata Aggarwal • Dr Jayanti Mala
• Dr Mukta Meel • Dr Pratibha Maan • Dr Prerana Choudhary
• Dr Batti Lal Meena • Dr Vandna Yadav • Dr Swapna Devi
• Dr Neha Gupta

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• Dr Rimjhim Shrimal • Dr Sunita Meena • Dr Sarla Saini
• Dr Sweta Meena

Senior Demonstrator of Pathology
• Dr Parul Gautam

Dr Sampurna Nand Medical College Jodhpur, Rajasthan
Senior Professor and Head of Pathology
• Dr Madhu Gupta

Senior Professors of Pathology
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• Dr Tarun Sharma

Assistant Professors of Pathology
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• Dr Swamlata • Dr Neha Bagga • Dr Mamta Choudhary
• Dr Shreya Agrawal • Dr Amrit Raj Kalla • Dr Bharti Varshney
• Dr Rishita Gehlot • Dr Anubha Gupta

Senior Demonstrator of Pathology
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• Dr Geeta Pachori

Senior Professors of Pathology
• Dr Neena Kasliwal • Dr Vandana Porwal

Professors of Pathology
• Dr Swaranlata Ajmera • Dr Seema Gupta • Dr Rashmi Sharma
• Dr Kalpana Sharma • Dr Deepali Jain

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• Dr Shweta Khandelwal • Dr Shilpa Jalan • Dr Kalpana Singh Beniwal

Assistant Professors of Pathology
• Dr Aparana Rathi • Dr Sonia Tanwar • Dr Leena Verma
• Dr Soniya Sanaadhya • Dr Pushpa Bai Bairwa • Dr Anita Kumari
• Dr Om Prakash Pachori • Dr Aditi Sirohi • Dr Neha Vijay
• Dr Hansa Choudhary • Dr Pankaj Kumar Jain • Dr Vimlesh
• Dr Sunil Kasana • Dr Sanju Bhati

Government Medical College Kota, Rajasthan

Senior Professor and Head of Pathology
• Dr RK Singh

Senior Professors of Pathology
• Dr Rajshree Bhati • Dr Sudha Pankaj Meena

Professors of Pathology
• Dr Akta Rasania • Dr Shaida Riyaz

Associate Professor of Pathology
• Dr Deepti Sukheeja

Assistant Professor of Pathology
• Dr Lakshmi Aggarwal

Sardar Patel Medical College Bikaner, Rajasthan

Senior Professor and Head of Pathology
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• Dr Vanita Kumar • Dr Dharam Pal Soni • Dr Nilima Arora
• Dr Nidhi Chitlangia • Dr Sunita Bika • Dr Sharda Dawan

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• Dr Nikita Manoj

Assistant Professors of Pathology
• Dr Ritika Agrawal • Dr Madhusudan Rankawat • Dr Ankita
• Dr Om Prakash Singh • Dr Durga Shankar Jaipal
• Dr Ajit Singh Beniwal

Rabinder Nath Medical College (RNT) Udaipur, Rajasthan

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• Dr Sandhya Bordia

Senior Professor of Pathology
• Dr Namita Goyal

Associate Professors of Pathology
• Dr Noratan Jatiya • Dr Sonal Bhati • Dr Pooja Kanwat
• Dr Gunja Dwivedi • Dr Vijay Rajak • Dr Priyanka Kumari

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• Dr Manjulata Gupta • Dr Seema Meena • Dr Jigyashu Joshi
• Dr Priya Parmar • Dr Dharmendra Kumar Garg • Dr Gunjan Bhatia
• Dr Samay Singh Meena • Dr Laxmi Kumari • Dr Abhilasha Garg
• Dr Jubeda Bano • Dr Hemant Kumar

All India Institute of Medical Sciences Jodhpur, Rajasthan

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• Dr Sudeep Khera

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Professors of Pathology
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• Dr Brajendra Shakyawal

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• Dr Yogendra Madan

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• Dr Deeksha Buliwal • Dr Deepika Jain

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Professor of Pathology
• Dr Hema Udawat

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• Dr Alka Mittal

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Professor of Pathology
• Dr Madhuri Agarwal

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• Dr Savita Soni

Geetanjali Medical College Udaipur, Rajasthan
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Professor of Pathology
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• Dr Sarita Tatawat • Dr Mansi Khamesara

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• Dr Abha Mathur
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• Dr Chetna Mehrol • Dr Deepak Garg • Dr Indu Chaudhary
• Dr Sony Mandal • Dr Sonia Agarwal
Assistant Professors of Pathology
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• Dr BRN Srivastav • Dr Kusum Mathur
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• Dr Indu Saxena • Dr Charu Tripathi • Dr Murari Lal Dhanetwal
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• Dr Ritu Meena
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• Dr Nikunj Bhimani • Dr Amit Yadav • Dr Anita Choudary
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• Dr Lavanya Raghavan
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• Dr Sidhi Periwal • Dr Ashwani S Deshmukh
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Professor of Pathology
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Section I

GENERAL PATHOLOGY

CHAPTER 1

Cellular Pathology and Biology of Aging

Vinay Kamal, Anubhav and Vigyat

LEARNING OBJECTIVES

CELL UNIT OF LIFE AND INTRODUCTION TO PATHOLOGY

- Cell unit of life
- Introduction to pathology

CELLULAR RESPONSE TO STRESS AND HARMFUL STIMULI

- Cellular adaptations
- Cell injury
  - Reversible cell injury
  - Irreversible cell injury
- Metabolic derangements

CELLULAR ADAPTATIONS

- Hyperplasia
- Hypertrophy
- Atrophy
- Metaplasia
- Dysplasia
- Reduction in size of organs in other pathologic processes

CELL INJURY AND CELL DEATH: OVERVIEW

- Causes of cell injury and cell death
  - Oxidative stress/hypoxia-induced cell injury
  - Ischemia-reperfusion-induced cell injury
  - Pathogen-induced cell injury
  - Physical agents induced cell injury
  - Chemical agents induced cell injury
  - Drug-induced cell injury

PATHOPHYSIOLOGY OF CELL INJURY, MORPHOLOGIC PATTERNS AND OUTCOME OF NECROSIS

- Reversible cell injury
  - Decreased ATP production in mitochondria
  - Plasma membrane Na^+/K^+ pump failure
  - Plasma membrane calcium pump failure
  - Plasma membrane bleb formation
  - Myelin figures
- Irreversible cell injury (cell death)
  - ATP depletion in mitochondria
  - Mitochondrial damage
  - Calcium influx and impaired calcium homeostasis
  - Reactive oxygen species production induced by cell stressors
  - Defects in permeability of plasma membrane and cell organelles
  - Rough endoplasmic reticulum disruption
  - Cytoskeleton disruption
  - Genetic apparatus disruption
- Morphologic patterns of necrosis
  - Coagulative necrosis
  - Liquefactive necrosis
  - Caseous necrosis
  - Fat necrosis
  - Gangrene
  - Fibrinoid necrosis
  - Gummatous necrosis
  - Subcutaneous necrosis in newborn
  - Zenker's necrosis in skeletal muscle
  - Postmortem autolysis
- Outcome of necrosis
  - Complete resolution
  - Injured tissue repair by fibrosis
  - Dystrophic calcification
  - Resorption of necrotic tissue

REGULATED CELL DEATH (RCD)

- Mitochondrial permeability transition pore driven necrosis
- Apoptosis
- Necroptosis
- Pyroptosis
- Ferroptosis
- Autophagy
- Lysosomal-dependent cell death

APOPTOSIS

- Three pathways of apoptosis
  - Extrinsic death receptor pathway of apoptosis
  - Intrinsic mitochondrial pathway of apoptosis
  - CD8+ cytotoxic T cells/NK cells (granzyme B/perforin) mediated pathway of apoptosis
- Family of cysteine proteins 'caspases'
  - Initiator/inducer caspases
  - Executioner/effector caspases
- Morphologic and biochemical changes in apoptotic cells
  - Histologic changes
  - Ultrastructural changes
  - Biochemical changes
- Apoptosis in health
  - Apoptosis during embryogenesis
  - Apoptosis during adult life
- Apoptosis in diseases
  - Apoptosis induced by viruses
  - Neuronal apoptosis and formation of red neurons in brain
  - Keratinocyte apoptosis in epidermis
  - Apoptosis of neutrophils in acute inflammation
  - Apoptosis of parenchymal organ's cells due to obstruction in ducts
  - Apoptosis of cells with damaged DNA
  - Apoptosis of cells with accumulated misfolded proteins
  - Apoptosis of cancer stem cells
- Laboratory diagnosis
  - Light microscopy
  - Electron microscopy
  - Electrophoresis
  - Flow cytometry
  - TUNEL assay
  - Immunohistochemistry

INTRACELLULAR ACCUMULATION OF SUBSTANCES

- Lipid accumulation
  - Triglyceride accumulation inducing fatty change in liver
  - Cholesterol and its esters accumulation inducing atherosclerosis, xanthomas and gallbladder cholesterolosis
- Glycogen accumulation
  - Glycogen storage diseases: types 1 to 10
- Protein accumulation
  - Increased protein droplets reabsorption in the proximal renal tubule in renal disease with proteinuria
  - Increased protein production by plasma cells forming Russell bodies
  - Accumulation of cytoskeleton proteins
  - α_1-Antitrypsin protein accumulation

- Pigment accumulation
  - Endogenous pigments
    - Lipofuscin pigment
    - Melanin pigment
    - Bilirubin pigment
    - Hemosiderin pigment
    - Hematin pigment
    - Homogentisic acid pigment
    - Hamazaki-Wesenberg bodies
  - Exogenous pigments
    - Coal pigment
    - Tattoo ink pigment

EXTRACELLULAR ACCUMULATION OF SUBSTANCES

- Intracellular and extracellular hyaline change
- Aggregation of misfolded proteins (amyloidosis)
- Fibrinoid necrosis of arterioles
- Hyalinization of glomenular basement membrane
- Dysgenetic hyalinization in seminiferous tubules

PATHOLOGIC CALCIFICATION

- Dystrophic calcification
- Metastatic calcification
- Pulmonary alveolar microlithiasis (PAM)

CELLULAR BIOLOGY OF AGING

- DNA damage theory of aging
- Cellular senescence
  - Telomere attrition
  - Activation of tumor suppressor genes
- Oxygen-derived free radicals theory linked to cellular aging
- Defective protein homeostasis and cellular aging
- Genetic disorders linked to premature aging
  - Hutchinson-Gilford progeria syndrome
  - Werner syndrome
  - Cockayne's syndrome (Weber-Cockayne's syndrome or Neill-Dingwall syndrome)
- Mammalian sirtuin system
  - Sirtuins in health and disease

CELL UNIT OF LIFE AND INTRODUCTION TO PATHOLOGY

Cell is defined as the most basic structural and functional unit of life. Normal cells handle physiological demands and maintains a steady state called homeostasis. More severe physiologic stresses stimuli may bring about a number of physiologic and morphologic cellular adaptations, during which new but altered steady states are achieved, preserving the viability of the cell. The adaptive response may consist of an increase in the size of the individual cell called hypertrophy and increase in the number of cells called hyperplasia. Conversely, **atrophy** is an adaptive response in which there is decrease in the size and function of cells.

CELL UNIT OF LIFE

Cell consists of cell membrane (plasma membrane), the nucleus and cytoplasm. The cell membrane is a phospholipid bilayer containing many different molecular components, including proteins and cholesterol, some with carbohydrate groups attached. A phospholipid molecule consists of a polar phosphate "head," which is hydrophilic and a nonpolar lipid "tail," which is hydrophobic. Unsaturated fatty acids result in links in the hydrophobic tails.

- The lipid bilayer provides the basic structure and serves as a relatively impermeable barrier to most water-soluble substances. Transmembrane proteins that span the phospholipid bilayer of cell membranes serve a variety of structural, transport, signaling and enzymatic functions.
- Cytoplasm is internal material between the cell membrane and nucleus of a cell. It consists of a clear, semi-fluid, water-based fluid called cytosol, within which are all specialized membrane-bound cell organelles (mitochondria and lysosomes) in the cell and cellular solute and suspended materials. Each organelle has its specific function, that is essential for cell survival.
- The cytoskeleton in cell is formed by rod-like proteins that support the cell's shape and provide other functions, locomotive abilities. Cytoskeleton consists of microtubules, microfilaments, and intermediate filaments, which plays an important role in maintaining cell shape and structure, in promoting cellular movement, and aiding cell division.
- Mitochondria are the energy-conversion factories of the cell. Mitochondrion, one of the cellular organelles, is bound by a double lipid bilayer membrane that functions primarily in the production of cellular energy (ATP).
- The endoplasmic reticulum (ER) is a winding network of thin interconnected membranous sacs found in close association with the cell nucleus.
- The smooth and rough endoplasmic reticula are very different in appearance and function.
  - Rough ER is studded with numerous ribosomes, which are sites of protein synthesis. Smooth endoplasmic reticulum is formed by a series of flattened, membrane-bound sacs that functions in protein modification, tagging, packaging, and transport within the other areas of cytoplasm. Some of these products are exported from the cell through exocytosis.
  - Enzymatic proteins are packaged and sent for fusion with existing lysosomes. Lysosomes and membrane-bound cellular organelles are originating from the Golgi apparatus and containing digestive enzymes.
  - Smooth ER synthesizes phospholipids, steroid hormones, regulates the concentration of cellular Ca^{++}, metabolizes some carbohydrates, and breaks down certain toxins.
- Peroxisome is a small membrane-bound organelle in the cytoplasm of many cells, which contains the reducing enzyme catalase and some oxidase for detoxifying harmful substances and lipid synthesis.

- The centrosome contains the small replicating organelle 'centriole' that provides the origin for microtubule growth and moves DNA during cell division.
- The nuclear envelope, a double membrane, surrounding the nucleus contains nuclear pores that control the movement of substances into and out of the nucleoplasm.
- Human DNA is described as a double helix that resembles a molecular spiral staircase. DNA has an antiparallel configuration with one strand arranged 5′ to 3′ in one direction and the other strand in the opposite direction. A purine is bound to pyrimidine by the hydrogen bonds: adenine:thymine and guanine:cytosine. The double helix of DNA is the result of bonds in the sugar-phosphate backbone. DNA is organized around histone proteins into nucleosomes, which are compacted and progressively coiled and finally supercoiled into 46 chromosomes. DNA is inactive in the coiled form and must be uncoiled for biological processes such as transcription and translation into proteins. The dense nucleolus is the site of ribosome production. The nucleus of a eukaryotic cell directs the cell's activities and stores DNA.

Pathology Pearls: Conditions Associated with Alteration in Cell Organelles

- Certain conditions are associated with alterations in cell organelles (lysosomes, smooth endoplasmic reticulum, and mitochondria) or the cytoskeleton.
- Lysosomal catabolism occurs by heterophagy or autophagy
- Smooth endoplasmic reticulum hypertrophy occurs due to induction of drug tolerance to barbiturates and alcohol
- Mitochondrial defects (number, size and shape)
- Cytoskeleton defects (phagocytosis and locomotion)
- Nucleus (pyknosis, karyorrhexis and karyolysis)
- Membranes (plasma membrane and subcellular membrane such as endoplasmic reticulum)

CELL ADHESION MOLECULES (CAMs)

Cell adhesion molecules are transmembrane-linked glycoproteins that mediate connections between cells or the attachment of cells to basement membrane. There are at least six groups of cell adhesion molecules: selectins, integrins, cadherins, members of immunoglobulin superfamily, mucins and lymphocyte homing receptors (CD44). Cell adhesion receptors enable cells to recognize and bind cell adhesion molecules on other cells or in the extracellular matrix. Cell adhesion receptors form homophilic adhesions (cadherin-cadherin) or heterophilic adhesions between different types of molecules (selectin-mucin). Major family of cell adhesion molecules (CAMs) is given in Table 1.1.

Table 1.1 Major family of cell adhesion molecules (CAMs)

Selectins (calcium-dependent)
Integrins (calcium-dependent)
Cadherins (calcium-dependent)
Members of immunoglobulin superfamily (calcium-dependent)
Lymphocyte homing receptor (CD44) (calcium-dependent)

Selectins

The selectins are a family of carbohydrate-binding transmembrane molecules found on the surface of endothelial cells, leukocytes and platelets. Members of selectin family include endothelial selectin (E-selectin), leukocyte selectin (L-selectin) and platelet selectin (P-selectin). E-selectin adheres to integrins, P-selectin interacts with platelets, and L-selectin interacts with leukocytes. Selectins mediate the adhesion of white blood cells and platelets to endothelial cells. In collaboration with other CAM families, selectins play important roles in leukocyte trafficking to the sites of inflammation.

Integrins

Integrins are the principal cell surface receptors that function mechanically by binding the cell microfilament (actin) of cytoskeleton to the extracellular matrix (ECM). The integrin family of proteins consists of α and β subtypes, which form transmembrane heterodimers. Integrins are responsible for transduction of external signals to the cytoskeleton.

Cadherins

Cadherins are calcium-dependent transmembrane proteins that constitute the major intercellular link at adherens junctions and bind to catenin and other proteins inside cell to link to the microfilament (actin) of the cytoskeleton. Different members of cadherin family are found in different locations: E-cadherin (epithelial cells), N-cadherin (neurons) and P-cadherin (placenta). Cadherins form homophilic adhesions (cadherin-cadherin). Failure of cadherin mediated cell-to-cell adhesion cascade is observed in breast lobular carcinoma.

Members of Immunoglobulin Superfamily CAMs

The immunoglobulin superfamily CAMs are calcium-independent transmembrane. Members of immunoglobulin superfamily include intercellular adhesion molecule (ICAM), vascular adhesion molecule 1 (VCAM-1), neural cell adhesion molecule (NCAM), and platelet endothelial cell adhesion molecule 1 (PECAM 1 or CD31). Nectins have been recently identified as new calcium-independent CAMs consisting of four members.

- Each immunoglobulin superfamily CAM has an extracellular domain, which contains several Ig-like intra-chain disulphide-bonded loops with conserved cysteine residues, a transmembrane domain, and an intracellular domain that interacts with the cytoskeleton.
- Immunoglobulin superfamily CAMs function by both homophilic and heterophilic binding. These are involved in recognition, binding or adhesion processes of cells.

Lymphocyte Homing Receptors

Lymphocyte homing receptor (CD44) is involved in lymphocyte adhesion to endothelial cells of venules and lymphocyte exit from the blood circulation. It may also be involved in hematogenous dissemination of malignant lymphoma.

CELL JUNCTIONS

At molecular level, intercellular junctions consist of three multiprotein components which differ depending on the type of junctions: transmembrane adhesion protein, cytoplasmic adapter protein and cytoskeleton proteins.

- Five types of cellular junctions are present between the epithelial cells: tight junctions (zona occludens), adherent junctions (zona adherens), desmosomes (macula adherens), gap junctions and hemidesmosomes.
- Cell junctions provide contact between neighboring cells or between a cell and the extracellular matrix. They also build up the paracellular barrier of epithelia and control the paracellular transport. Functional classification of cell junctions is given in Table 1.2.

Table 1.2 Functional classification of cell junctions

Occluding Junctions
Tight junctions (zona occludens)
Anchoring Junctions

▪ Intermediate filament attachment sites
  • Desmosomes (cell-to-cell junction)
  • Hemidesmosomes (cell-extracellular matrix junction)
▪ Actin filament attachment sites
  • Zonula adherens (cell-to-cell junctions)
  • Focal adhesions (cell-extracellular matrix junction)

Communicating Junctions
Gap junctions
Signal Relaying Junctions
▪ Chemical synapses in the nervous system ▪ Immunological synapses in the immune system ▪ Transmembrane ligand-receptor cell-to-cell signaling contacts

Zona Occludens (Tight Junctions)

Zona occludens is a membrane protein complex composed of occludens and claudins, which are attached to three proteins called ZO1, ZO2 and ZO3, which help in holding occludens and claudins properly in their positions. Occludens and claudins exit cell membrane and interact with each other and seal the surface of two adjacent cells. Zona occludens is present in the lateral surfaces of epithelial cells, which prevents floating of protein channels from apical surface to basal surface. Zona occludens regulates fluid movement through paracellular route.

Zonula Adherens (Cell-to-Cell Junctions)

Zonula adherens is a cell-to-cell adherens junction that forms a belt in the apical most region of the lateral cell surface of many epithelia.

Desmosomes

Desmosomes are specialized adhesive proteins that localize intercellular junctions of epithelia and cardiac muscle. Desmosomes resist mechanical stress and maintain the mechanical integrity of tissues.

Hemidesmosomes

Hemidesmosomes are the only cellular junctions present on the basal surface of the epithelium, which link the cell to the basal lamina and through integrin attaches to extracellular matrix. Intermediate filaments are present inside hemidesmosomes.

Gap Junction Channels

Gap junction channels are formed by docking of two connexons, that are present at cell-to-cell appositions. Gap junction channels are responsible for direct intercellular transfer of ions and small molecules including propagation of inositol triphosphate-dependent calcium waves.

Signal Relaying Junctions

Chemical synapses are specialized junctions through which neurons signal to each other and to non-neuronal cells such as those in the glands and muscles. Chemical synapses allow neurons to form circuits within the central nervous system. Their key feature is the presence of synaptic vesicles at presynaptic terminals, which are filled with one or more neurotransmitters, which act as messengers between the communicating neurons.

CYTOSKELETON

Cytoskeleton of a cell represents network of protein filaments in the cytosol that maintains cell shape, cell movement, cell division, intracellular organization and intracellular transport of organelles and molecules.

- There are three main components of the cytoskeleton: microtubules, microfilaments and intermediate filaments, along with other proteins that support these components. All three components of cytoskeleton interact with each other noncovalently.
- Cytoskeleton helps cells in maintaining their shape and internal organization, and also provides mechanical support that enables cells to perform essential functions like cell division and cell movement. Hypoxic injury may cause damage to cytoskeleton.
- Blebs are observed on cell surface, most likely caused by disorderly function of the cellular cytoskeleton. Components of cytoskeleton, their arrangement, functions and disorders are given in Table 1.3.

Pathology Pearls: Disorders of Cytoskeleton

Disorders of Microfilaments

The membrane skeleton composed of spectrin, actin, and protein provides structural integrity to the cell membrane of red blood cells. Congenital spherocytosis, an autosomal dominant disease, is an example of a defect in spectrin resulting in a hemolytic anemia.

Disorders of Microtubules

- **Male sterility:** Defects in microtubules inhibit sperm motility results in sterility.
- **Kartagener's syndrome (immotile cilia syndrome):** It refers to immobilization of cilia of respiratory epithelium causes interference of clearance of pathogens resulting in bronchiectasis.
- **Dysfunctional leukocytes:** Colchicine drug used in acute attacks of gout binds to tubulin and prevents the assembly of microtubules, thus prevents migration and phagocytosis of leukocytes in response to urate crystals.
- **Chédiak-Higashi syndrome:** It is autosomal recessive disorder due to defect in the assembly (polymerization) of microtubules in the cytoplasm. It is characterized by defective degranulation of neutrophils, impaired microbial killing, and recurrent *Staphylococcus aureus* bacterial infections forming soft tissue abscess. This disorder results from a mutation in the '**LYST gene**' on chromosome 1q42 that encodes a protein for microtubules involved in intracellular trafficking of proteins.

Disorders of Intermediate Filaments

- **Mallory hyaline:** In chronic alcoholic persons, Mallory hyaline demonstrated in hepatocytes. It is an example of intermediate filaments (cytokeratins) abnormality.
- **α_1-Antitrypsin deficiency:** Structurally abnormal α_1-antitrypsin molecules accumulate in the liver.
- **Parkinson disease:** α-Synuclein accumulates in neurons in the substantia nigra of patients with Parkinson disease.

Microfilaments

Microfilaments or actin filaments (6–8 nm) are the thinnest, linear and strong filaments of the cytoskeleton; predominantly of a contractile protein called actin subunits. These are protein filaments primarily composed of polymers of actin and found in the cytoplasm of eukaryotic cells, which interact with numerous other proteins in the cell.

- Microfilaments are intimately involved in many plasma and internal membrane functions. Functions of microfilaments include cytokinesis, cell motility, amoeboid movement leukocytes, changes in cell shape, endocytosis (phagocytosis) and exocytosis, cell contractility, transmembrane signaling and mechanical stability.

Table 1.3 Components of cytoskeleton, their arrangement, functions and disorders

Cytoskeleton Component Size	Arrangement	Functions	Disorders
Microfilaments			
8 nm	Microfilaments thin, twisted strands of protein molecules	Movement of pigment granules	Impairment of leukocytes
Microtubules			
25 nm	Microtubules hollow, tough and durable fibers have spiral arrangement of protein subunits	▪ Maintenance of shape of cells, intracellular transport, movement of organelles and chromosomes ▪ Colchicine drug disrupts microtubules hence impairs movement of chromosomes	▪ Male sterility ▪ Kartagener's syndrome ▪ Dysfunctional leukocytes ▪ Chédiak-Higashi syndrome
8–10 nm	Intermediate filaments are thick hollow tubes, twisted protein strands (e.g. keratin, glial filaments, desmin and vimentin)	In most of the cells, intermediate filaments are forming a basket around the nucleus and present in cell-to-cell junction	▪ Mallory's hyaline ▪ α_1-Antitrypsin deficiency ▪ Parkinson disease

- Microfilaments in leukocytes participate in leukocytic movement and phagocytosis. Phalloidin toxin (*Amanita phalloides*) inhibit actin filaments resulting in disruption of phagocytic activity of the cells.

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome protein (WASp) regulates remodeling of actin filaments of cytoskeleton to regulate cell movement, cell signaling and cell division.

- Abnormality of microfilaments is linked to Wiskott-Aldrich syndrome, a X-linked recessive immunodeficiency disorder characterized by recurrent bacterial sinopulmonary infections, eczema (atopic-like), platelet dysfunction (thrombocytopenia), bloody diarrhea (secondary to low platelet count) and T cell defect.
- Wiskott-Aldrich syndrome is treated with stem cell transplant by using bone marrow, peripheral blood or umbilical cord blood from a healthy suitably tissue matched donor.

Microtubules

Microtubules are the major components of the cytoskeleton and formed by the polymerization of a dimer of two globular proteins, tubulin α and β subunits, assembled into linear protofilaments that can then associate laterally to form hollow tubes, the microtubules with a diameter of 20–25 nm.

- Microtubules are capable of undergoing rapid assembly and disassembly in the cytosol. Microtubules function in maintenance of cell shape, intracellular organelle motility, structural support, keeping cell organelles in place and chromosome segregation during cell division (mitosis and meiosis, i.e. movement of chromosomes and creation of the mitotic spindle).
- Microtubules are essential for leukocytic migration and phagocytosis. Cytochalasin B drug prevents the assembly of microtubules also results in disruption of phagocytic activity of the cells.
- Microtubule abnormalities are linked to male sterility, Kartagener's syndrome (immotile cilia syndrome), dysfunctional leukocytes and Chédiak-Higashi syndrome (defective degranulation of neutrophils, impaired microbial killing).
- Male infertility can be caused by low sperm production, abnormal sperm function or blockages that prevent the delivery of the sperm. A sperm (spermatozoon) has three parts: head, neck, midpiece and tail (exoneme).

Clinical Pearls: Structure of Spermatozoon

- **Head of spermatozoon:** The head of the sperm contains nucleus with haploid number of chromosomes, that holds DNA of the cell. The head also contains numerous enzymes such as hyaluronidase, corona penetrating enzyme and zona lysine (acrosin) collectively called 'spermatolysins', which help the sperm breakthrough the cell membrane of ovarian ovum.
- **Neck of spermatozoon:** Neck of the sperm contains proximal and distal centrioles that form the cilium of the sperm. And after fertilization form the major microtubule-organizing center of the zygote.
- **Midpiece of spermatozoon:** Midpiece of the sperm contains mitochondria.
- **Tail of spermatozoon:** The structure of tail (exoneme) of sperm consists of 9+2 microtubules, molecular motors (flagellar dyneins), and their regulatory linker proteins. Microtubules are the prime component of the cytoskeleton, which are vital for organelle transport and cellular division during spermatogenesis, sperm motility and functional capacity of sperm. Tail (exoneme) of the sperm is well preserved. Defects in the microtubules in axonemal structure cause defects in sperm abnormality (teratozoospermia, oligospermia, oligozoospermia, asthenozoospermia or even azoospermia), sperm motility and often leads to male infertility.

Kartagener's Syndrome (Immotile Cilia Syndrome)

Kartagener's syndrome, also called immotile cilia syndrome, is an autosomal recessive ciliary microtubule associated disorder and characterized by triad of situs inversus, chronic sinusitis and bronchiectasis. The basic defect lies in the defective movement of cilia lining nose, paranasal sinuses, and bronchus. Eustachian tube and fallopian tube that cause interference of clearance of pathogens leading to recurrent infections of upper respiratory tract, otitis media and infertility. These patients are treated with colchicine drug.

Dysfunctional Leukocytes

Gout is caused by excessive uric acid in blood resulting from breakdown of purine. Defective metabolism of uric acid causes arthritis especially of smaller bones of the feet, due to deposition of uric acid crystals. Gout is characterized by sudden, severe episodes of acute pain, swelling and tenderness in the small joints, often at the base of the big toe resulting from dysfunctional leukocytes due to microtubule defects.

- Colchicine drug works by decreasing swelling and lessening the buildup of uric acid crystals that cause severe acute pain in the affected joints. Colchicine is a classical antimitotic drug which blocks mitotic cells in metaphase.

- Colchicine binds to tightly to ends of unpolymerized tubulin and forms a colchicine-tubulin complex resulting in inhibition of microtubule polymerization essential for cell mitosis.
- Colchicine may enhance activation, migration and phagocytic activity of neutrophils to the site of inflammation in response urate crystals.

Chédiak-Higashi Syndrome

Chédiak-Higashi syndrome is an autosomal recessive disorder caused by mutation in the 'LYST gene' on chromosome 1q42 that encodes a protein essential for assembly of microtubules in neutrophils.

- Defect in assembly of microtubules in neutrophils results in impaired chemotaxis, defective degranulation of neutrophils, impaired microbial killing, and recurrent *Staphylococcus aureus* bacterial infections forming soft tissue abscess with fatal outcome.
- Neutrophils contain giant granules due to aberrant organelles. Accelerated phase of the syndrome results in fatal outcome due to multiorgan failure in 85% of cases.

Intermediate Filaments

Intermediate filaments are composed of a variety of proteins. These are 8–10 nm diamtere filaments highly stable cytoskeletal component expressed in different types of cells. Examples of intermediate filaments are keratin, microfilaments, glial filaments, desmin and vimentin. Immunochemical stains utilizing monoclonal antibodies against individual intermediate filaments (e.g. desmin to identify muscle) are useful in identifying the origin of neoplasms.

- The most important function of intermediate filaments is to provide mechanical support for the plasma membrane where it comes in contact with other cells and extracellular matrix for maintenance of cell shape.
- Unlike microtubules and microfilaments, intermediate filaments do not participate in cell motility.
- Abnormalities of intermediate filaments are linked to Mallory hyaline formation in liver diseases, accumulation of α-synuclein in neurons in the substantia nigra in Parkinson disease, and other diseases like epidermolysis bullosa simplex (EBS) and desmin myopathy.

Mallory Hyaline (Mallory-Denk Body)

In histopathology, Mallory hyaline also known as Mallory-Denk body or Mallory body, is an irregular eosinophilic intracytoplasmic inclusion, that represents aggregates of intermediate filaments (cytokeratins 8 and 18). The cytokeratins form a filamentous support within the hepatocytes. Liver disorders associated with Mallory-Denk bodies in hepatocytes are given in Table 1.4.

Table 1.4 Liver disorders associated with Mallory-Denk bodies

Alcoholic liver disease
Biliary atresia
Extrahepatic bile duct obstruction
Primary biliary cirrhosis
Primary sclerosing cholangitis
α_1-Antitrypsin deficiency
Abetalipoproteinemia
Hepatic adenomatosis in glycogen storage disease type 1a (von Gierke disease)
Wilson disease
Ethanol-induced hepatocellular injury
Amiodarone-induced hepatocellular injury
Indian childhood cirrhosis
Nonalcoholic steatohepatitis
Hepatic adenoma
Hepatocellular carcinoma
Focal nodular hyperplasia of liver

Neuronal Intermediate Filaments Related Neurodegenerative Diseases

Neuronal intermediate filaments (NIFs) are most abundant cytoskeleton component in mature neurons. These are composed of different protein units encoded by separate genes such as neurofilament light chain (NFL), neurofilament medium chain (NFM), neurofilament heavy chain (NFH), α-internexin and peripherin.

- Neuronal intermediate filament proteins give cells shape, determine axonal caliber, which control signal conduction, and regulate the transport of synaptic vesicles and modulate synaptic plasticity by binding to neurotransmitter receptors.
- Mutations in genes encoding neuronal intermediate filaments result in their aggregation in neurons responsible for neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, Charcot-Marie-Tooth disease, dementia with Lewy bodies and giant axonal neuropathy.
  - **Parkinson disease:** α-Synuclein is normally wavy-like presynaptic neuronal protein. In Parkinson disease, α-synuclein protein misfolds and forms toxic aggregates, which damage neurons in the part of the brain called the substantia nigra. Normally,

neurons in this part of brain are responsible for production of a chemical called dopamine. Neuronal damage in this part of brain results in reduction of dopamine production. An antibody that binds the misfolded α-synuclein can be used to intercept the protein as it passes between neurons.

- **Alzheimer's disease:** It is a neurodegenerative disorder characterized by brain pathology of intracellular neurofibrillary tangles made of hyperphosphorylated R-protein and extracellular amyloid plaques present in cortical and limbic areas of human brain. It is characterized by memory loss and progressive neurocognitive dysfunction.
- **Amyotrophic lateral sclerosis (ALS):** It is also known as motor neuron disease or Lou Gehrig's disease affecting 50–60 years elderly persons. Peripherin is an intermediate filament protein detected in spheroids, a hallmark of amyotrophic lateral sclerosis. Increased levels of peripherin mRNA have been observed in some cases of amyotrophic lateral sclerosis. Disorder is characterized by death of neurons controlling voluntary muscles. Patient presents with muscle stiffness, muscle twitching and gradually worsening muscle weakness. Life expectancy is 2–4 years.
- **Charcot-Marie-Tooth disease type 2:** Neurofilaments (NFs) are the major intermediate filaments of mature neurons. Charcot-Marie-Tooth disease type 2 is a hereditary disorder involving motor and sensory neuropathies of the peripheral nervous system characterized by progressive skeletal muscle weakness and decreased touch sensation across various parts of the body. Recently, two mutations in the neurofilament light subunit have been detected in families affected by Charcot-Marie-Tooth disease type 2.
- **Dementia with Lewy bodies:** In dementia, Lewy bodies are intracytoplasmic inclusion bodies composed of the neurofilament (intermediate filament) protein, ubiquitin, α-synuclein and α-crystallin. Lewy bodies may occasionally be surrounded by neurofibrillary tangles.
- **Giant axonal neuropathy:** Giant axonal neuropathy (GAN) is an autosomal recessive slowly progressive neurodegenerative disorder caused by mutation in GAN gene that encodes gigaxonin, a member of the BTH/Kelch family of E3 ligase adaptor proteins. The disease is characterized by the aggregation of intermediate filaments of cytoskeleton. Patient presents with progressive motor and sensory peripheral neuropathy, central nervous system involvement (including cerebellar and pyramidal signs), and kinky hair in most cases.

Epidermolysis Bullosa Simplex

Epidermolysis bullosa simplex (EBS) is an inherited skin disorder caused by mutation in the keratin 5 (KRT5) and keratin 14 (KRT14). Genes with fragility of basal keratinocytes resulting in epidermal cytolysis and blistering produced with even a slight mechanical stress. Cells with severe mutations in KRT5 and KRT14 are more sensitive to osmotic stress and take longer time to recover from it.

Desmin Myopathy

Desmin myopathy is an autosomal dominant or autosomal recessive disorder caused by mutation in desmin or α-crystallin B. Patient presents with lower limb muscle weakness slowly spreading to involve truncal, neck-flexor, facial, bulbar and respiratory muscles. Histologic examination of tissue section shows abnormal fiber area containing amorphous eosinophilic deposits seen as granular or granulofilamentous material. Immunohistochemical examination demonstrates positive staining for desmin in each region containing abnormal structures.

MITOCHONDRIA

Mitochondria are cylindrical-shaped double membrane-bound organelle with inner part being folded inwards to form cristae and found in the cytoplasm of eukaryotic cells. Numerous mitochondria are found in human liver cells, with 1000–2000 mitochondria per cell, making up one fifth of the cell volume.

- Mitochondria utilize cytoplasmic proteins to degrade sugars and produce cellular energy in the form of ATP (i.e. phosphorylation of ADP) through cellular respiration for regulation of cellular metabolism. The set of reaction involved in ATP production are collectively known as the citric acid cycle, or the Krebs cycle.
- The Krebs cycle occurs in the mitochondrial matrix and generates chemical energy (ATP, NADH, and $FADH_2$) from the oxidation of pyruvate, the end product of glycolysis. When acetyl-CoA is oxidized to carbon dioxide in the Krebs cycle, chemical energy is released and captured in the form of ATP, NADH, and $FADH_2$.
- $FADH_2$ is high energy electron carrier used to transport electrons generation in glycolysis and Krebs cycle to the electron transport chain.

Mitochondrial Dysfunction

Mitochondrial dysfunction results in cell injury and apoptosis. Mitochondrial dysfunction has been indicated as a potential player of development of cardiac hypertrophy. Mitochondrial number is increased in hypertrophy and decreased in atrophy.

Mitochondrial Hypertrophy

Hypertrophy of mitochondria termed megamitochondria can be induced by a variety of processes. By electron microscopy, megamitochondria demonstrate normal cristae and matrix density; and associated with normal oxidative phosphorylation. It should be differentiated from swollen mitochondria, which have swollen cristae and irregular densities in the matrix; and associated with uncoupling of oxidative phosphorylation and reduction in ATP production induced by NSAIDs and tolcapone.

- Mild to severe hypertrophy of mitochondria is seen with disruption of mitochondrial β-oxidation of drug-induced hepatocellular injury. By electron microscopy, the amount of mitochondrial matrix increases and becomes electron lucent. By light microscopy, these changes may be observed as cytoplasmic vacuolation.
- Microvesicular steatosis, inflammation and necrosis occur in human beings as a feature of severe interference with mitochondrial β-oxidation of free fatty acid.
- Hypertrophy of mitochondria (megamitochondria) may be seen in alcohol liver disease, mitochondrial myopathies, certain nutritional diseases and benign tumors arising from oncocytes of salivary glands, thyroid, parathyroid and kidneys (known as oncocytes).

Mitochondrial Gene Mutations Associated Disorders

Mitochondrial disorders may be caused by inherited or acquired mutations in mitochondrial DNA or nuclear genes that code for mitochondrial components. Acquired mitochondrial dysfunction can be caused by drugs, infections and environmental factors. Mitochondrial gene mutations and associated disorders are given in Table 1.5.

- Mitochondrial diseases caused by mutations in mitochondrial genes include mitochondrial myopathies, cardiomyopathy, exercise intolerance, diabetes mellitus and deafness (DAD), Kearns-Sayre syndrome (KSS), Leigh syndrome, mitochondrial depletion syndrome (MDS), mitochondrial encephalopathy, subacute sclerosing encephalopathy, Leber's hereditary optic neuropathy (LHON); and neuropathy, ataxia, retinitis pigmentosa and ptosis (NARP).
- Even autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and Sjögren syndrome appear to have a mitochondrial basis. Mitochondrial diseases can occur at any age.
- Mitochondrial diseases can be diagnosed by biochemical tests on blood, urine and cerebrospinal fluid, skeletal muscle biopsy to examine the mitochondria and test enzyme levels, magnetic resonance imaging of the brain and spinal cord.
- Prognosis is variable in mitochondrial diseases. Minimally affected individuals live a normal life. Severely affected persons manifest with mitochondrial disease. The progression of mitochondrial disease is unpredictable and different for each individual.

Table 1.5 Mitochondrial gene mutations and associated disorders

Mitochondrial myopathies
Cardiomyopathy
Exercise intolerance, diabetes mellitus and deafness (DAD)
Kearns-Sayre syndrome (KSS)
Leigh syndrome
Mitochondrial depletion syndrome (MDS)
Mitochondrial encephalopathy
Subacute sclerosing encephalopathy
Leber's hereditary optic neuropathy (LHON)
Neuropathy, ataxia, retinitis pigmentosa and ptosis (ANRP)

LYSOSOMES

Lysosomes are sphere-shaped important membrane-enclosed cytoplasmic organelles, that contain numerous acid hydrolases (acid phosphatase, glucuronidase, sulfatase, ribonuclease, and collagenase). The pH in lysosomal lumen ranges between 4.5 and 5.0. Lysosomes degrade macromolecules (proteins, carbohydrates, some lipids) taken up into the cells by endocytosis (heterophagy). Major proteins present in lysosomes are given in Table 1.6. Lysosomal disorders are given in Table 1.7.

- Lysosomal proteases make up a great contribution to the overall process of intracellular degradation of most of proteins and carbohydrates. However, some lipids remain undigested. When examined under electron microscope, newly formed 'primary lysosomes' are more uniform in size, with an amorphous electron-opaque content. After fusion of lysosomes with phagocytic vesicles or other cell contents, these form 'secondary lysosomes', whose size and internal density is more variable. Lysosomes

Table 1.6 Major proteins present in lysosomes

Lysosomal Luminal Proteins
Acid hydrolases (enzymes) or their activators
Lysosomal Integral Proteins
▪ Structural proteins (amino acids and lipid transporters) ▪ Ion channels like calcium channels ▪ Trafficking and fusion machinery ▪ Vesicular ATPase which functions both as a proton pump and nutrient sensor
Lysosomal Associated Proteins

Table 1.7 Lysosomal disorders

Categories	Diseases
Sphingolipidosis	Niemann-Pick disease: types A, B and C, Gaucher's disease: types 1, 2 and 3, Fabry disease (classic and late-onset types), metachromatic leukodystrophy, globoid leukodystrophy, multiple sulfatase deficiency, GM1 gangliosidosis: types 1, 2, GM2 gangliosidosis: Tay-Sachs disease, and Sandhoff disease and GM2 activator deficiency
Mucopolysaccharidosis	Hurler syndrome, Scheie syndrome, Hunter syndrome, Sly syndrome, Morquio syndrome: types A, B, Sanfilippo syndrome: types A, B, C and D, and Maroteaux-Lamy syndrome
Oligosaccharidosis	Schindler disease, fucosidosis, α-mannosidosis and aspartylglucosaminuria
Neuronal ceroid lipofuscinosis	Neuronal ceroid lipofuscinosis 1 through neuronal ceroid lipofuscinosis 14 (lipofuscin pigment)
Sialic acid disorders	Galactosialidosis, infantile sialic acid storage disease, salla disease and sialuria
Mucolipidosis	Mucolipidosis I (sialidosis I and II), mucolipidosis II (I cell disease), mucolipidosis III (pseudo-Hurler polydystrophy), and mucolipidosis IV
Miscellaneous disease	Pompe disease (glycogen storage disease type 2), Dannon disease (glycogen storage disease), cystinosis and lysosomal acid lipase deficiency (infantile and childhood/adult types—accumulation of cholesterol esters, triglycerides)

containing indigestible material often remain in the cytoplasm as residual bodies (e.g. lipofuscin pigment in elderly persons).

- Lysosomes participate in removal of damaged cell organelles during cell injury and the cellular remodeling of differentiation, and atrophic cells due to nutrient deprivation. Chloroquine antimalarial drug raises the intracellular pH of the lysosomes, thus inactivating its enzymes reduces tissue damage in inflammatory reactions.
- Certain substances are not metabolized by cells resulting in abnormal accumulation of glycogen and phospholipids in lysosomes and impairment of functions. Deficiency of enzyme results in accumulation of endogenous substances and causes lysosomal storage diseases. Insoluble endogenous pigments such as lipofuscin and melanin accumulate in the cells. Exogenous particulate particles accumulate such as silica, carbon and asbestos in the lungs.

Pathology Pearls: Autophagy and Heterophagy

Autophagy

- Autophagy usually inhibits apoptosis; however, if uncontrolled, it can cause lysosomal digestion of the cell's own components. Autophagy participates in removal of damaged cell organelles during cell injury and the cellular remodeling of differentiation, and atrophic cells due to nutrient deprivation.
- Chloroquine antimalarial drug raises the intracellular pH of the lysosomes, thus inactivating cell enzymes reduces tissue damage in inflammatory reactions.
- Autophagy is categorized as macroautophagy, microautophagy (direct pinocytosis by the lysosomes) and chaperone-assisted autophagy.
- In macroautophagy, portions of cytosol and cell organelles are enveloped in a double-membrane autophagosome, which subsequently fuses with a lysosome to form a single-membrane bound autophagolysosome resulting in degradation of the cell's own cytoplasmic organelles by releasing proteolytic enzymes.

Heterophagy

- Heterophagy is the process of lysosomal degradation of materials ingested from the extracellular environment by the general process of endocytosis.
- Ingestion of particulate particles by the cells is known as phagocytosis, and uptake of soluble smaller macromolecules is called pinocytosis.
- Extracellular materials are endocytosed into phagosomes, which eventually fuse with lysosomes to form phagolysosomes, where the engulfed extracellular material is degraded.
- Heterophagy is the most common in the professional phagocytes, such as neutrophils and macrophages. Examples of heterophagy include the uptake and degradation of bacteria by neutrophils and the removal of apoptotic cells by macrophages.

ENDOPLASMIC RETICULUM

The smooth endoplasmic reticulum is a multifunctional membranous organelle found in most eukaryotic cells, which lacks ribosomes. It participates in lipid biosynthesis, steroid hormones synthesis and detoxification of harmful metabolic byproducts, protein folding and processing; and storage of calcium ions in the cell.

- Smooth endoplasmic reticulum is especially abundant in mammalian liver and gonad cells. When cells exposed to chemical agents, the endoplasmic reticulum shows hypertrophy as an adaptive response.
- Proper functioning of endoplasmic reticulum is disturbed by a number of physiologic, pathologic conditions and pharmacologic agents resulting in impairment of protein folding and risk of proteotoxicity.

- Smooth endoplasmic reticulum stress is triggered by intracellular alterations (e.g. calcium or redox imbalances), microenvironmental conditions (e.g. hypoxia, hypoglycemia, and acidosis), high sugar-diet, high-fat diet, natural compounds (e.g. tunicamycin and geldanamycin), and several drugs (e.g. bortezomib (Velcade®), Celebrex and nelfinavir). The cell reacts to smooth endoplasmic reticulum stress by initiating a defensive cellular mechanism, known as the unfolded protein response (UPR) aimed at cellular adaptations and safeguarding cellular survival.
- A malfunction of the endoplasmic reticulum stress response induced by aging, genetic mutations, or environmental factors can result in various diseases such as diabetes mellitus type 2, obesity, inflammation, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, atherosclerosis and nonalcoholic fatty liver. Obesity also induces endoplasmic reticulum stress. Hyperglycemia and free fatty acids disrupt endoplasmic reticulum homeostasis and induce pancreatic β cell death through C/EBP homologous protein, which has a central role in apoptotic execution pathways triggered by endoplasmic reticulum stress.
- Endoplasmic stress triggers a protective cellular mechanism, which includes the unfolded protein response (UPR). When activation of the UPR is severe and prolonged, the final cellular outcome is pathologic apoptotic cell death. Stress that impairs endoplasmic reticulum homeostasis leads to accumulation of unfolded or misfolded proteins such as amyloid β (Aβ) peptide, the major component of amyloid plaques in Alzheimer's disease.
- Endoplasmic reticulum stress has been implicated in the pathogenesis of Parkinson's disease. Accumulation of a substrate of Parkin in the endoplasmic reticulum activates endoplasmic reticulum stress. Human ubiquitin ligase HRD1 is associated with protection against endoplasmic reticulum stress. Human ubiquitin ligase HRD1 also prevents apoptosis of neurons.
- Dysfunction in protein handling in endoplasmic reticulum has been implicated in the pathogenesis of amyotrophic lateral sclerosis. Mutation in Cu/Zn-superoxide (SOD1) induces endoplasmic reticulum and motor neuron death resulting in amyotrophic lateral sclerosis (ALS). Exact mechanism of SOD1 induced death is unknown.
- Prolonged endoplasmic reticulum stress has been implicated in the pathogenesis and progression of atherosclerosis. Oxidized phospholipids, homocysteinemia and cholesterol loading induce death of macrophages, vascular smooth muscle cells and endothelial cells via CHOP.
- Disruption of endoplasmic reticulum homeostasis, often termed endoplasmic reticulum has been observed in liver and adipose tissue with nonalcoholic fatty liver disease and/or obesity. Endoplasmic reticulum contributes to hepatic SREBP-Ic activation and lipid accumulation in fructose-evoked nonalcoholic fatty liver disease (NAFLD).

INTRODUCTION TO PATHOLOGY

A disease is an alteration from the normal function/structure of an organ or system, which manifests as a characteristic of signs and symptoms. Disease can be congenital (genetic or nongenetic) or acquired. Pathology emphasized three aspects of disease process: (a) mechanism of development of disease (pathogenesis), (b) the alterations of structure and forms (morphology), and (c) functional alterations (pathophysiology).

- Etiopathogenesis refers to study the causes such as genetic defects, environmental including iatrogenic or idiopathic.
- Pathogenesis is defined as the mechanism that leads to a diseased state by progression of processes of cellular lineage, maturation, migration, and eventual morphogenesis of both individual cells and their architecture in formation of a tissue or organ.
- Pathophysiology refers to the study of functional changes in body functions that are the causes, consequences or concomitants of disease processes.
- Most important aspects in clinical practice of medicine and surgery are diagnosis, prognosis, treatment and prophylaxis.

PATHOLOGY

Pathology is the scientific study of disease, that identifies etiology, mechanism and functional changes in cells, tissues and organs that underlie disease. Pathology lays a strong foundation of every aspect of patient care from diagnostic testing and treatment. Alterations at the molecular level and cellular level correlate with the clinical manifestations of the disease. Understanding the processes of the disease helps in the accurate recognition, diagnosis (biochemical investigations, hematologic tests, surgical pathology and immunohistochemistry) and treatment of diseases.

Pathology Divisions

Pathology is divided into following clinical discipline: general pathology, systemic pathology, chemical pathology and forensic pathology. There are three main subtypes of pathology: anatomic pathology, clinical pathology, and molecular pathology.

General Pathology

General pathology is the study of the reactions of cells or tissues to injury with a focus on the mechanisms of that response, which involves all aspects of pathology. It deals with clinical history, examination of patient, diagnosis and management of the disease by use of laboratory medicine and diagnostic techniques.

Systemic Pathology

Systemic pathology is the pathology of systems in the human body. Anatomic pathology is the study of tissues, organs and tumors. Systemic pathology is not a separate discipline from general pathology, but a different approach at the level of the tissue or organ or even the entire body. Pathologists are specialists in the disciple of pathology. Although general pathology and systemic pathology are educationally useful divisions of pathology discipline. Pathologists can be specialists in a particular organ system.

Histopathology

Histopathology is the study of histologic abnormalities of diseased cells and tissues under a microscope, which enables pathologists to look for changes in cells or tissues that explain the actual cause of the patient's illness. Pathologists are able to reach a diagnosis by examining a biopsy (punch, wedge, excision) from various organs. Histopathology is essential as it broadens and progresses treatment options.

Cytopathology

Cytopathology is the study of cellular changes related to cells and mainly used to diagnose or screen for cancer. Cell samples can be obtained during routine diagnostic procedures, such as bronchoscopy and cystoscopy or fine needle aspiration from specific body's sites for diagnosis. Cytopathology is also used to screen for fetal abnormalities and Papanicolaou smears are prepared from cells taken from the cervix to diagnose cervical cancer and infectious organisms. Two methods can be used in a Papanicolaou test: conventional and automated liquid tests.

Immunopathology

Immunopathology is a branch and manifestation of conditions concerned with immune responses associated with the production of diseases through the analysis of humoral and cellular immune function. There is a great deal of synergy between the adaptive immune system and its innate immune system, and defects in either of immune system can provoke disease such as inappropriate inflammation, autoimmune diseases, immunodeficiency diseases and hypersensitivity reactions.

Molecular Pathology

Molecular pathology is the study of disease at the molecular level by testing deoxyribose nucleic acid (DNA), ribonucleic acid (RNA) and proteins found in tissues, organs, and even body fluids within a clinical context. The applications of molecular diagnostics span a range of human disorders including hereditary, neoplastic and infectious diseases. Molecular-based *in vitro* biological assays such as polymerase chain reaction enzyme-linked immunosorbent assay (PCR-ELISA) or fluorescence *in situ* hybridization (FISH) are used to detect a molecule, often in low concentrations, that is a marker of disease or risk in a sample from a patient.

Hematology

Hematology is a branch of internal medicine in relation to health and disease, that deals with the pathophysiology, diagnosis, treatment, prognosis and prevention of blood-related disorders such as anemias, leukemias, lymphoma, bleeding and coagulation disorders. Hematology tests are performed on the blood, blood proteins and hematopoietic organs. Full blood count is a routine test that evaluates three major components found in the blood: red blood cells, white blood cells and platelets.

Chemical Pathology

Chemical pathology is the study of biochemical abnormalities associated with disease, which involves biochemical investigations of body fluids such as blood, urine and cerebrospinal fluid.

Forensic Pathology

Forensic pathology refers to perform autopsies and legal pathology tests. Autopsy room sessions can provide students with an excellent opportunity to correlate the gross and histopathologic features with the natural history of the disease.

Basis of Pathology Study

Understanding the processes of the disease help in the accurate recognition, diagnosis (biochemical investigations, hematological tests, surgical pathology and immunohistochemistry) and treatment of diseases.

Etiology

The word etiology refers to the scientific study of disease process, which generally falls into three main categories: intrinsic, extrinsic and idiopathic (unknown cause).

- Pathologic change that has occurred from inside the body as a result of intrinsic factors such as inherited, metabolic, neoplastic and immune system disorders. Hemophilia is an example of inherited disorder that

leads to excessive bleeding. Diabetes mellitus is a metabolic and endocrine disorder that causes high blood sugar.

- Pathologic change that has occurred from outside as a result of extrinsic factors include: (a) infectious agents such as bacteria, viruses, fungi and parasites, (b) animal bites or stings, (c) physical trauma, chemical agents, electricity burns and radiation; and (d) iatrogenic causes resulting from medical professional's actions or within a medical setting, and (e) idiopathic of unknown cause.
- Reactive oxygen species (ROS) is a group of extremely reactive peroxides and oxygen-containing radicals that may contribute to cellular damage.
- Autophagy refers lysosomal breakdown of a cell's own components. Autolysis refers to breakdown of cells by their own enzymatic action.

Risk Factors

A risk factor confers an increased risk of developing a disease. For example, tobacco smoking is a risk factor for lung cancer and obesity is a risk factor for heart disease.

- Types of risk factors include tobacco smoking, alcoholism, nutritional, physical inactivity, prolonged exposure to sunlight, not having certain vaccination and unprotected sexual activity leading to sexual transmitted diseases.
- The risk factors of chronic diseases are modifiable for men and women such as unhealthy diet, physical inactivity and tobacco use. These risk factors are expressed through the intermediate risk factors of raised blood pressure, raised blood glucose levels, abnormal lipids, overweight and obesity.
- Chronic diseases are the major causes of mortality and disability worldwide, which include heart disease (coronary artery disease, ischemic heart disease), cerebral stroke, chronic respiratory diseases (chronic obstructive pulmonary disease, bronchial asthma), bone and joint disorders, genetic orders and neoplastic disorders.

Predisposition

Predisposition is a term applied for patients having an increased susceptibility to develop a disease. A genetic predisposition is an increased likelihood of developing a genetic disorder. For example, familial adenomatous polyposis (FAP) patients have a mutated APC gene associated risk of developing colorectal carcinoma resulting from succession of mutations in one or more polyps. Other examples of genetic disorders include Down syndrome, thalassemia, cystic fibrosis and sickle cell disease.

Pathogenesis

Pathogenesis is the pathologic mechanism which results in clinically evident disease. For example, the way in which the interaction between *Mycobacterium tuberculosis* and the host immune system produces the caseating epithelioid granulomatous lesion in tuberculosis.

- Atherosclerosis is a chronic inflammatory disease, which begins with fatty streak due to accumulation of lipid laden foam cells in the intimal layer of large elastic and medium-sized arteries and then progresses to formation of atheromatous plaque.
- The atheromatous plaque has cellular component (e.g. smooth muscle cells and inflammatory cell), a fibrous component composed of connective tissue, and central core fat component of lipids. Major risk factors of atheromatous plaque are hypertension, diabetes mellitus, dyslipidemia, tobacco smoking, obesity, sedentary lifestyle and family history.

Morphologic Changes in Cells and Tissues

Morphologic changes are characterized by structural alterations in the cells and tissues and altered cellular functions such as cell–pathogen interaction, tumor formation and stem cell differentiation.

- Cellular adaptation is the ability of cells to respond to various types of stimuli and adverse environmental changes. If cells are unable to sustain to the adverse injurious insults, cell undergoes apoptosis or cell death in the form of necrosis.
- Morphology is a branch of life science dealing with the study of gross structure of diseased tissue and microscopic examination of tissue.

Functional Derangements and Clinical Manifestations

External and internal factors adversely affecting cell, tissue, organ or person cause structural and functional changes and cellular adaptation and response from cellular to whole person level resulting in symptoms and signs.

- For example, *Streptococcus pneumoniae* causes acute inflammatory response (consolidation is structural change in lung parenchyma that becomes solid, reduced exchange of gases in alveoli is functional change) resulting in symptoms (cough, breathlessness, hemoptysis) and signs such as reduced chest movements, dull percussion notes and radiologic changes.
- Injury to the cells and extracellular matrix results in tissue and organ injury, which determines the morphologic and clinical patterns of the disease.

Diagnosis

Understanding the processes of the disease help in the accurate recognition, diagnosis (biochemical investigations, hematological tests, surgical pathology and immunohistochemistry) and treatment of diseases.

CELLULAR RESPONSE TO STRESS AND HARMFUL STIMULI

Normal cells are the structural and functional units of tissues, which remain in a state of homeostasis with the extracellular fluid and respond to changes in their environment. The cells are capable of adjusting their structure and functions in response to various physiologic and pathologic mild to severe stimuli throughout life. Genetic or acquired metabolic defects and chronic cell injury cause intracellular accumulation of glycogen, proteins and pigments; and pathologic dystrophic and metastatic calcification. Cellular response to injurious stimuli is given in Table 1.8. Cellular response to injurious stimuli is shown in Fig. 1.1.

- Altered physiologic stimuli and some nonlethal stimuli produce cellular adaptations: atrophy, hypertrophy, hyperplasia and metaplasia. Intracellular accumulations occur due to altered metabolism.
- Cellular stress beyond the level of adaptive response results in cell injury. Cellular injury occurs when a stress exceeds the cells ability to adapt due to altered physiologic stimuli, reduced oxygen supply, extremes of temperature, electrical injury, radiation, biologic agents, and nutritional deficiency, metabolic alteration and cumulative aging.
- Cell injury depends on injurious stimuli: (a) type, duration and severity of injury, (b) ability of the tissues to regenerate (e.g. labile or dividing cells/stable cells/permanent cells), (c) metabolic needs of cell, (d) adaptability of cell, and (e) genetic constitution. Neurons are highly susceptible to ischemic injury; whereas skeletal muscle is relatively more resistant to ischemic injury.
- Depending on severity of cell injury, various cellular changes occur: (a) subcellular alterations (intracellular accumulation of biomolecules and calcium), (b) reversible cell injury, (c) irreversible cell injury

Table 1.8 Cellular response to injurious stimuli

Cellular Adaptations
▪ Hyperplasia ▪ Atrophy ▪ Hypertrophy ▪ Metaplasia
Cell Injury
▪ Reversible cell injury ▪ Irreversible cell injury (necrosis and apoptosis)
Intracellular Accumulations
▪ Normal endogenous substances (e.g. fatty liver and protein droplets in renal tubules) ▪ Normal endogenous substances due to enzyme defects (e.g. glycogen storage diseases and lipid storage disorders) ▪ Mutated gene products (misfolded proteins) ▪ Abnormal exogenous products (e.g. silicosis and anthracosis)
Subcellular Changes
▪ Lysosomal catabolism (heterophagy or autophagy) ▪ endoplasmic reticulum hypertrophy (induction of drug tolerance to barbiturates and alcohol) ▪ Mitochondrial defects (number, size and shape) ▪ Cytoskeleton defects (phagocytosis and locomotion) ▪ Membranes (plasma membrane and subcellular membrane such as endoplasmic reticulum) ▪ Nucleus (pyknosis, karyorrhexis and karyolysis)

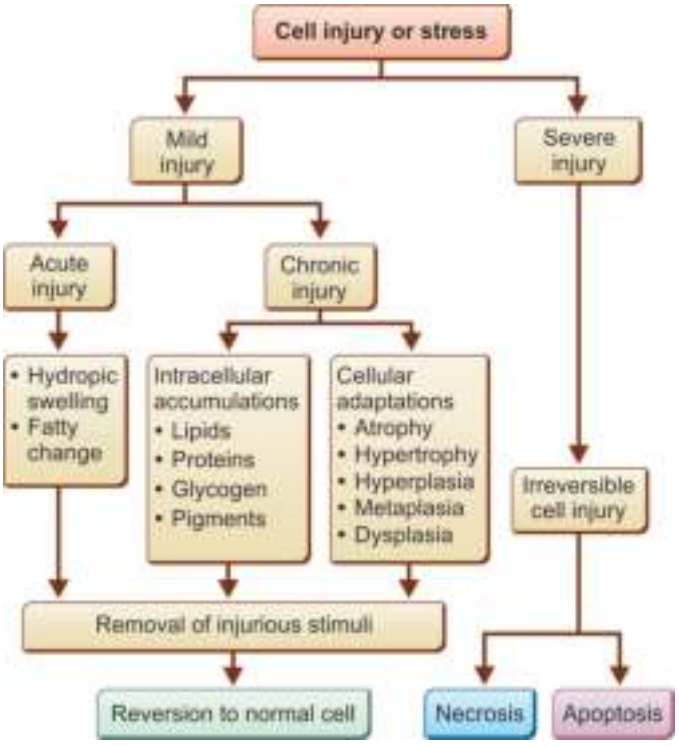


Fig. 1.1: Cellular response to injurious stimuli. Cellular adaptations, reversible injury and cell death may be stages of progressive impairment following different types of insults depending on type and duration of injurious stimuli. Acute cell mild injury can cause hydropic change and fatty change. Chronic cell injury can cause intracellular accumulation of biomolecules and cellular adaptations. Severe cell injury can cause necrosis and apoptosis.

and (d) ischemic/reperfusion injury. Initially, cellular injury may be reversible, but prolonged or severe stress leads to irreversible cell injury (necrosis or apoptosis). Reversible cell injury is the ability to heal without permanent damage.

- Ultrastructural signs in both reversible and irreversible cell injury include: (a) cellular swelling occurs due to diminished activity of the sodium/potassium pump in the cell membrane causing influx of sodium along with water and efflux of potassium, (b) mitochondrial swelling results in reduced adenosine triphosphate (ATP), aerobic respiration (oxidative phosphorylation), (c) dilatation and degranulation of rough endoplasmic reticulum results in cessation of protein synthesis, and (d) autophagocytosis occurs due to ingestion of damaged cell organelles by the lysosomes.

CELLULAR ADAPTATIONS

Cellular adaptations are usually reversible changes that result in increased, decreased, or altered functions of cells, tissues and organs, which can be physiologic or pathologic and have many causes. Cellular adaptations encompass several processes: (a) hyperplasia, (b) hypertrophy, (c) atrophy, (d) metaplasia, and (e) dysplasia. Cellular adaptations are shown in Fig. 1.2.

- **Hyperplasia:** Hyperplasia is the enlargement of the tissue/organ caused by an increase in the reproduction rate of its cells. Physiologic hyperplasia occurs in normal cells: (a) hormones-driven increase in the thickness of endometrium during menstrual cycle, lactating breast and uterus during pregnancy, (b) tissue damage-driven proliferation of connective tissue cells in wound healing, and (c) hepatocellular regeneration after partial resection. Pathologic hyperplasia is linked to cancer in endometrium and breast.
- **Hypertrophy:** Hypertrophy is defined as an increase in the size of the cell due to the synthesis of structural components and expression of embryonic genes triggered by mechanical and trophic factors (growth factors and stretch receptors). Physiologic hypertrophy occurs due to increased functional demands placed on the cell (e.g. skeletal muscle hypertrophy from weight lifting). Pathologic hypertrophy occurs when the limit of cardiac hypertrophy is exceeded and the cells can longer compensate for the increased burden (e.g. congestive heart failure and systemic hypertension).
- **Atrophy:** Atrophy refers to reduction in size of cell and tissues. Physiologic atrophy of skeletal muscles occurs in bed-ridden persons. Pathologic atrophy occurs due to loss of stimulus to a specific region due to diminished blood supply, loss of hormone stimulation, and neurogenic diseases (poliomyelitis, Guillain-Barré syndrome, Charcot-Marie-Tooth disease, amyotrophic lateral sclerosis).
- **Metaplasia:** Metaplasia is the conversion of one adult tissue type into another in same tissue. It is an adaptive response to a hostile environment that increase risk of development lung carcinoma in tobacco smokers (glandular to squamous epithelium conversion in respiratory mucosa) and esophageal adenocarcinoma in 'Barrett's esophagus' (squamous to glandular epithelium conversion).
- **Dysplasia:** Dysplasia is used to describe acquired disorderly growth and maturation of cells that are abnormal but not obviously malignant under a light microscope. This type of preneoplastic condition is

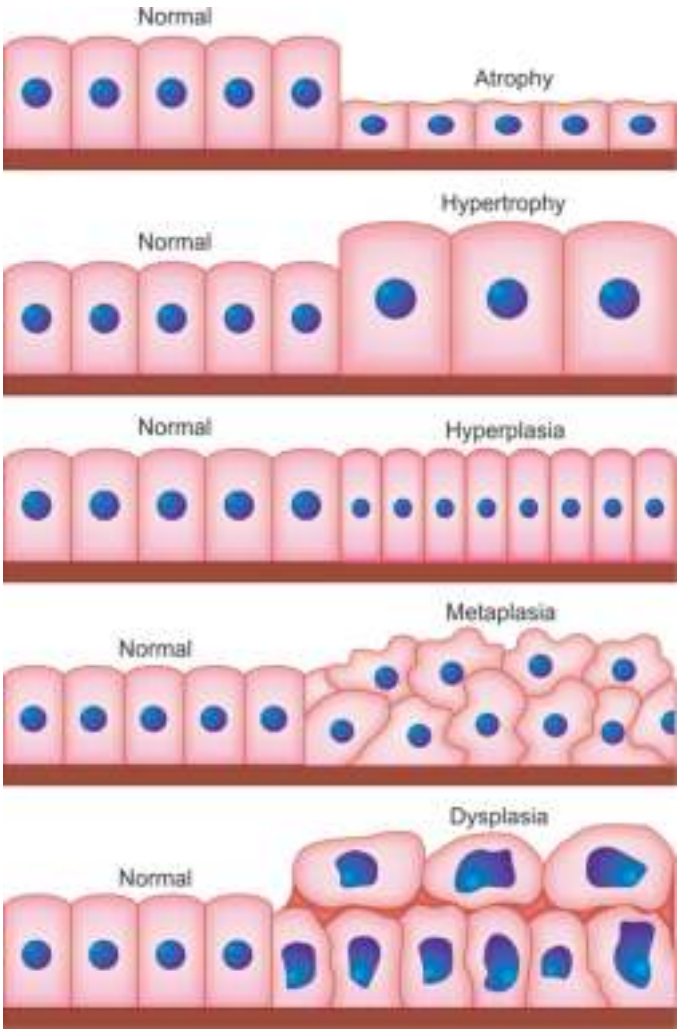


Fig. 1.2: Cellular adaptations. In cell biology and pathophysiology, cellular adaptation refers to alteration made by a cell in response to adverse or varying environmental changes. Cellular adaptations may be physiologic or pathologic (atrophy, hypertrophy, hyperplasia, metaplasia and dysplasia).

a precursor of invasive neoplasia. High-risk human papillomavirus (HPV 16, 18) is linked to cervical intraepithelial neoplasia (CIN) that may progress to invasive cervical carcinoma.

CELL INJURY

Cell can be injured by a variety of injurious stimuli such as hypoxia (oxygen deficiency), ischemia (lack of blood flow), physical or mechanical injury, chemical injury, radiation, biological injury (viruses, pathogens), and nutritional injury. Depending on the type of injurious agent and severity, cell injury may be reversible and irreversible process.

- **Mild and transient sublethal cell injury:** Mild and transient sublethal cell injury results in reversible injury, which can be repaired and cell returns to normal function by removal of damaged cell components by autophagy. In sublethal injury, cell shows swelling of endoplasmic reticulum and some mitochondria; and loss of ribosomes.
- **Severe progressive lethal cell injury:** Severe progressive lethal cell injury is irreversible, that results in cell death by necrosis or apoptosis. In lethal cell necrosis, initial cellular changes are loss of nucleolus, loss of ribosomes, swelling of all mitochondria and swelling of endoplasmic reticulum. Later cellular changes are nuclear condensation, plasma membrane blebs and holes, lysosomal rupture and fragmentation of all inner membranes of cell organelles and degradation of nucleus (pyknosis, karyorrhexis and karyolysis).

REVERSIBLE CELL INJURY

Reversible cell injury is acute sublethal injury with a hallmark of morphologic changes in cytoplasmic organelles sparing nucleus. These morphologic changes in reversible cell injury can be reversed if injurious noxious stimulus is removed.

- One global response in reversible cell injury is cellular swelling, that is a result of misfunctioning ion channels ($Na^+/K^+/Cl^-/Ca^{++}$ pumps) in the plasma membrane. Another global response in reversible cell injury is fatty change (large lipid vacuoles in cytoplasm) in the organs. Reversible cell injury occurs in the metabolically active cells of liver, heart and kidneys. Examples of reversible cell injury are hydropic change and fatty change in organs.
- In reversible cell injury, cell swelling occurs as a result of misfunctioning ion channels ($Na^+/K^+/Cl^-/Ca^{++}$ pumps) in the plasma membrane. Subcellular changes in the reversible injured cells include endoplasmic reticulum cisternae swelling, mitochondrial swelling, plasma membrane blebbing due to disordered cytoskeleton, segregation of fibrillary and granular components of nucleolus in the nucleus and intracellular myelin figures. Mitochondrial swelling results in reduced oxidative phosphorylation, downregulation of adenosine triphosphate (ATP), and decreased glycogen.
- Plasma membrane blebbing occurs due to disordered cytoskeleton. Myelin figures are derived from damaged membranes of mitochondria, or rough endoplasmic reticulum and the plasma membrane; demonstrated in cytoplasm on electron microscopy.

IRREVERSIBLE CELL INJURY

Severe and progressive cell injury results in irreversible cell injury (necrosis) induced by prolonged ischemia, viral infection, chemical agents and immunologic response. Following severe cell injury, cell and its organelles start to disintegrate resulting in rupture of the cells and inducing acute inflammatory response. On the other hand, apoptosis is a process of programmed cell death that occurs in multicellular organisms. Apoptosis can be triggered through either intrinsic pathway or extrinsic pathway.

Necrosis

Necrosis is fundamentally a cytoplasmic driven uncontrolled process in response to prolonged or severe injurious stimuli resulting in damage to plasma membrane and membrane of organelles resulting in depletion of ATP, increased acidosis, leakage of lysosomal hydrolytic enzymes in the cellular environment, protein digestion, loss of basophilia, increased cytoplasmic eosinophilia and nuclear changes (pyknosis, karyorrhexis, karyolysis due to endonucleases), calcification, release of cellular breakdown products and cytokines incites an inflammatory reaction.

- Morphologic patterns of necrosis are coagulative necrosis, liquefactive necrosis, gangrenous necrosis, caseous necrosis, fat necrosis and fibrinoid necrosis.
- The tissue in coagulative necrosis has firm texture, maintenance of general tissue architecture, ghost outlines of necrotic cells lacking nuclei for weeks before undergoing phagocytosis.
- In liquefactive necrosis, normal tissue architecture is rapidly transformed into a liquid mass due to autolysis and heterolysis leading to liquefactive necrosis in brain and pyogenic lung abscess with suppurative (neutrophil-rich) inflammation.
- In fat necrosis, fat degradation with possible saponification occurs most commonly due to release of enzymes from pancreas.

- Fat necrosis occurs due to physical trauma to the female breasts, radiation to a particular area of tissue, history of surgery to a particular area and history of removal of breast implants.
- Caseous necrosis occurs in tuberculosis and certain fungal infections.

Apoptosis

Apoptosis is a process of programmed cell death mediated by caspases. Apoptosis features nuclear shrinkage, DNA fragmentation by endonucleases, cytoskeleton breakdown by proteases, membrane blebbing; and membrane-bound apoptotic bodies, which are phagocytosed by macrophages and adjacent cells without generating inflammatory response.

- Apoptosis is an essential physiologic mechanism triggered by normal healthy processes in the body to regulate embryonic development, cell differentiation and tissue turnover. Apoptosis of embryonic cells in the limb beds results in formation of fingers and toes. Hormone-induced apoptosis of endometrial cells occurs at the end of menstrual cycle. In pathologic state, apoptosis can be triggered by radiation, chemotherapeutic agents and cell deprived of growth factors. Hepatitis virus-induced hepatocellular injury results in formation of acidophilic Mallory bodies.
- Primary pathways of apoptosis include: extrinsic (death receptor) pathway, intrinsic (mitochondrial) pathway and CD8+ cytotoxic T cell/NK cell's granzyme B/perforin-mediated pathway.
  - **Intrinsic (mitochondrial) pathway:** Intrinsic pathway of apoptosis is induced by chemotherapy and/or radiotherapy. Intrinsic pathway works by increasing mitochondrial permeability and releasing cytochrome c into cytoplasm, which combines with APAF-1 (apoptosis activating factor 1) to activate caspases, which cleave DNA into fragments, if analyzed on a gel electrophoresis will form a **'ladder pattern'**. In contrast, in necrosis, DNA breakdown will form smear pattern.
  - **Extrinsic (death receptor) pathway:** Extrinsic pathway of apoptosis is initiated through the stimulation of the transmembrane death receptors, such as the Fas receptors located on the cell membrane. In Fas-Fas ligand binding, the Fas ligand binds to a member of tumor necrosis factor family known as the Fas receptor. The activated Fas receptor in turn activates FADD (Fas-associated death domain), which in turn activates caspases.
  - **CD8+ cytotoxic T cell/NK cell's granzyme B/perforin-mediated pathway:** CD8+ cytotoxic T cell/NK cell's granzyme B/perforin-mediated pathway induces apoptosis releasing two types of preformed cytotoxic proteins such as granzyme B and perforin. Granzyme B induces apoptosis of target cell, and the pore forming perforin protein, which punches holes in the target cell membrane through which the granzyme B can enter the target cell to induce apoptosis.
- Apoptosis has two phases: (a) initiation phase (activation of caspases) and (b) execution phase (caspases induced cell death). Apoptosis can be triggered through either intracellular (mitochondrial) pathway or extracellular pathway. Both pathways share similar endpoints, culminating with the use of caspases and prevention of inflammatory reaction.
- Steps of apoptosis are cell shrinkage, chromatin condensation and DNA fragmentation by endonuclease, formation of apoptotic bodies and phagocytosis by macrophages and surrounding epithelial cells.
  - Examples of physiologic apoptosis include: (a) programmed death of cells in the limb buds with formation of fingers and toes, (b) predetermined death of cells on the surface of the intestinal mucosa and (c) hormone-induced death of endometrial cells at the endo of menstrual cycle.
  - Examples of pathologic apoptosis include: (a) hepatitis virus-induced death of hepatocytes (acidophilic bodies), (b) corticosteroid-induced atrophy of the neonatal thymus, (c) immune-mediated injury-related skin keratinocytes (Civatte bodies).

Pathology Pearls: Necrotic Cell versus Apoptotic Cell

- Necrotic cells release danger-associated molecular patterns (DAMPs), whereas apoptotic cells lack release of DAMPs.
- Injurious stimuli that induce necrosis (severe cellular damage), which results in rapid cell rupture with consequent release of intracellular DAMPs. DAMPs then cells of the immune system and can promote inflammation.
- On the other hand, injurious stimuli that initiate apoptosis are typically physiologic and relatively mild in nature, apoptotic cells do not rupture and their removal is coordinated by macrophage and other cells the innate immune system, before release of DAMPs can occur. For this reason, apoptosis process is not typically associated with activation of the immune system.

METABOLIC DERANGEMENTS

Stresses of different may induce changes in cells and tissues other than cellular adaptations, cell injury and cell death. Metabolic derangements in cells and sublethal chronic injury may be associated with intracellular accumulation of biochemical molecules such as carbohydrates, lipids and proteins as a result of derangements in cell metabolism because of defect in enzymatic mechanism and transport of biomolecules.

INTRACELLULAR ACCUMULATIONS

Intracellular accumulation occurs by two mechanisms: (a) it occurs due to overproduction of normal endogenous substance at increased rate and inadequate metabolism (fatty changes in liver), and (b) it occurs due to accumulation of normal endogenous substance and inadequate metabolism as a result of lack of enzyme that blocks metabolic pathway. Three categories of intracellular accumulations are given in Table 1.9.

Lipid Accumulation

Lipid accumulates in the liver in alcoholic patients and obese persons. Excessive fat accumulation in various organs such as liver, heart or the kidneys can occur due to toxins, malnutrition, diabetes mellitus, obesity, anorexia and alcohol consumption. Cholesterol accumulates within atheromatous plaque.

Table 1.9 Three categories of intracellular accumulations

Accumulation of Constituents of Normal Cell Metabolism
▪ Lipids ▪ Proteins ▪ Carbohydrates
Accumulation of Abnormal Substance of Cell Metabolism
▪ Storage disease ▪ Inborn error of metabolism
Accumulation of Pigments
▪ Endogenous pigments ▪ Exogenous pigments

- Gross examination of fatty liver reveals enlarged, bright yellow and soft greasy. Calcium is often deposited at the sites of cell death. Calcification is buildup of calcium in the injured tissues such as soft issues, arteries and other regions.
- Light microscopy shows lipid vacuoles in the cytoplasm displacing the nucleus to the periphery of the cell. Rarely, lipid laden cell rupture and enclosed lipid globules coalesce and form fatty cyst.

Glycogen Accumulation

Glycogen accumulates in the renal tubular cells in diabetic patients, liver and skeletal muscles in inborn errors of metabolism caused by enzymatic defect in synthesis and breakdown of glycogen.

Protein Accumulation

Protein accumulates as protein droplets in the proximal renal tubules in renal disease with heavy protein leakage across the glomerular basement membrane.

Pigments

Pigment that can accumulate in various cells include lipofuscin (wear and tear pigment, a product of lipid peroxidation and deposited in lysosomes of organs in elderly persons), anthracotic pigment in alveolar macrophages, melanin (found in melanocytes and melanomas), hemosiderin (iron-rich brown pigment derived from hemolyzed red blood cells). Abnormal endogenous substances accumulate due to alteration in protein folding and transport.

CELLULAR ADAPTATIONS

Cells are the structural and functional units of tissues. **Hemostasis** is the state of internal equilibrium at which normal physiologic demands of a cell are met. Pathologic state results when injurious stimuli sufficiently disrupt homeostasis. Cellular response to injury depends on the type, duration and severity of injury.

- Some diseases represent spontaneous alterations in the ability of a cell to proliferate and function normally, and in other cases, disease occurs when an external injurious stimulus induces alterations in the cell's environment that make it impossible for the cell to maintain new steady state 'homeostasis'. In such situations, cells must adapt and become compatible with their viability in the new environment.
- Cellular adaptations include hyperplasia (increased number of cells in an organ or tissue), hypertrophy (increased number of cells in an organ or tissue), atrophy (shrinkage of cells in an organ or tissue), and metaplasia (transformation or replacement of one adult cell type with another), which can be physiologic or pathologic, depending on whether the stimulus is normal or abnormal.
- A cell can adapt to certain point, but the stimulus continues beyond this point resulting in failure of the cell and hence the organ. If the cells are unable to adapt to the pathologic injurious stimulus, the cells can die. This chapter will discuss cellular adaptation, cell injury, cellular accumulations and cellular aging.
- Mild and transient cell injury results in reversible injury. Severe and progressive cell injury leads to irreversible injury (e.g. necrosis or apoptosis). Genetic or acquired metabolic defects and chronic injury cause intracellular accumulation of glycogen,

proteins and pigments; and pathological calcification (e.g. dystrophic and metastatic types). Cumulative sublethal injury causes cellular aging.

- In cellular biology, labile cells multiply constantly throughout life. Labile cells are alive for only a short period of time. Due to this, labile cells can end up reproducing new stem cells and replace functional cells.
  - Labile cells include bone marrow epidermis, epithelial lining of gastrointestinal tract, bronchi and vagina; and epithelial lining of excretory ducts (salivary glands, pancreas, and biliary tract).
  - Stable cells multiply only when needed. These cells spend most of the time in the quiescent G0 phase of the cell cycle, but can be stimulated to enter the cell cycle when needed. Stable cells include hepatocytes, proximal tubules of kidney, endocrine glands, fibroblasts, vascular endothelium, cartilage and bone.
  - Permanent cells are incapable of regeneration and considered to be terminally differentiated and nonproliferative in postnatal life. Examples of permanent cells are brain cells, neurons, cardiac myocytes, skeletal muscle, renal glomeruli and red blood cells. Cells based on proliferative activity in the context of the cell cycle are given in Table 1.10.

HYPERPLASIA

Hyperplasia is defined as an increase in number of cells as an adaptive response to stress, usually resulting in increased volume of an organ or tissue. Cells must be capable of mitotic division. Hyperplasia involves the production of new cells from stem cells. Hyperplasia can only occur in tissues containing labile or stable cells. Common cause of hyperplasia is growth factor-driven proliferation of mature cells in tissues having lot of stem cells. Only cells that can divide will undergo hyperplasia. It is worth mentioning that hyperplasia of the terminal differentiated cells such as myocytes in the heart and neurons in the brain does not occur. Schematic representation of hyperplasia is shown in Fig. 1.3. Physiologic, pathologic and compensatory hyperplasias are shown in Table 1.11.

Table 1.10 Cells based on proliferative activity in the context of the cell cycle

Labile Cells	
▪ Bone marrow	
▪ Epidermis	
▪ Epithelium lining (gastrointestinal tract, bronchi and vagina)	
▪ Epithelium lining excretory ducts (salivary glands, pancreas, and biliary tract)	
Stable Cells	
▪ Hepatocytes	▪ Vascular endothelium
▪ Proximal tubules of kidney	▪ Cartilage
▪ Endocrine glands	▪ Bone
▪ Fibroblasts	
Permanent Cells	
▪ Central nervous system	▪ Skeletal muscle
▪ Sensory organs	▪ Renal glomeruli
▪ Cardiac muscle	▪ Red blood cells

PHYSIOLOGIC HYPERPLASIA

Physiologic hyperplasia occurs due to a normal sensor. Examples of physiologic hyperplasia include increase in the size of breasts during pregnancy (as a result of increased circulating hormones), increase in the thickness of endometrium during menstrual cycle (as a result of increased circulating hormones), bone marrow hyperplasia at high altitude, thyroid hyperplasia during puberty and pregnancy, proliferation of epidermis in wound healing by primary intention, and liver hyperplasia after partial resection.

Endometrial Hyperplasia during Menstrual Cycle

The normal thickness of the endometrium changes throughout a person's life from childhood, through to sexual maturity, fertile years and after menopause. Estrogen and progesterone participate in the regulation of menstrual cycle.

- The endometrium is at its thinnest during menstrual phase, when it usually measures between 2 and 4 mm in thickness.
- At end of the menstrual cycle, estrogen plays important role in proliferation of endometrial cells; during proliferative phase endometrium begins to thicken and measures 5–7 mm in thickness.
- As the menstrual cycle progresses and moves towards ovulation, the endometrium in early secretory phase grows thicker, up to about 11 mm. About 14 days into a woman menstrual cycle, hormones trigger the release of an ovum.
- During the secretory phase, endometrial thickness is at its greatest and can reach 16 mm. In case fertilization does not take place, progesterone signals shedding of endometrium.

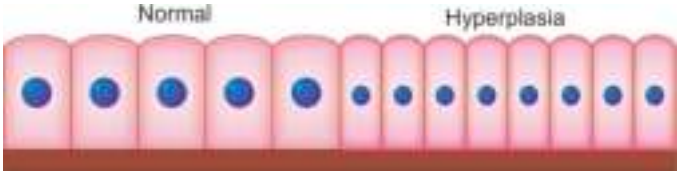


Fig. 1.3: Schematic representation of hyperplasia. Hyperplasia refers to an increase in the number of cells in the tissue.

Table 1.11 Physiologic, pathologic and compensatory hyperplasias

Hyperplasia of Organs	Mechanism
Physiologic hyperplasia	
Uterine endometrial hyperplasia	Estrogen hormone
Uterine muscle hypertrophy and hyperplasia	Estrogen hormone during pregnancy
Breast glandular epithelial hyperplasia	Hormones synthesis during pregnancy
Bone marrow hyperplasia	Higher altitude
Islet β-cells hyperplasia of fetus	Diabetic mother
Pathologic hyperplasia	
Ductal hyperplasia of breast in fibrocystic disease	Hormonal imbalance
Cystoglandular or adenomatous or atypical hyperplasia of endometrium	Hormonal imbalance
Thyroid follicular hyperplasia	Increased TSH level
Hyperplasia of surface epithelium of skin	Wound-healing by primary intention
Skin epidermal cell hyperplasia	Psoriasis
Compensatory hyperplasia	
Hyperplasia of hepatocytes	TGF-α, TGF-β, HGF synthesis following partial resection of liver

Breast Hyperplasia during Pregnancy

Female breasts show structural and functional variations during puberty, pregnancy, lactation, the normal menstrual cycle, and at the menopause. Pregnancy affects levels of estrogen and progesterone hormones.

- Estrogen stimulates growth of breast ductal and lobular cells and synthesis of prolactin.
- Progesterone supports the formation and growth of milk-producing cells within the glands of the breasts.
- Prolactin stimulates breast enlargement and milk production. Breast histology from a woman of 30 weeks pregnancy shows the lobular acini lined by cells containing secretory vacuoles and with pink secretions in their lumens.

Thyroid Gland Hyperplasia

During puberty, changes in thyroid functions and an increase in thyroid volume occur as an adaptation to body and sexual development. Benign physiologic changes of the thyroid are known to occur during pregnancy due to increased demand of thyroid hormones. TSH stimulates thyroid follicular cells and produce benign hyperplasia (adenomatous change) with diffuse enlargement of thyroid glands.

Bone Marrow Hyperplasia at High Altitude

Hypoxia at high altitudes can lead to increased production of red blood cells in bone marrow through the hormone erythropoietin (EPO).

- Erythropoietin is synthesized by kidney (89–90%), and liver (<15%). Normal plasma concentration is 2–25 IU/L.
- Erythropoietin stimulates BFU-E and CFU-E to divide and mature, increases rate of mRNA and protein (hemoglobin) synthesis, decreases normoblasts maturation time, increases rate of extrusion of nucleus from normoblasts and stimulates early release of bone marrow reticulocytes.
- It is well known that the body's hemoglobin concentration increases due to the hypoxic environment of high altitude.

Beta Cells of Islets of Langerhans Hyperplasia in Fetus in Severe Maternal Diabetes Mellitus

Severe maternal diabetes mellitus poses short- and long-term consequences for the infant. Maternal hyperglycemia increases transport of glucose, amino acids, and fatty acids via placenta to fetus resulting in β cell hyperplasia mainly due to formation of numerous small islets of Langerhans during intrauterine period followed by synthesis of excess of insulin (hyperinsulinemia) and thus hypoglycemia at birth in newborn. Hypoglycemic can cause shakiness, blue tint to the skin, breathing and feeding problems.

PATHOLOGIC HYPERPLASIA

Pathologic hyperplasia occurs due to an abnormal stressor either excessive hormonal stimulation or abnormal production of hormonal growth factors.

- Proto-oncogenes code for growth factors, growth factor receptors, signal transducers (e.g. mitogen-induced protein kinases), and DNA transcription factors to initiate cell mitosis. It may be associated with increased risk for cancer.

- Examples of pathologic hyperplasia include growth of adrenal glands due to production of adrenocorticotropic hormone (ACTH) by pituitary adenoma, proliferation of endometrium and breast due to prolonged estrogen stimulation, proliferation of thyroid follicles due to increased TSH, proliferation of epidermis in psoriasis. Women with pathologic endometrial hyperplasia can progress to dysplasia and eventually endometrial carcinoma.

Endometrial Hyperplasia

Endometrial hyperplasia refers to abnormal proliferation of endometrial glands caused by estrogenic stimulation. It occurs in anovulatory cycles as a result of estrogenic effect not opposed by progesterone.

- Excess of estrogens also occurs in women with polycystic ovary syndrome, estrogen-secreting ovarian tumors, i.e. granulosa cell tumor, estrogen replacement therapy and ovarian cortical stromal hyperplasia.
- Hormonal imbalance may lead to cystoglandular, adenomatous and atypical hyperplasia of endometrium, that may progress to endometrial carcinoma and manifest with postmenopausal bleeding. Young women manifest with menorrhagia and anovulation.

Benign Nodular Hyperplasia of Prostate Gland

Benign nodular hyperplasia of prostate gland is also known as benign prostatic hypertrophy (BPH), which primarily affects periurethral and transitional zones of men after 40 years of age. It is the most common cause of urinary tract obstruction.

- Digital rectal examination reveals a firm, enlarged, nodular prostate. It is an androgen-dependent disorder. Dihydrotestosterone (DHT) is a major growth factor for prostate gland derived from testosterone by the action of 5-reductase enzyme, that stimulates prostate glandular acini leading to prostatic hyperplasia.
- Patient presents with frequency of micturition, difficulty in starting urination, nocturia, dribbling, incomplete emptying of urinary bladder and dysuria. Persistent obstruction to urine outflow may result in urinary bladder distension, muscular hypertrophy, diverticulum formation and trabeculae formation.
- Histologically, benign nodular hyperplasia of prostate gland is diagnosed on samples like transurethral resection of prostate (TURP chips), simple or radical prostatic specimens. Benign nodular hyperplasia of prostate gland is composed of hyperplasia of glands and stroma in variable proportions forming papillary buds, infoldings and cysts. The lining epithelium ranges from flat to columnar, sometimes facing each other in the same gland. Basal layer is demonstrated immediately above a well-developed epithelium. The glands are dilated or even cystic and containing an inspissated secretion of glycoprotein nature (corpora amylacea), which is sometimes calcified. Histology of benign nodular hyperplasia of prostate gland is shown in Fig. 1.4.

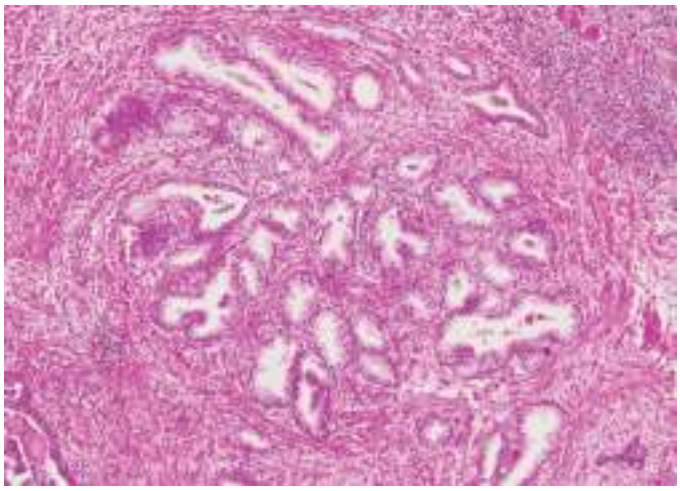


Fig. 1.4: Histology of benign nodular hyperplasia of prostate gland. It is benign nodular lesion with proliferation of glandular and stromal components predominantly located in the transition zone of the prostate gland. The acini are relatively uniform and evenly spaced are lined by columnar secretory cells. The basal cell layers may be inconspicuous, but may be highlighted by high molecular weight cytokeratin immunohistochemical stain (400X).

Thyroid Gland Hyperplasia in Graves' Disease

Graves' disease is an autoimmune disorder which primarily affects thyroid gland. It is most common cause of hyperthyroidism accounting for 60–80% of hyperthyroidism cases, which may affect eye and skin.

- Graves' disease is caused by thyroid stimulating immunoglobulin, that binds with thyroid-stimulating hormone receptor on the thyroid follicular cell membrane and stimulates the action of the thyroid stimulating hormone resulting in both thyroid hormone synthesis, hyperthyroidism and thyroid follicular epithelial hyperplasia causing thyromegaly.
- Graves' oculopathy occurs due to inflammation, cellular proliferation and increased growth of extraocular muscles and retro-orbital connective tissue and adipose tissue by the action of thyroid stimulating antibodies and cytokines synthesized by cytotoxic T cells.
- Thyroid stimulating autoantibodies and cytokines activate periorbital fibroblasts and preadipocytes causing excessive growth of retro-orbital adipose tissue and hydrophilic glycosaminoglycans leading to extraocular muscle swelling by trapping water.

- Changes in ocular and extraocular muscles give rise to proptosis, diplopia, congestion and periorbital edema.
- Graves' disease is diagnosed by thorough clinical history, physical examination, thyroid function tests, thyroid autoantibodies such as thyroid stimulating immunoglobulin (TSI) and thyrotropin-binding inhibiting (TBI) immunoglobulin or thyrotropin-binding inhibitory immunoglobulin (TBII)) and radioactive iodine uptake scan with ^{123}I or ^{131}I.

Defect in Primary Intention of Wound Healing

The epidermis is a stratified epithelium composed of several layers of keratinocytes. It provides a physical barrier between the environment and the microorganisms, thereby protecting it from external injurious agents and pathogens and limiting the loss of fluids.

- Keratinocytes of epidermis are responsible for restoring the epidermis after injury through a process of epithelialization. Epithelialization is defined as a process of covering denuded epithelial surface.
- The cellular and molecular processes involved in the initiation, maintenance and completion of epithelialization are essential for successful closure of skin wound.
- There are three distinct epidermal stem cell (ESC) niches identified to date: (a) bulge of the hair follicle (HF), (b) the base of the sebaceous gland, and (c) the basal layer of the interfollicular epidermis (IFE). In response to epidermal injury, both the HF and HFE niches participate in the re-epithelialization of the skin wound defect.
- A wound cannot be considered healed in the absence of re-epithelialization. The epithelialization process is impaired in all types of chronic skin wounds.

Psoriasis

Psoriasis is a chronic nonpruritic disease of the dermis and epidermis and characterized by persistent epidermal hyperplasia resulting in sharply demarcated erythematous plaques covered with a silvery scale commonly on the elbows, knees, scalp, umbilicus and lumbar region.

- Psoriasis may be caused by defective epidermal cell surface receptors and altered intracellular signaling. Cw6 gene mutation has the strongest association with psoriasis.
- Scratching of scaly lesion causes rupture of small blood vessels in suprapapillary thinned area resulting in pinpoint hemorrhages known as 'Auspitz sign'. Histology in a case of psoriasis is shown in Fig. 1.5.

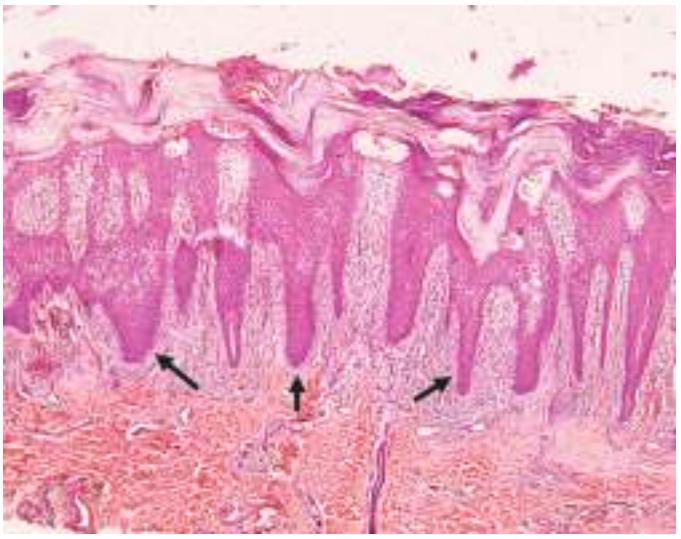


Fig. 1.5: Histology in a case of psoriasis. Hematoxylin and eosin-stained section shows downward elongation of the rete ridges (arrows) with thinning of overlying stratum granulosum, with parakeratosis above this. Small aggregates of neutrophils with surrounding spongiform change are seen in the superficial epidermis. Capillaries within dermal papillae are brought close to the surface (400X).

COMPENSATORY HYPERPLASIA OF HEPATOCYTES FOLLOWING PARTIAL RESECTION

The liver has a remarkable capability to regenerate after injury. Within a week after partial hepatectomy of two-thirds of the liver, the hepatocytes regenerate and also transdifferentiate into biliary epithelial cells and endothelial cells; and the liver retains back essentially to what it was during normal health prior to surgery.

- DNA synthesis is initiated in these cells within 10–12 hours after surgery and essentially ceases in about 3 days. Cellular proliferation begins in the portal triads (i.e. periportal region) and proceeds toward the centers of the hepatic lobules.
- Proliferative hepatocytes initially form clumps, which get transformed into classical plates. Similarly, proliferative endothelial cells develop into the type of fenestrated cells of those observed in hepatic sinusoids.
- Proliferating hepatocytes appear to at least partially revert to a fetal phenotype and express markers such as α-fetoprotein. Transforming growth factor-β is the main cytokine involved in this process. Other cytokines such as transforming growth factor-α and human growth hormone (HGH) also participate in the regeneration of hepatocytes.

HYPERTROPHY

Hypertrophy is defined as an increase in cell size of an organ or tissue due to synthesis of cellular structural

components in response to various stimuli (hormones and growth factors), without an increase in the number of cells.

- It is worth mentioning that both hyperplasia and hypertrophy result in an increase in organ size, therefore both cannot always be distinguished on gross examination, hence histologic examination is required to distinguish the two entities.
- Both hypertrophy and hyperplasia can occur due to upregulation or downregulation of receptors and induction of new proteins synthesis, which include transcription factors (e.g. c-Jun, c-Fos), contractile proteins (e.g. myosin light chain), and embryonic proteins (e.g. β-myosin heavy chain).
- Hypertrophy may be physiologic, pathologic, compensatory and subcellular. Schematic representation of hypertrophy is shown in Fig. 1.6. Physiologic, pathologic, compensatory and subcellular hypertrophies are given in Table 1.12. Differences between hypertrophy and hyperplasia are shown in Table 1.13.

PHYSIOLOGIC HYPERTROPHY

Physiologic hypertrophy occurs due to normal stressor. It is usually adaptive response that improves cell function. For example, hypertrophy of skeletal muscle occurs due to exercise and physical labor. Uterine smooth muscle hypertrophy occurs during pregnancy.

Physiologic Skeletal Muscle Hypertrophy

Exercise, hard physical labor or weight lifting stimulate skeletal muscle fibers to increase in diameter as a result of increased synthesis of more structural contractile proteins. **Phosphoinositide-3 kinase pathway** is postulated to play role in exercise-induced skeletal muscle hypertrophy. Skeletal muscle shows increased number of myofilaments and mitochondria; abundant endoplasmic reticulum, mild nuclear enlargement, which leads to synthesis of more membranes, enzymes, ATP and higher levels of aerobic respiration.

Physiologic Uterine Hypertrophy during Pregnancy

The adaptive uterine hypertrophy is a critical event that involves changes in cellular phenotypes throughout pregnancy. In early pregnancy, uterine hypertrophy involves the endometrial glandular epithelial cells and/or the myometrial muscle cells during pregnancy induced by circulating estrogens. The glandular epithelial cells become tall columnar with increase in cytoplasm to nucleus ratio, basophilic cellular cytoplasm and mitotic figures. Mechanical stress by growing fetus and placenta regulates hypertrophic phenotype of the myometrium during pregnancy; however, major component of myometrial hypertrophy occurs after mid-gestation.

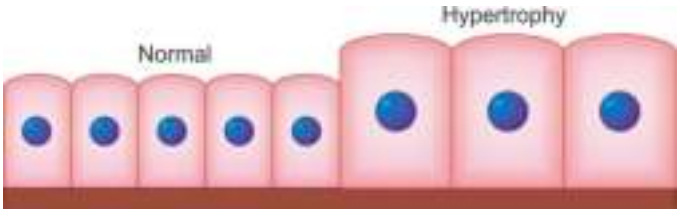


Fig. 1.6: Schematic representation of hypertrophy. Hypertrophy refers to enlargement of cells without cell division.

Table 1.12 Physiologic, pathologic, compensatory and subcellular hypertrophies

Hypertrophy of Organs	Mechanism
Physiologic hypertrophy	
Skeletal muscle hypertrophy	Physical labor and weight training
Uterus hypertrophy in pregnancy	Hormonal response
Pathologic hypertrophy	
Cardiac hypertrophy	Systemic hypertension and valvular disease
Adrenal gland hypertrophy (zona glomerulosa)	Increased ACTH synthesis
Focal hypertrophy of retinal pigment epithelium	Following retinal detachment
Compensatory hypertrophy	
Hypertrophy of residual cardiac myocytes	Following myocardial infarction
Renal hypertrophy	Following nephrectomy of opposite kidney, congenital absence of one kidney or nonfunctional kidney
Subcellular hypertrophy of cell organelles	
Subcellular hypertrophy of liver organelles such as endoplasmic reticulum	Hepatotoxic agents such as phenobarbital, alcohol and other anticonvulsant drugs

Table 1.13 Differences between hypertrophy and hyperplasia

Hypertrophy	Hyperplasia
Increase in cell size and cytoplasm without cell division results in increase in size of organ that does not induce neoplastic transformation	Cell size usually remains normal, cytoplasm may be reduced with cell division that may not induce neoplastic transformation
Physiologic state	
A demand for additional work met by skeletal muscle hypertrophy	▪ Hormone-induced hyperplasia in female breast ▪ Uterus during pregnancy ▪ Erythroid bone marrow hyperplasia at altitude ▪ Compensatory hyperplasia after partial hepatectomy and nephrectomy
Pathologic state	
▪ Cardiac hypertrophy (hypertension, valvular disease) ▪ Urinary bladder hyperplasia during obstruction to urine outflow due to prostate enlargement	▪ Uterus hypertrophy during pregnancy ▪ Cystic glandular and adenomatous hyperplasia of endometrium ▪ Atypical hyperplasia of the endometrium and endometrial carcinoma ▪ Prostatic hyperplasia (prostatic glands and stromal smooth muscle cells) ▪ Thyroid gland hyperplasia (toxic or nontoxic goiter), hyperplasia of epidermis in wound healing

Physiologic Myocardial Hypertrophy

Physiologic myocardial hypertrophy is characterized by normal organization of cardiac structure and normal or increased cardiac function. It can ensue as a result of exercise or pregnancy, and is deemed mild and/or reversible.

PATHOLOGIC HYPERTROPHY

Pathologic hypertrophy occurs due to abnormal stressor and is commonly associated with upregulation of fetal genes, fibrosis, organ dysfunction and increased mortality. Cardiac hypertrophy is caused in the presence of chronic stressful conditions such as systemic hypertension, valvular disease, hypertrophic cardiomyopathy and congenital heart disease.

- Cardiac hypertrophy is usually associated with upregulation of fetal genes, excessive increase in ventricular dimensions, accompanied by fibrosis, myocardial dysfunction and increased mortality.
- Ventricular hypertrophy is considered as a predictor of cardiovascular morbidity such as cardiac arrhythmias, myocardial infarction, cerebrovascular events, and sudden death. The most common symptoms of left ventricular hypertrophy are shortness of breath, chest pain after activity, dizziness and rapid heartbeats.
- Since the cardiac muscle is composed of terminally differentiated myocytes that cannot divide. Increased demand for action can be met only by increased size of the cardiac myocytes.
- Three stages of myocardial hypertrophy are recognizable: (a) initiation of myocardial hypertrophy, (b) stable function of the hypertrophied heart, and (c) deterioration of cardiac function associated with degeneration of hypertrophied cardiac myocytes. Histologic examination of myocardial hypertrophy shows enlarged myocytes with large nuclei.

Pathology Pearls: Molecular Basis of Cardiac Hypertrophy

- The molecular basis of cardiac hypertrophy reflects increased expression of growth-promoting genes (proto-oncogenes) such as c-Jun, c-Fos, c-Myc and RAS (nuclear transcription factors). It leads to increase synthesis of cellular proteins and number of intracellular organelles (e.g. mitochondria, Golgi apparatus). Only labile and stable cells are able to undergo hypertrophy as well as hyperplasia.
- Mechanical stretch induces production of growth factors such as TGF-β, FGF, insulin-like growth factor 1 and vasoactive agents (angiotensin II and endothelin), which bind to G protein-coupled receptors, induce signal to synthesize increased synthesis of contractile proteins, embryonic genes (e.g. cardiac α-actin and ANF) and growth factors.
- Cardiac hypertrophy resulting from transcriptional regulation leads to increased synthesis of mRNA, rRNA, and protein, which increase the strength and work capacity of heart.
- Cardiac hypertrophic muscles show increased cytosol, number of organelles and DNA content. Gross morphology of left ventricular hypertrophy is shown in Fig. 1.7.

Pathologic Adrenal Gland Hypertrophy

Pathologic adrenal gland hypertrophy may be bilateral and diffuse and focal. Bilateral diffuse cortical hypertrophy is usually caused by increased synthesis of ACTH; when associated with increase in thickness

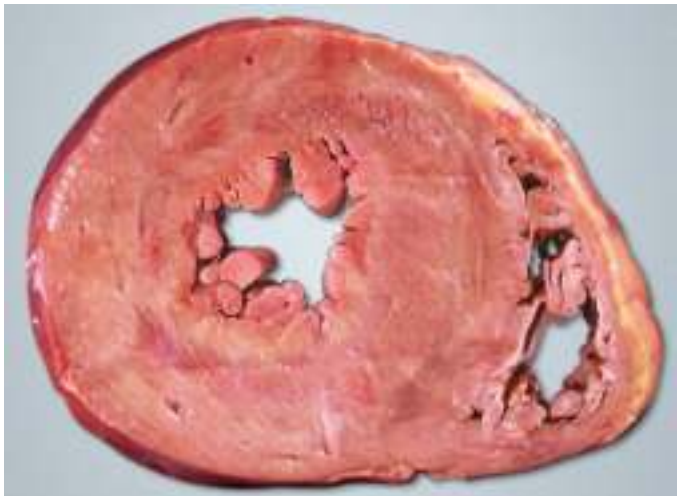


Fig. 1.7: Gross morphology of left ventricular hypertrophy. It is the enlargement and hypertrophy (increase in thickness of the wall) of left ventricle. This thickening may result in elevation of pressure within the heart and sometimes poor pumping action. The most common cause is severe hypertension. Normal thickness of wall of left ventricle is 1.2–1.5 cm.

of cortex and weight of adrenal gland. Zona fasciculata is usually increased in thickness, whereas the zona reticularis increases to a lesser extent. The cytoplasm of the affected cells is usually eosinophilic and finely granular with or without clear lipid vacuoles.

COMPENSATORY HYPERTROPHY IN RESIDUAL CARDIAC MYOCYTES

Compensatory hypertrophy in residual cardiac myocytes can take place only if some portion of the original structure is left to react to the loss. The growth can be a result of increased cell size or increase in cell division or both. Compensatory hypertrophy occurs in residual cardiac myocytes following myocardial infarction and renal hypertrophy of opposite side following nephrectomy.

- For instance, if one kidney is surgically removed, the cells of the other kidney divide at an increased rate. Compensatory renal hypertrophy can also occur on opposite side due to congenital absence of one kidney or nonfunctional kidney.
- Hypertrophy can occur as a compensatory response to injury when parenchymal cardiac myocyte cells are irreversibly damaged following myocardial infarction, in which residual cardiac myocytes increase in size.

SUBCELLULAR ENDOPLASMIC RETICULUM HYPERTROPHY IN LIVER

Subcellular endoplasmic reticulum hypertrophy occurs in liver in response to hepatotoxic agents.

Endoplasmic Reticulum Hypertrophy in Liver by Phenobarbital

Ingestion of administration of lipid soluble drugs, e.g. phenobarbital and other anticonvulsant drugs induces the hepatocytes to increase synthesis of cytochrome P450 oxidase enzyme available to detoxify the drugs. The process of synthesis of drug metabolizing enzyme takes place in the endoplasmic reticulum is a sequential event and can be divided into three stages as demonstrated on electron micrographs: (a) increased production of rough endoplasmic reticulum and its hypertrophy in the cytoplasm, (b) increased synthesis of cytochrome P450 oxidase enzyme available to detoxify the drugs, and (c) shedding of attached ribosomes of rough endoplasmic reticulum converting into smooth endoplasmic reticulum with the same attached enzymes and accumulation in the cytoplasm.

Endoplasmic Reticulum Hypertrophy in Liver by Excessive Alcohol Consumption

Excessive alcohol consumption induces numerous pathologic stress responses, part of which is endoplasmic reticulum. Alcohol crosses the biological membrane and disturbs numerous biological pathways and causing damage to liver, brain, pancreas, heart and immune system.

- In addition to conversion of alcohol into acetaldehyde in the circulation by aldehyde dehydrogenase, cytochrome P450 activities, mainly CYP2E1 in the endoplasmic reticulum of hepatocytes also oxidize alcohol to acetaldehyde and hydrogen peroxide (H_2O_2).
- There is excessive accumulation of unfolded proteins in the endoplasmic reticulum of hepatocytes.

ATROPHY

Atrophy is reduction in size of the cell (organ or tissue) due to loss of structural components of the cells, that has at one time been of normal size. The number of cells remain same as before the atrophy occurred, but the size of some fibers is reduced.

- Atrophy occurs as a result of insufficient blood flow, disuse of organs, denervation, or reduced endocrine stimulation. Cell attempts to reduce demand to match reduced energy supply. The entire tissue/organ diminishes in size when enough cells are involved.
- Decrease in cell size occurs via ubiquitin-proteosome degradation of the cytoskeleton and autophagy of the cellular components. In ubiquitin-proteosome degradation, intermediate filaments of the cytoskeleton are 'tagged' with ubiquitin and degraded by proteosomes. Autophagy of cellular components involves generation of autophagic vacuoles. These vacuoles

fuse with lysosomes whose hydrolytic enzymes breakdown the cellular components.

- Uterus returns to nongravid state after parturition is an example of physiologic atrophy. Hypoxia, loss of innervation, disuse of organ and aging are examples of pathologic atrophy.
- Schematic representation of atrophy is shown in Fig. 1.8. Physiologic and pathologic (systemic or local) atrophies are given in Table 1.14.

PHYSIOLOGIC ATROPHY

Physiologic atrophy is associated with the natural aging process. Examples of physiologic atrophy include thymus atrophy during puberty, embryonic structures atrophy (notochord, thyroglossal duct and patent ductus arteriosus), atrophy of female breasts, uterus and vagina during menopause, brown atrophy of organs (heart, liver, adrenal gland and ganglion cells) and brain atrophy during advancing age.

Embryonic Structures Atrophy

During eighth weeks of fetal development, embryonic folding converts a flat sheet of cells into a hollow, tube-like embryonic structures such as notochord, thyroglossal duct, and patent ductus arteriosus derived from the ectoderm, mesoderm and endoderm, undergo atrophy.

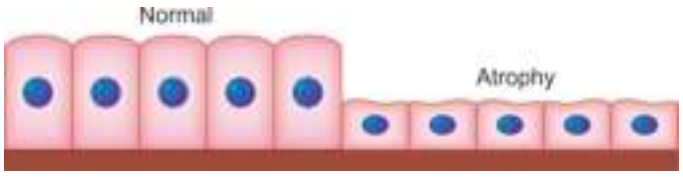


Fig. 1.8: Schematic representation of atrophy. Atrophy refers to reversible reduction in the size of the cells.

Female Breasts, Uterus and Vaginal Atrophy

During reproductive period, estrogen hormone plays key role in normal metabolism and function of breast and reproductive organs. After menopause, the ovaries, uterus, vagina and breasts normally undergo a degree of atrophic changes. In postmenopausal women, the walls of the vagina become thinner (atrophic vaginitis). After menopause the body have no further functional biological need to maintain the reproductive system.

Brain Atrophy in Advancing Age

Brain atrophy in advancing age is defined as the loss of neurons, which destroys the connections that help the cells to communicate. Focal brain atrophy affects cells in certain areas of the brain and results in loss of function in those specific areas. Generalized brain atrophy affects cells all over the brain. In old age, frontal cortex undergoes marked atrophy revealing thinning of the gyri and widening of the sulci.

Brown Atrophy of Organs in Advancing Age

Lipofuscin is the name given to fine yellow-brown pigment electron dense granules composed of lipid-containing residues of lysosomal digestion. Lipofuscin pigment is considered to be one of the aging or 'wear-and-tear' pigments, that accumulates progressively over time in lysosomes of postmitotic cells, such as cardiac myocytes, liver, kidney, adrenal glands, nerve cells and ganglion cells. The intracellular accumulation of lipofuscin-like material may be the result of an imbalance between formation and disposal mechanisms.

Table 1.14 Physiologic and pathologic (systemic or local) atrophies

Atrophy of Organs	Mechanism
Physiologic atrophy	
Atrophy of thymus gland	Physiologic phenomenon
Atrophy of embryonic structures (notochord, thyroglossal duct and patent ductus arteriosus)	Physiologic phenomenon
Female breasts, uterus and vagina during menopause	Decreased estrogens in menopausal women
Brown atrophy of heart, liver, adrenal gland and ganglion cells	Lipofuscin pigment (wear and tear pigment), important indicator of free radical induced injury
Brain atrophy	Aging process
Pathologic generalized atrophy	
Atrophy of thyroid gland and adrenal gland	Hypopituitarism
Pathologic local atrophy	
Skeletal muscle atrophy	Denervation
Renal cortex atrophy	Nephrolithiasis
Brain atrophy	Alzheimer disease
Exocrine gland atrophy	Cystic fibrosis

The lipofuscin pigment is found at the periphery of the lobule in hepatocytes and some bile ductular cells. The adrenal cortex accumulates lipofuscin granules with advancing age.

PATHOLOGIC ATROPHY

Pathologic atrophy occurs due to an abnormal stressor. In general, pathologic atrophy occurs due to the loss of stimulus to the organ such as insufficient blood flow and nutrients, disuse of organs, denervation, or reduced endocrine stimulation. The organ is smaller than usual. Atrophy occurs in a once normally developed organ. If the organ was never of normal size and does not develop normally, the condition is called hypoplasia.

- **Systemic pathologic atrophy:** Systemic pathologic atrophy occurs in cases of insufficient nutrition, chronic infection, disorders of endocrine glands, and disorders of central nervous system.
- **Local pathologic atrophy:** Local pathologic atrophy can occur in the settings of: (a) trophic nerves involvement results in atrophy of skeletal muscles in poliomyelitis, (b) atherosclerotic plaques in carotid arteries cause insufficient blood supply to brain leading to brain atrophy, (c) dysfunctional atrophy of optic nerve occurs after removal of diseased eye, (d) prolonged immobilization of extremities due to fracture results in atrophy of skeletal muscles, and (e) application of iodine preparation causes thyroid gland atrophy.

Brown Atrophy of Organs in Disorders

Lipofuscin pigment accumulation is also demonstrated in adrenocortical tumors associated with Cushing syndrome, particularly those with **PRKAR1A gene** mutations, such as in primary pigmented nodular adrenocortical disease (PPNAD).

- Lipofuscin accumulation in the eye can cause macular degeneration and inherited juvenile form of macular degeneration in Stargardt disease, an inherited disorder. Abnormal accumulation of lipofuscin in the peripheral nervous system can cause lipofuscinosis.
- Batten disease is a familial neurodegenerative disorder showing neuronal ceroid lipofuscinosis. Pathologic accumulation of lipofuscin has been implicated in Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, melanosis coli and lipid myopathy. Calorie reduction, vitamin E and increased glutathione appear to reduce production of lipofuscin pigment.

Brain Atrophy in Disorders

Diseases that cause cerebral atrophy in adults include cerebral stroke due to interruption of blood supply, traumatic brain injury, Alzheimer's disease, Pick disease, frontotemporal dementia, cerebral palsy (abnormal development of brain during gestation period), multiple sclerosis, Huntington's disease and AIDS. Alzheimer disease is secondary to extensive cell death resulting in marked atrophy of the frontal lobe, thinning of gyri and widening of sulci. Patient with multiple sclerosis can have close to a normal life span if treated effectively.

Skeletal Muscle Atrophy in Disorders

Normally, skeletal muscle is maintained by multiple pathways regulating turnover of cell and proteins. There are three types of skeletal muscle atrophy: physiologic, pathologic and neurogenic. Physiologic atrophy of skeletal muscle is caused by not using the muscles for long time.

- Pathologic atrophy of skeletal muscle occurs due to reduced workload, loss of innervation, reduced blood supply, protein calories malnutrition, osteoporosis, immobilization of skeletal fracture in a plaster cast for prolonged period, loss of endocrine stimulation and aging.
- Activation of proteolytic system leads to removal of contractile proteins, cytoplasm and cell organelles resulting in shrinkage of skeletal muscle fibers and thus skeletal muscle atrophy.
- Excessive removal of skeletal muscle mass is associated with poor prognosis in myopathies and muscular dystrophies as well as diabetes mellitus, cancer cachexia, sepsis, AIDS, heart failure, chronic renal failure and aging.
- The cells exhibit autophagic granules, which are intracytoplasmic vacuoles containing debris from degraded organelles.
- In skeletal muscle, the ubiquitin-proteasome system is required to remove sarcomere proteins upon changes in muscle activity.
- Atrophy of skeletal muscle mass is associated with (a) increased conjugation of ubiquitin to muscle proteins; (b) increased proteasomal ATP-dependent activity; (c) increased breakdown of proteins; and (d) upregulation of transcripts encoding ubiquitin; some ubiquitin-conjugating enzymes (E2), a few ubiquitin-protein ligases (E3) and several proteasome subunits.

Endocrine Glands Atrophy in Disorders

A destructive or atrophic lesion affecting the pituitary gland with loss of its hormones (hypopituitarism) leads to atrophy of the thyroid gland, adrenal glands and gonads and in turn brings atrophic changes to their target. The decrease in size of the endocrine glands may be extreme.

Cachexia (Irreversible Wasting Syndrome)

Cachexia is a multiorgan and multifactorial and irreversible wasting syndrome associated with cancer and other chronic illnesses such as chronic heart failure, chronic renal failure, chronic obstructive pulmonary disease and chronic inflammatory diseases.

- Cancer cachexia is characterized by systemic inflammation, negative protein and energy balance and involuntary marked muscle wasting and anorexia.
- Multiple mechanisms are reported in the development of cachexia, with a number of cytokines including TNF-α postulated to play important role in the etiology of the persistent catabolic state.
- Cachexia is treated with dietary supplements and/or exercise and therapeutic agents, such as megestrol acetate, medroxyprogesterone, ghrelin, omega-3-fatty acids and erythropoietin.

Renal Atrophy in Disorders

Renal atrophy may be associated with antiphospholipid syndrome, tuberculosis, metabolic syndrome, renal artery stenosis, atheromatous plaques in renal artery, sickle cell disease, obstruction of the urinary tract due to urolithiasis and cancer. Atrophic kidney has impaired renal functions. Patient presents with changes in urination, drowsiness, nausea, vomiting, anorexia, muscle cramps, increased serum creatinine and electrolytes imbalance.

Exocrine Glands Atrophy in Cystic Fibrosis

Normally, cystic fibrosis transmembrane conductance regulator (CFTR) gene located on long arm of chromosome 7 codes for a membrane protein that regulates chloride channel in epithelial cells and thus participates in the movement of chloride and other ions across membranes.

- Cystic fibrosis is autosomal recessive disorder, that occurs due to mutation of cystic fibrosis transmembrane conductance regulator/CFTR gene resulting in thick secretions in the respiratory tract and exocrine glands and causes pressure atrophy on the ducts.
- Patient develops recurrent respiratory infections, meconium ileus and sterility in males. Sweat chloride test is an important diagnostic tool.

METAPLASIA

The epithelium normally presents at a site cannot handle the new microenvironment induced by persistent stimuli (chronic inflammation or irritation), so one adult cell type is transformed to another adult cell type, in response to persistent injury (chronic inflammation or irritation).

- Metaplasia is a reversible phenomenon. New metaplastic cells are better able to handle the new stressor.
- Examples of pathologic metaplasia include Barrett esophagus, squamous metaplasia in the tracheobronchial mucosa, metaplasia in ocular and exocrine gland ducts in vitamin A deficiency, myositis ossificans, apocrine metaplasia in breast.
- Schematic representation of metaplasia is shown in Fig. 1.9. Schematic representation of squamous metaplasia is shown in Fig. 1.10. Differences between

Fig. 1.9: Schematic representation of metaplasia. Metaplasia refers to replacement of an adult cell with another adult cell as a response to chronic inflammation or irritation.

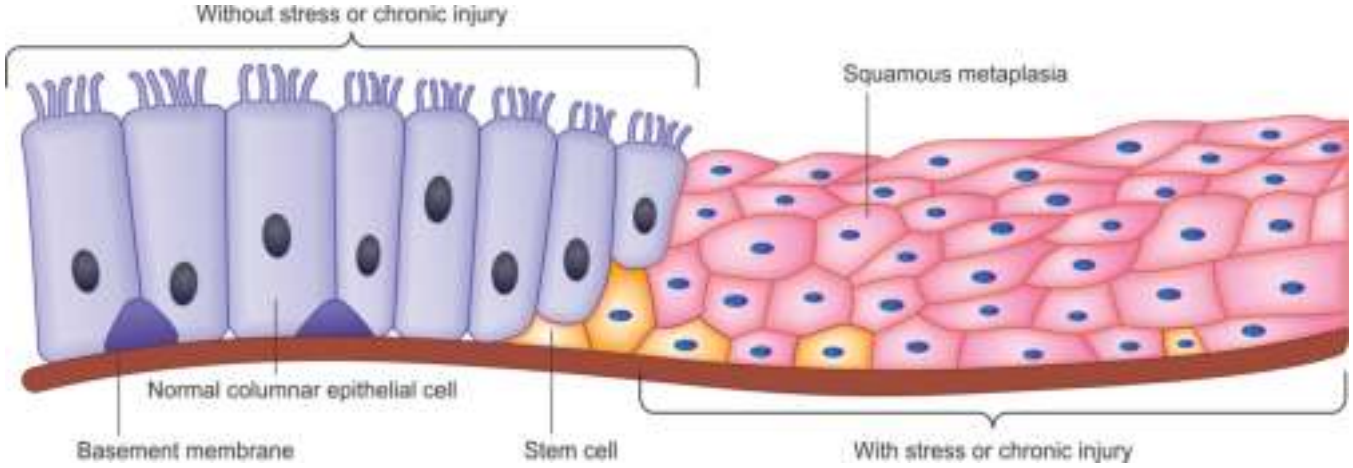


Fig. 1.10: Schematic representation of squamous metaplasia. Squamous metaplasia is used to designate the focal or extensive replacement of mucus-secreting glandular epithelium by stratified squamous epithelium in response to chronic irritation.

Table 1.15 Differences between physiologic metaplasia and pathologic metaplasia

Original Tissue	Stimulus	Replaced by Metaplastic Lining/Tissue
Physiologic metaplasia		
Monocytes in blood circulation	Physiologic state	Tissue macrophages
Pathologic metaplasia		
Ciliated columnar epithelium in bronchial tree	Tobacco smoking	Squamous metaplasia
Columnar glandular epithelium lining ducts pancreas	Vitamin A deficiency	Squamous metaplasia
Transitional epithelium in urinary bladder	Renal stones	Squamous metaplasia
Squamous epithelium in Barrett's esophagus	Gastric acid (gastroesophageal reflux disease)	Columnar metaplasia
Transitional epithelium in urinary bladder	Chronic inflammation prostatitis	Glandular epithelium (cystitis glandularis)
Fibrocollagenous tissue	Traumatic injury	Osseous metaplasia
Myeloid metaplasia and agnogenic myeloid metaplasia (myelofibrosis, polycythemia vera)	Agnogenic myeloid metaplasia, myelofibrosis, polycythemia vera	Extramedullary hematopoiesis in liver and spleen
Metaplasia in benign neoplasm	Benign neoplastic process	Cartilaginous and bony tissue in pleomorphic adenoma
Squamous or osseous metaplasia in malignant neoplasm	Malignant neoplastic process	Breast carcinoma

physiologic metaplasia and pathologic metaplasia are given in Table 1.15.

PHYSIOLOGIC METAPLASIA

Physiologic metaplasia is generally a normal transient response to changing conditions. For example, in the body's normal response to inflammation, monocytes migrate to inflamed tissues and transform into macrophages.

PATHOLOGIC METAPLASIA

Pathologic metaplasia is usually a response to chronic irritation (e.g. tobacco smoking or chronic inflammation). This process is usually reversible. Examples of pathologic hyperplasia are described as under.

Squamous Metaplasia in Tissues/Organs

Squamous metaplasia is a benign non-neoplastic change of surface lining epithelial cells to a squamous morphology. Common sites of squamous metaplasia include urinary bladder, bronchi, cervix, and ducts of exocrine gland.

Squamous Metaplasia in Cervix

The uterine cervix measures 3–4 cm in length and 2.5 cm in diameter. However, it varies in size and shape depending on age, menstrual status and parity of the women.

- Ectocervix is most often readily visible portion of cervix and lined by stratified squamous epithelium consisting of multiple layers of cells.
- Endocervix is invisible and lies proximal to the external os and lined by columnar epithelium consisting of single layer of cells.
- The location of squamocolumnar junction in relation to the external os varies depending on age, menstrual status, parity and contraceptive use.
- Squamous metaplasia in cervix occurs in the transformation zone, in which the endocervical epithelium is replaced by subcolumnar reserve cells, which differentiate into immature and then mature squamous epithelium. Squamous epithelium overlies endocervical glands, may replace glands.

Squamous Metaplasia in Urothelium Lining Urinary Bladder

Squamous metaplasia is defined as the transformation of the normal urothelium into nonkeratinized or keratinized stratified squamous epithelium due to urolithiasis in trigone region.

Squamous Metaplasia in Bronchi Lined by Columnar Epithelium

Although metaplasia is considered to be a protective mechanism, yet it may be harmful. Columnar epithelium of tracheobronchial mucosa undergoes epithelial squamous metaplasia in response to chronic tobacco smoke exposure. Squamous metaplasia also impairs the production of mucus and ciliary clearance of debris. It is widely acknowledged that continuous exposure to tobacco smoke is linked to the development of chronic obstructive pulmonary disease (COPD). A history of cigarette smoking is independent risk factor for lung

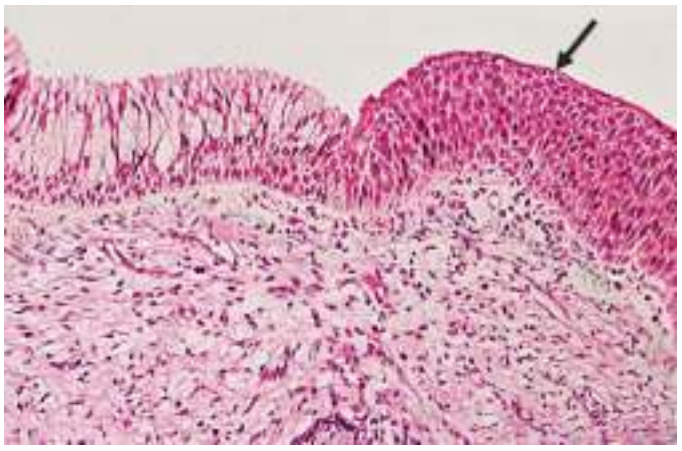


Fig. 1.11: Histology of squamous metaplasia. Squamous metaplasia is preneoplastic change of the bronchial columnar epithelium. It is observed in the bronchi in response to toxic injury induced by tobacco smoke. It is a multistage process, which may eventually lead to full neoplastic transformation, i.e. bronchogenic carcinoma (arrow) (100X).

cancers. Squamous metaplasia is a preneoplastic change of the bronchial columnar epithelium is shown in Fig. 1.11.

Squamous Epithelium Metaplasia in Conjunctiva

Vitamin A deficiency can also result in squmous metaplasia. Vitamin A is essential for differentiation of specialized epithelial surfaces such as the conjunctiva covering eye. In vitamin A deficiency, the thin squamous epithelium lining the conjunctiva undergoes metaplasia into stratified keratinizing squamous epithelium. The different ocular signs of vitamin A deficiency in children, as graded by the WHO, are: night blindness, conjunctival xerosis and Bitot's spots.

Squamous Epithelium Metaplasia in Pancreatic Ducts

Vitamin A deficiency can cause squamous metaplasia of epithelium lining pancreatic duct, since vitamin A maintains orderly differentiation of epithelia.

Squamous Epithelium Metaplasia to become Columnar Epithelium in Barrett's Esophagus

Barrett esophagus is classic example of metaplasia. Esophagus is normally lined by nonkeratinizing squamous epithelium, that is suited to handle friction of a food bolus.

- Barrett esophagus occurs due to gastroesophageal reflux of gastric contents into the esophagus, which causes the epithelium type to convert from squamous to intestinal type columnar epithelium, which can handle the stress of gastric acid. Metaplasia is thought to arise from reprogramming of stem cells present in adult tissue. Metaplasia is reversible with removal of the driving stressor.
- Treatment of gastroesophageal reflux may reverse Barrett disease. Under persistent stress, metaplasia can progress to dysplasia and eventually lead to cancer. Barrett esophagus may progress to adenocarcinoma. A notable exception is apocrine metaplasia of breast, which carries no increased risk for cancer.

Transitional Epithelium Metaplasia to Glandular Epithelium

Metaplasia of transitional epithelium to glandular epithelium is seen in patients with chronic inflammation of the bladder (cystitis glandularis).

Osseous Metaplasia in Myositis Ossificans

Mesenchymal tissues can also undergo metaplastic change. Osseous metaplasia is the formation of bony trabeculae within striated muscle at the site of repetitive traumatic injury.

- Fibrocollagenous tissue transforming to osseous tissue is known as myositis ossificans. It is worth mentioning that dystrophic calcification occurring at injury site does not lead to the formation of bone trabeculae.
- Most of the times, myositis ossificans occurs in the large skeletal muscles of the arms or the legs in young adults and athletes. Regardless of the cause, myositis ossificans happens when the body makes an error in the healing process. Patient may notice that pain worsens with time instead of getting better.

Myeloid Metaplasia

Myeloid metaplasia occurs due to proliferation of hematopoietic cells at the site other than bone marrow, such as liver and spleen. It is known as extramedullary hemopoiesis. Various causes of myeloid metaplasia are agnogenic myeloid metaplasia, myelofibrosis and polycythemia vera.

Metaplasia in Neoplasms

Metaplasia is seen in pleomorphic adenoma of salivary gland. Tumor shows areas of cartilage and bone. Breast carcinoma may show squamous or osseous metaplasia in some cases. Prognosis of metaplastic breast carcinoma is poor.

DYSPLASIA

Dysplasia means loss of regular appearance of cells in epithelium. It is characterized by variations in size and shape of dividing cells with mitotic activity arranged in disordered fashion with loss of cell maturation as cells progress to the surface, as a result of chronic irritation or inflammation. It may revert to normal, if stimulus is removed. If stimulus persists, it can precede malignant change. Differences between metaplasia and dysplasia are given in Table 1.16.

Table 1.16 Differences between metaplasia and dysplasia

Metaplasia	Dysplasia
Definition	
Transformation of one type of cell (epithelial or mesenchymal) into another adult cell type	Disordered cell growth, that may progress to development of cancer
Type of change	
Adaptive change and reversible if injurious stimuli removed	Nonadaptive change and irreversible even injurious stimulus removed
Cellular changes	
▪ Pleomorphism in cells absent ▪ Normal nuclei ▪ Few mitotic figures ▪ Normal architecture of cells in tissue maintained	▪ Pleomorphism in cells present ▪ Hyperchromatic nuclei ▪ Numerous mitotic figures ▪ Architecture of cells in tissue lost
Examples	
▪ Ciliated columnar to squamous metaplasia in trachea and bronchi (tobacco smoking) ▪ Columnar to squamous metaplasia due to stones in the ducts of salivary gland, pancreas and biliary system; and vitamin A deficiency ▪ Squamous to columnar metaplasia in Barrett's esophagus caused by chronic irritation by gastric juices in gastroesophageal reflux ▪ Transitional to squamous metaplasia of urinary bladder due to trauma ▪ Fibrocollagenous tissue to osseous metaplasia in myositis ossificans ▪ Tumor metaplasia (adenosquamous cell carcinoma, stromal mucinous metaplasia in pleomorphic adenoma, and squamous or osseous metaplasia in breast carcinoma)	▪ Cervical dysplasia due to human papillomavirus 16 ▪ Dysplasia of the bronchus due to cigarette smoking causing squamous metaplasia progressing to squamous dysplasia ▪ Skin dysplasia due to exposure to sun ultraviolet light causing skin dysplasia

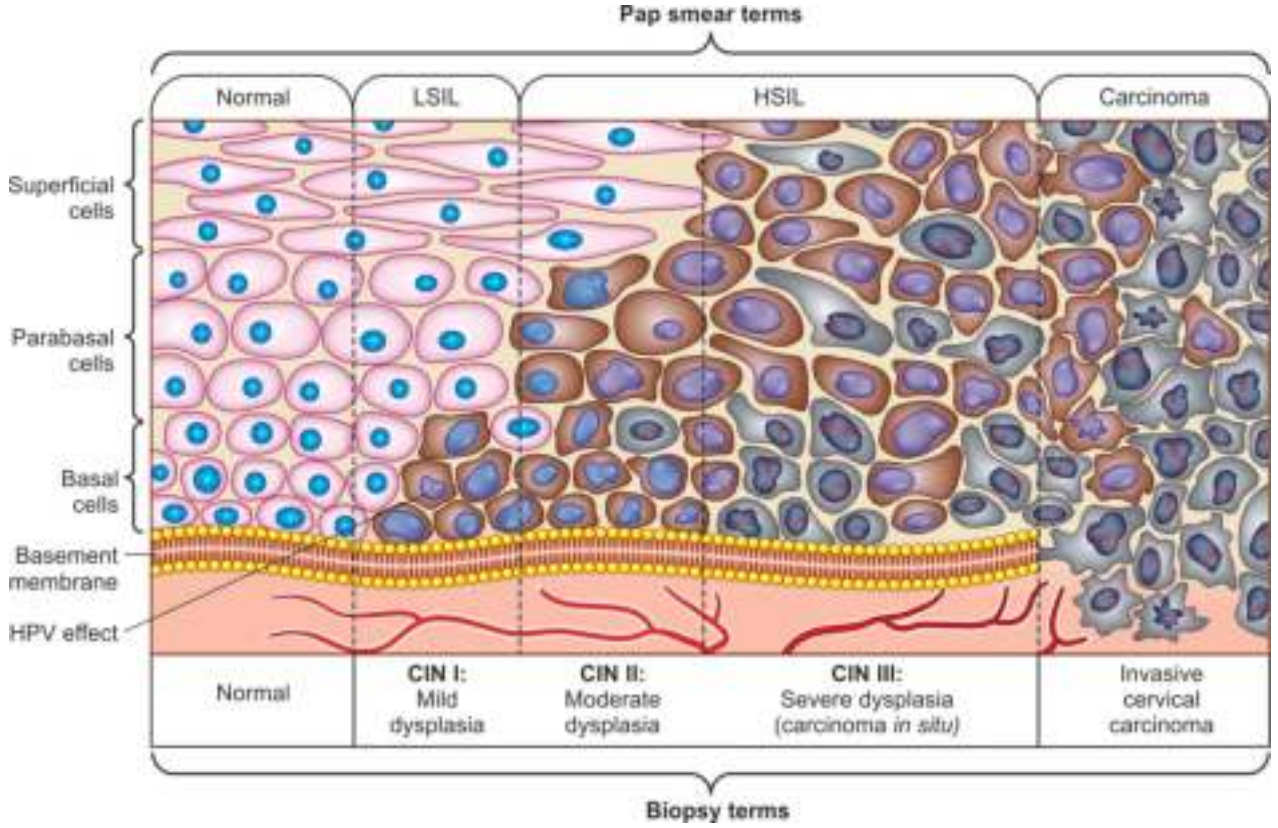


Fig. 1.12: Schematic representation of development of cervical intraepithelial neoplasia (CIN I, CIN II, CIN III) and invasive cervical carcinoma.

- **Cervical intraepithelial dysplasia:** Human papillomavirus 16, 18, 31, 33, 35, 39, 45 and 51 causes cervical intraepithelial dysplasia. The distinction between severe dysplasia and early cancer of the cervix is a common diagnostic problem for the pathologist. Schematic representation of development of cervical intraepithelial neoplasia (CIN I, CIN II, CIN III) and invasive cervical carcinoma is shown in Fig. 1.12. CIN III (cervical intraepithelial neoplasia) is shown in Fig. 1.13.
- **Dysplasia in bronchial epithelium:** Tobacco smoking produces dysplasia in respiratory epithelium. It is potentially reversible if the patient stops tobacco smoking. It may progress to bronchogenic carcinoma in heavy tobacco smokers.

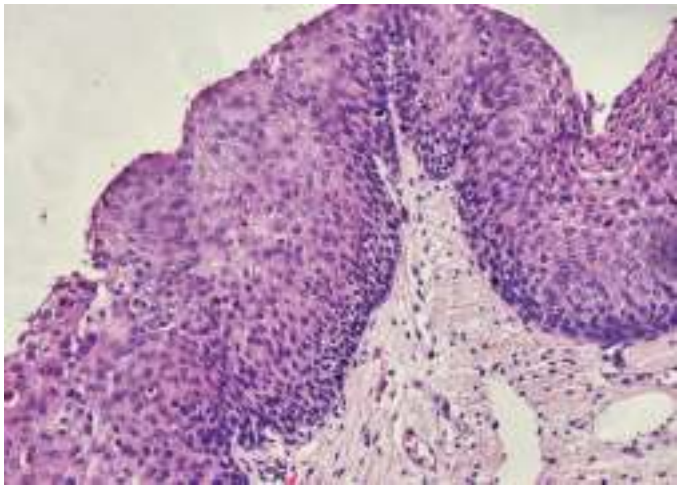


Fig. 1.13: Histology of CIN III. This is cervical squamous dysplasia at high magnification extending from the center to the right. The epithelium is normal at the left. Note how the dysplastic cell nuclei are larger and darker, and the dysplastic cells have a disorderly arrangement (400X).

- **Skin dysplasia:** Exposure to sun ultraviolet light causes skin dysplasia, which may lead to squamous cell carcinoma.
- **Actinic keratosis:** It is a form of dysplasia in sun-exposed skin. Histologic examination reveals atypical cells, varying in size and shape without, signs of regular maturation as the cells move from the basal layer of the epidermis to the surface.

REDUCTION IN SIZE OF ORGANS IN OTHER PATHOLOGIC PROCESSES

Pathologic processes other than atrophy associated with reduced organ size include agenesis, aplasia and hypoplasia.

- **Agenesis:** Agenesis refers to absence of organ resulting from failure to develop during embryonic development (e.g. renal agenesis). Renal agenesis differs from atrophy, in which a decrease in the size of an organ results from a decrease in preexisting cells. Bilateral renal agenesis is incompatible with life. Unilateral renal agenesis causes compensatory hypertrophy of opposite kidney.
- **Aplasia:** Aplasia is failure of cell production during embryogenesis. During fetal development, there is no development of adrenal cortex. During postnatal period, aplasia may occur due to permanent loss of bone marrow precursor cells as seen in aplastic anemia.
- **Hypoplasia**: Hypoplasia refers to incomplete development of an organ due to decrease in cell production. It may also occur due to partial lack of growth and maturation of gonadal structures in Klinefelter's syndrome and Turner syndrome.

CELL INJURY AND CELL DEATH: OVERVIEW

Homeostasis refers to the ability of an organism to maintain the vital internal environment within limits that allow it to survive. The fundamental pathogenesis of cell injury is a perturbation of homeostasis.

- Cell injury is defined as a change in cell structure, metabolism, physiochemical properties, and function which leads to impairment of its vital activity.
- Cell injury occurs due to defects in membrane permeability, mitochondrial damage with ATP depletion, accumulation of oxygen-derived free radicals (oxidative stress) and influx of intracellular calcium and loss of calcium homeostasis.
- Cell injury is initiated at the molecular level. Cell injury may be reversible or irreversible process depending on the type, severity and duration of cell injury. In reversible cell injury, cells can recover to their normal function. If the stress remains severe, cell injury becomes irreversible and leads to cell death.
- Cell injury can be caused by several intrinsic or extrinsic stresses. The basic mechanisms of cell injury can be categorized: (a) adenosine triphosphate (ATP) depletion, (b) permeabilization of plasma membrane and organelles with membranes, (c) disruption of biochemical pathways (impaired synthesis of structural protein and enzymes), and (d) DNA damage (pyknosis, karyorrhexis, karyolysis). Injury to the components of the cell can lead to disease as the cells lose their ability to adapt.

- Cell injury occurs when a stress exceeds the cells ability to adapt. Cell continues to function by adaptation despite mild to severe stress through its life. Cellular stress beyond the level of adaptive response results in cell injury, which may progress through a reversible cell injury stage and culminate in cell death.
- Basic principles of cell injury depend on nature of injurious stimuli, duration and severity of injury, type, ability of the tissues to regenerate (labile cells/stable cells/permanent cells), metabolic needs of cell, adaptability of the injured cells, genetic constitution and biochemical mechanisms acting on the several essential cellular components.
- In reversible cell injury, initially mitochondrial oxidative phosphorylation is disrupted that leads to decreased ATP production. Increase in cellular glycolysis to increase ATP stores leads to decreased in glycogen store and lactic acid accumulation resulting in decrease in intracellular pH. Decrease in ATP stores impairs membrane Na^+/K^+/calcium pumps that lead to accumulation of sodium and calcium; and diffusion of potassium leading to cellular swelling. Continued oxygen depletion leads to cytoskeleton disruption, mitochondrial changes, dilatation of the endoplasmic reticulum, distortion of microvilli, loosening of intercellular junctions, and blebbing of plasma membrane. Acute cell swelling is an example of reversible cell injury.
- Oncotic necrosis is the term currently used for nonapoptotic, accidental cell death. It is generally regarded as a severe injury to cell membrane integrity. The term 'oncotic necrosis' is defined as cell death with swelling (oncosis), that can be caused by ischemia, and toxic agents that interfere with ATP generation by mitochondria and plasma membrane pumps failure ($Na^+/K^+/Cl^-/Ca^{++}$).
  - Oncotic necrosis is characterized by cell swelling (oncosis), organelles swelling, plasma cell blebbing, loss of plasma membrane integrity, increased membrane permeability, dispersion of ribosomes, chromatin fragmentation (pyknosis, karyorrhexis, karyolysis), release of lysosomal enzymes into extracellular spaces, eosinophilic cytoplasm, leakage of proteins (creatine kinase, troponins, myoglobin, cellular enzymes) into blood, inflammation, vascularization and tissue repair. Necrosis is always pathologic as a result of irreversible cell injury.
- Irreversible cell injury results in a loss of cell functions. Myocardial cell injury results in loss of heart contraction. Motor neuron injury leads to skeletal muscle paralysis. Irreversible injury to islets of Langerhans results in diabetes mellitus. Pathophysiology of oxidative stress-induced cell death is shown in Fig. 1.14.
  - Neurons are highly susceptible to ischemic injury, that begins to die within three to five minutes after the oxygen supply has been cut off. Myocardial infarct develops within 20–30 minutes of interruption of blood supply to myocardium.
  - Watershed areas in brain and bowel are susceptible to oncotic necrosis. Skeletal muscles are relatively more resistant to ischemic injury. Fibroblasts are resistant to ischemia.

Pathology Pearls: Reversible and Irreversible Cell Injury

Reversible Cell Injury

The initial phase of cell injury is reversible. The hallmark of reversible cell injury is cellular swelling: (a) cytosol swelling results in loss of microvilli and membrane blebbing; and (b) swelling of the rough endoplasmic reticulum results in dissociation of ribosomes and decreased protein synthesis.

Irreversible Cell Injury

- Eventually, prolonged cell injury results in irreversible cell injury. The hallmark of irreversible cell injury (cell death) is membrane damage.
- Plasma membrane damage results in leakage of cytosolic enzymes and proteins into the blood (cardiac troponins in myocardial infarction, leakage of liver enzymes in hepatocellular injury); and influx of additional calcium occurs into the cell.
- Mitochondrial membrane damage results in loss of the electron transport system located in inner mitochondrial membrane; and leakage of cytochrome c into the cytosol; that activates apoptosis.
- Lysosomal membrane damage results in leakage of hydrolytic enzymes into the cytoplasm, which in turn, are activated by the high cytosolic calcium. The end result of irreversible injury is cell death.
- The morphological hallmark of cell death is degradation of the nucleus, which occurs via nuclear condensation (pyknosis), fragmentation (karyorrhexis), and dissolution (karyolysis). The death of large group of cells is followed by acute inflammation.

CAUSES OF CELL INJURY AND CELL DEATH

Cell injury can be caused by several intrinsic or extrinsic stresses. Causes of cell injury are given in Table 1.17.

- Intrinsic stressors of cell injury include genetically determined metabolic imbalances, genetic abnormalities, gross malformations, hypersensitivity reactions, oxidative stress (hypoxia), and ischemia-perfusion injury, hypoxia is the most important cause of cell injury.

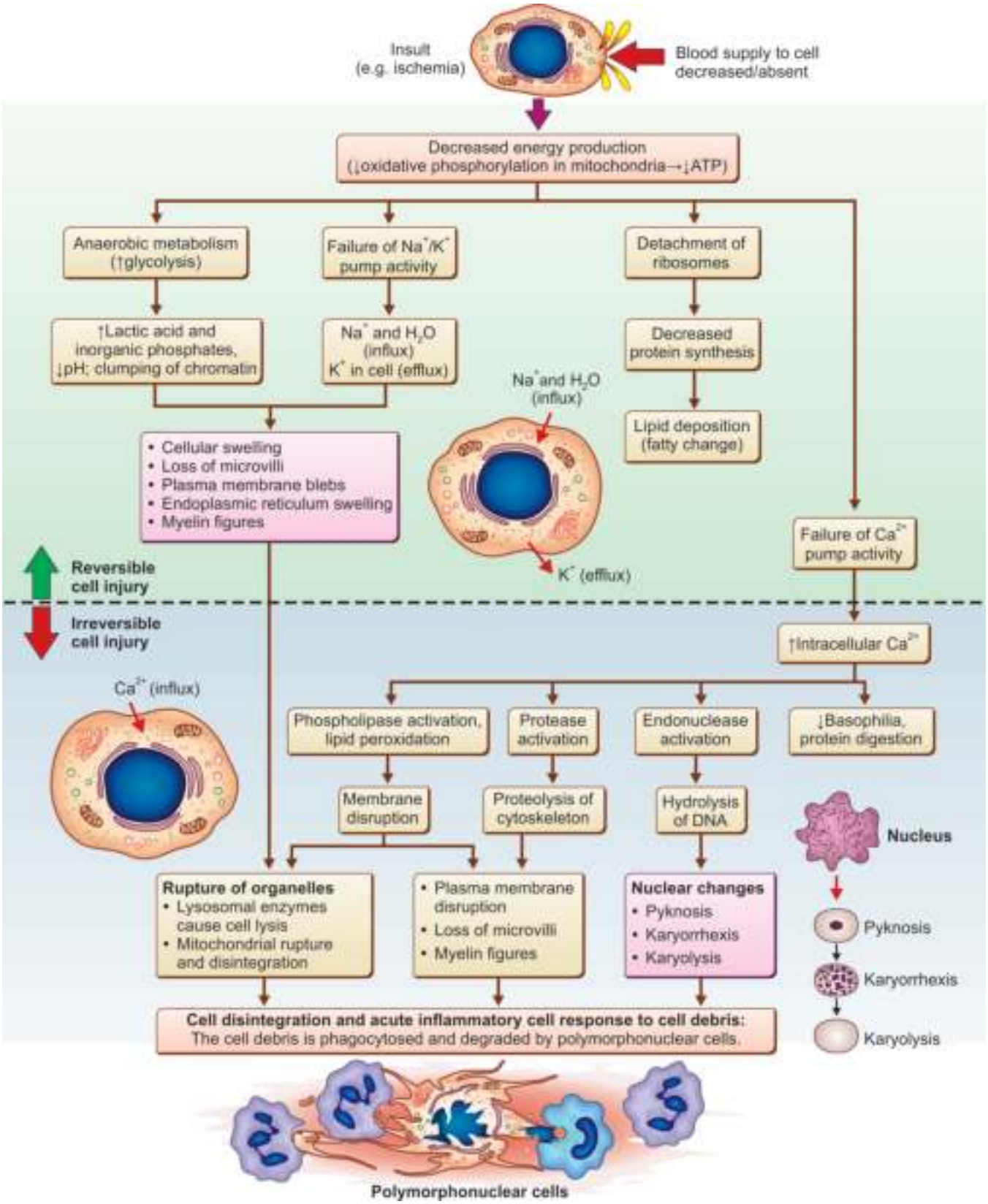


Fig. 1.14: Pathophysiology of oxidative stress-induced cell death. Oxidative stress-induced cell injury that occurs when there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense systems in the body. The reactive oxygen species comprises many molecules that have divergent effects on cellular function leading to disintegration of cell membranes and nucleus and inflammatory response.

Table 1.17 Causes of cell injury

Mechanism	Cell Injury
Oxidative stress (oxygen deprivation → hypoxia/ischemia)	Pneumonia, cardiopulmonary failure (hypotensive shock), anemia, carbon monoxide (CO) poisoning, relative ischemia-induced cell injury (poor blood supply to organs), complete cessation of blood supply induced cell injury causing organs infarcts (heart, kidney, spleen, lung, intestine)
Ischemia-reperfusion injury in various organs	Myocardial infarction, cerebral stroke, peripheral vascular disease
Infectious agents	Bacteria, viruses, parasites, fungi
Physical injury	Mechanical trauma, extreme of temperature (burns and severe cold), electric shock, radiation, sudden changes in atmospheric pressure
Chemical agents	Mercury, cyanide, arsenic, carbon monoxide, strong acids, strong alkali
Drugs	Alcohol, narcotic drugs, therapeutic drugs
Immunological reactions	Anaphylaxis, autoimmune diseases
Genetic defects (DNA alterations)	Congenital malformations, inborn errors of metabolism
Nutritional imbalance	Starvation, protein energy malnutrition disorder (marasmus and kwashiorkor), obesity, vitamins deficiency
Cellular aging	Cellular degeneration, cellular senescence

- Extrinsic stressors of cell injury include physical agents (mechanical trauma, extremes of temperature—burns and deep cold), electrical injury, radiation, sudden fluctuations of the barometric pressure, chemical agents, alcohol consumption, carbon monoxide, lead, drugs, chemotherapeutic agents, immunosuppressive drugs, infectious agents (bacteria, viruses, fungi and protozoa interfere in maintaining cell integrity and its function), venoms, poisons, insecticides, carbon monoxide, asbestos, nutritional deficiency, and immunological reactions.

OXIDATIVE STRESS/HYPOXIA-INDUCED CELL INJURY

The ability to maintain oxygen homeostasis is essential to the survival of cells in each organism. Normally, O_2 diffuses down a gradient from the atmosphere to the alveoli, to plasma, and into the red blood cells (RBCs), where O_2 attaches to heme groups. Oxygen is essential for aerobic respiration.

- Hypoxia literally means 'low-oxygen' but is defined as a deficiency in the amount of oxygen that reaches the tissues/organs of the body.
- Hypoxia prevents normal oxidative phosphorylation process, and thus reducing the capacity of mitochondria to generate adenosine triphosphate (ATP). Without ATP, the cell is unable to maintain its vital functions.

Causes of Hypoxia

Hypoxia can result from inadequate blood supply of oxygen (atherosclerosis, hypotensive shock), obstruction of airways, inadequate oxygenation of blood in the lungs (lung diseases), inadequate oxygen transport (e.g. anemia), inadequate perfusion of blood in the tissues (e.g. heart failure) and inhibition of cellular respiration (e.g. inhibition of oxygen utilization by cyanide poisoning of respiratory enzymes).

- Hypoxemia refers to a low partial pressure of oxygen in the blood due to high altitude, hypoventilation or diffusion defect. Decreased venous drainage can also impair adequate oxygen to the tissues.
- Hypoxia can be classified as acute or chronic, with acute meaning a rapid onset, and chronic meaning that hypoxia has been ongoing for some time.
- Decreased oxygen carrying capacity occurs due to hemoglobin loss or dysfunction in the settings of anemia and carbon monoxide poisoning.
- Carbon monoxide (CO) is a harmful gas produced from firewood smoke and motor vehicles exhaust. When carbon monoxide is breathed, it binds hemoglobin more avidly than oxygen and forms carboxyhemoglobin leading to deprivation of oxygen to heart, brain and other vital organs. Early signs and symptoms in carbon monoxide poisoning are headache, and cherry-red appearance of skin. Significant exposure to carbon monoxide results in loss of consciousness and death.
- Methemoglobinemia is a condition of elevated methemoglobin (normal range 1%) in the circulating red blood cells. It can occur due to inherited disorder or acquired causes such as benzocaine, dapsone or nitrates. The underlying mechanism involves some of the iron in hemoglobin being converted from ferrous (Fe^{++}) to the ferric (Fe^{+++}) form, that

cannot bind oxygen that leads to decreased oxygen supply to the tissues/organs. **Cyanosis** occurs, when methemoglobin level in the red blood cells reaches around 15%. Symptoms may include headache, dizziness, shortness of breath, nausea, poor muscle coordination and blue-colored skin (cyanosis). Complications of methemoglobinemia may include seizures and cardiac arrhythmias. Methylene blue is an effective treatment for reducing methemoglobin, however, it is associated with adverse reactions in glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals. Abnormal hemoglobins inducing hypoxia are given in Table 1.18.

Mechanism of Hypoxia-induced Cell Injury

Hypoxia implies damage to the cells/tissues resulting from reduced oxygen availability due to poor hemoglobin saturation, inadequate ventilation and hemolysis. Causes of hypoxia are ischemia, hypoxemia, and decreased oxygen carrying capacity of blood.

- Ischemia is a state of significantly reduced blood flow due to thrombotic occlusion and trauma which leads to tissue damage if not reversed.
- Oxygen deprivation is the most common cause of cell injury. Oxygen is the final electron acceptor in the electron transport chain of oxidative phosphorylation in mitochondria.
  - Early consequences of ischemia injury include transient shift of anaerobic glycolysis, disturbed ionic and fluid balance and inhibited β-oxidation of fatty acids.
  - Late consequences of ischemic injury include lysosomal activation and leakage of proteins such as creatine kinase, troponins, myoglobin, cellular enzymes into blood.
- Reduced oxygen availability impairs oxidative phosphorylation in mitochondria resulting in decreased ATP (essential source of energy) production. Adenosine triphosphate (ATP) depletion results in failure to energizes several important homeostatic processes leading to ischemia-induced necrotic cell death.
- During ischemia, the affected cells become dependent on anaerobic glycolysis for their ATP supply, which leads to an accumulation of lactate and NAD^+, and therefore causes a drop in cytosolic pH.
- Calcium and reactive oxygen species (ROS) are common players in necrotic cell death irrespective of the stimulus. ATP depletion results in additional cell damage by causing failure of energy-dependent enzymes, especially the cell membrane ATP ion pumps ($Na^+/K^+/Cl^-/Ca^{++}$) that control cell volume and electrolyte balance, which results in rapid intracellular increase of calcium, sodium and chloride leading to cell swelling.
- Increased cytosolic calcium results in the activation of signaling kinases such as mitogen-activated protein kinase (MAPK or MAP kinase) and calmodulin kinase, which act as transcription factors. Elevated cytosolic calcium levels result in modification of cytoskeleton, plasma cell membrane blebbing, activation of phospholipases, proteases and endonucleases, which seem to trigger cell membrane damage through phospholipase, proteins degradation through protease and DNA degradation through endonuclease.
- Mitochondria are the major site of ATP production and also one of the most vulnerable organelles of the cell. Mitochondrial injury leads to ATP depletion and increased permeability of mitochondrial membranes with resultant loss of calcium homeostasis and activation of cellular enzymes such as phospholipases, proteases and endonucleases. Activated cellular enzymes inflict damage to membranes (plasma membrane, mitochondrial membrane and other organelles membranes), structural molecules (lipids, proteins) and enzymatic proteins, and nucleic acids.
- The excessive reactive oxygen species (ROS) such as superoxide, hydroxyl radical, hydrogen peroxide formed in the mitochondria cause damage to the lipids, proteins, DNA, membrane integrity (plasma membrane and organelles) consequently results in $Na^+/K^+/Cl^-/Ca^{++}$ pump failure, ionic balance deregulation, mitochondrial dysfunction and cell swelling.

Table 1.18 Abnormal hemoglobins inducing hypoxia

Hemoglobin	Acquired Alteration	Abnormal Function	Spectroscopy Hemoglobin Maximum Absorption Band
Methemoglobin	Hemoglobin iron in ferric form	Methemoglobin cannot with oxygen	630 nm (chocolate color)
Sulfhemoglobin	Sulphur combines with hemoglobin	Sulfhemoglobin has 1/100 oxygen affinity of HbA	620 nm
Carboxyhemoglobin	Carbon monoxide combined with hemoglobin	Affinity of carboxyhemoglobin is 200 times higher than oxygen	541 nm

Table 1.19 Vulnerability of cells (organs) to ischemic irreversible cell injury

Cells/Tissues Vulnerable to Ischemia	Duration of Irreversible Injury
Neurons (cerebellum's Purkinje cells and hippocampus area most susceptible to injury)	3–5 minutes
Myocardium	20–30 minutes
Hepatocytes	1–2 hours
Intestine	8–16 hours
Skeletal muscle	24–72 hours
Fibroblasts	Resistant to ischemic injury

Watershed areas are present mesenteric arteries and cerebral arteries.

- Reactive oxygen species (ROS) can react with the nucleic acids attacking the nitrogen bases and the sugar phosphate backbone and evoke single-stranded or double-stranded DNA breaks. The change of nuclear morphology upon induction of necrosis includes pyknosis, karyorrhexis and karyolysis.
- Furthermore, necrotic cells release immunomodulatory factors that result in recognition and engulfment of necrosed cells by phagocytes and the subsequent immunologic response.
- Hypoxia adversely affects function of brain, heart and liver. Hypoxic cell injury depends on type of tissues undergoing necrosis. Vulnerability of cells (organs) to ischemic irreversible cell injury are given in Table 1.19.
- Ischemia-reperfusion injury occurs when blood flow is restored, cells that survived the initial ischemia may now be irreversibly injured by process initiated by oxygen-derived free radicals, inflammatory cells, or activation of the complement cascade.

Hypoxia-induced Brain Injury

Border zone or watershed infarcts are ischemic lesions that occur in characteristic locations at the junction between two main arterial territories.

- Progressive hypoxia of brain in the setting of hypotension and hypovolemia progresses and eventually becomes irreversible within 3–5 minutes of ischemia except if oxygenation is restored.
- The most vulnerable regions to oxygen deprivation (hypoxia) seem to be the brainstem especially cerebellum's Purkinje cells, hippocampus and cerebral cortex.
- Prolonged brain hypoxia can cause coma, seizures and even brain death. Histologically, these can be wedges of cortical and subcortical infarction.
- Extracellular brain adenosine levels are low under normal conditions, but increase substantially in response to hypoxia.
- Adenosine acts as an inhibitory neurotransmitter in the brain through the activation of four specific G protein-coupled receptors (A1, A2B and A3 receptors), that facilitates sleep and dilates the blood vessels probably to ensure good oxygen supply to brain during sleep.
- In contrast, a robust and prolonged activation of the G protein-coupled receptor A3 adenosine receptor has been observed to trigger cell death by either necrosis or apoptosis.
- During brain hypoxia, low oxygen quickly results in a fall of ATP production and consequent increase in adenosine. Both these alterations prevent the hypoxia-induced membrane depolarization resulting in reduction of Ca^{++} influx and K^{+} efflux. Adenosine acts as a neuroprotective agent in cerebral hypoxia or ischemia by suppressing neurotransmitter through synaptic receptors.
- The regulation and generation of extracellular adenosine in response to oxidative stress are critical in tissue protection.

Hypoxia-induced Myocardial Infarction

Myocardial infarction develops within 20–30 minutes of complete cessation of blood flow to a portion of the myocardium. It is closely associated with coronary artery disease induced by modifiable risk factors (tobacco smoking, hypertension, hyperlipidemia, diabetes mellitus, abdominal obesity, lack of physical activity) or non-modifiable risk factors (advanced age, male gender, genetics).

- When the coronary artery is occluded due to atheromatous plaques, the myocardium is deprived of the oxygen.
- Prolonged deprivation of oxygen supply to heart leads to the myocardial cell death and necrosis. Myocardial infarction may be associated with ECG changes and elevated biochemical markers such as cardiac troponins and enzymes (CK-MB, LDH and SGOT).
- Patient presents with crushing chest pain lasting for more than 20 minutes that can radiate to the neck, jaw, shoulder or arm, breathlessness, nausea, palpitations or silent without symptoms. As myocardial infarction is associated with several serious complications. These patients are best managed in intensive coronary care unit.

Hypoxia-induced Hepatocellular Injury

A diverse set of ischemic, metabolic, toxic and hepatotropic viruses, inflammatory insults result in hepatocellular injury. Interruption of blood supply to liver

leads to irreversible hepatocellular injury after 1–2 hours of hypoxia.

- Necrosis and apoptosis are the most widely recognized forms of hepatocyte cell death.
- Necrosis occurs due to ATP depletion during ischemia or hypoxic cell injury, excessive formation of reactive oxygen species (ROS) during reperfusion of ischemic tissue, acetaminophen toxicity, xenobiotics toxicity, and sustained increase in intracellular calcium.
- Oncotic necrosis is characterized by cell swelling, organelle swelling, formation of blebs in plasma membrane, cell rupture, release of lysosomal enzymes into extracellular environment, and inflammatory response.

Bowel Ischemia

Bowel ischemia can affect small intestine or large intestine as a result of reduction in arterial blood flow to these regions within 8–16 hours.

- Intestinal tract receives dual blood supply from two most distal branches of superior mesenteric artery and inferior mesenteric artery. The celiac artery has also collateral branches to supply intestine.
  - Superior mesenteric artery supplies the bowel from lower part of the duodenum to proximal two-thirds of the transverse colon.
  - Inferior mesenteric artery supplies the bowel from the distal one-third of the transverse colon to the rectum.
- Watershed area is the region in intestine where blood supply does not overlap and thus is susceptible to hypoxia injury.
  - Two main watershed regions between two major arteries that supplying colon include splenic flexure (Griffiths point) and rectosigmoid junction (Sudek's point), which are prone to ischemia.
  - Splenic flexure is the watershed area between superior mesenteric and inferior mesenteric artery, and the rectosigmoid junction is the watershed region between inferior mesenteric artery and superior rectal artery.
- Patients may develop small intestine (mesenteric) ischemia or colon ischemia. Clinical manifestations in a case of colon ischemia are abdominal pain, tenderness, rectal bleeding or bloody diarrhea. An abdominal computed tomography (CT) scan is done in hemodynamically stable patients who present with acute abdominal pain. CT angiography and MRI scan are performed to diagnose highly suspicious intestinal ischemia.

Hypoxia-induced Rhabdomyolysis in Skeletal Muscle

Skeletal muscle ischemia, damage and eventual necrosis lead to rhabdomyolysis within 24–72 hours. The classic presentation in a case of rhabdomyolysis includes myalgia, weakness and dark urine. Physical examination demonstrates skeletal muscle tenderness. Creatine phosphokinase activity is the most sensitive indicator of skeletal muscle damage; it may continue to increase for several days.

- Calcium may be deposited in damaged skeletal muscle. Lactic acidosis and anion gap metabolic acidosis can result from release of other organic acids from cells.
- Rhabdomyolysis is particularly common in people with diabetes mellitus or sickle cell anemia.
  - Complete interruption of blood supply to skeletal muscle causes irreversible cell death within many hours. In uncontrolled diabetes mellitus patients, skeletal muscle ischemia occurs from thrombosis of medium or small arterioles in the setting of atherosclerosis.
  - Skeletal muscle ischemia leads to severe pain with a palpable mass with or without swelling of especially vastus thigh skeletal muscle and calf skeletal muscle. The ischemia may start in the calf skeletal muscle and progress to the vastus skeletal muscles.
  - Bilateral lower limbs involvement is observed in more than one-third of cases. More than 50% of cases have coexistent diabetic nephropathy, neuropathy and retinopathy.

Cardiac Fibroblasts Resistant to Hypoxia

Fibroblasts are mesenchymal cells, abundantly distributed in connective tissues of most organs, which synthesize the extracellular matrix and collagen types 1, 3 and 4, including proteoglycans, glycosaminoglycans, fibronectin, laminins, metalloproteinases. Fibroblasts produce the structural framework for tissues (skin, lung, heart, kidney, liver, eye and other organs). These also play a critical role in wound healing.

- Cardiac fibroblasts represent the largest population of interstitial cells in myocardium surrounding cardiomyocytes.
  - Cardiac fibroblasts are resistant to hypoxia, which rather protect cardiomyocytes against lethal ischemia-reperfusion injury and so play important roles in the cascade of events after ischemic injury.
  - Cardiac fibroblasts transform into myofibroblast produce α-smooth muscle actin.

- Myofibroblasts play critical role in cardiac remodeling by forming collagen-rich scar that allows the infarcted region to maintain structural integrity after cardiomyocyte death.

ISCHEMIA-REPERFUSION-INDUCED CELL INJURY

In the clinical settings of myocardial infarction, cerebral stroke, organ transplantation and peripheral vascular disease, restoration of blood flow to the diseased organs triggers further ischemic cellular damage, this paradoxical effect is known as reperfusion injury. It is a challenge for the clinicians to control further cell damage and restore organ functions.

- The role of nitric oxide (an endothelial-derived relaxing factor) as a cardioprotective agent against reperfusion injury, has been demonstrated as nitric oxide works to inactivate reactive oxygen species, thereby, ameliorating the process of reperfusion injury.
- The extent of cell/tissue injury is directly related to the extent of reduction in blood flow and duration of ischemic period, which influence the exchange of ions across cell membrane (sodium, potassium, hydrogen, chloride and calcium), oxidative phosphorylation in mitochondria and drop in intracellular pH (tissue acidosis occurs).
- By impairing ATPase-dependent ion transport across cell membrane, ischemia causes increased calcium levels (calcium overload) in the cellular cytoplasm and mitochondria. Cell volume regulatory mechanisms are also disrupted due to lack of ATP production, which induce lysis of cell organelles and plasma membranes.
- It is essential to restore the blood flow to hypoxia-induced tissue/organ injury. For example, percutaneous coronary angioplasty and fibrinolytic therapy are performed to restore blood flow to limit myocardial infarct size. The restoration of blood flow to ischemic tissues/organs causes additional myocardial damage, which is termed reperfusion injury. All tissues are susceptible to reperfusion injury, but susceptibility varies between tissues/organs.
- Ischemia-reperfusion injury influences the outcome of patients after myocardial infarction, cerebral stroke, organ transplantation and cardiovascular surgery. Reperfusion of the tissues produces paradoxical tissues/organs response that fuel to the production of reactive oxygen species (ROS) through the xanthine oxidase pathway released from activated neutrophils, sequestration of proinflammatory cells (neutrophils, macrophages and T cells) in ischemic tissue, endoplasmic reticulum stress and development of post-ischemic capillary absence of reflow as a result of decreased nitric oxide (vasodilator) production resulting in amplification of tissue injury.
- These pathologic events culminate in opening mitochondrial permeability transition pores in the inner mitochondrial membrane as a common end-effector of ischemia-reperfusion-induced ionic loss homeostasis, matrix swelling, outer membrane rupture, cell lysis and death.
- It is evident that therapeutic approaches will be effective only when multiple pathologic processes are targeted in the management of perfusion injury to tissues/organs. Pathophysiologic mechanisms of ischemia-reperfusion-induced myocardial injury are given in Table 1.20.

Table 1.20 Pathophysiologic mechanisms of ischemia-reperfusion-induced myocardial injury

Mechanism of Ischemia-reperfusion-induced Myocardial Injury	Biochemical Alterations	
Accumulation of ions	▪ Intracellular calcium workload ▪ Increased intracellular sodium	▪ Drop in intracellular pH with rapid normalization with upon reperfusion
Dissipation of mitochondrial membrane potential essential component in the process of energy storage during oxidative phosphorylation	Dysfunctional mitochondrial transition pore potential may result in sustained transport of ions and proteins essential for normal functioning of mitochondria	
Reactive oxygen species (ROS)	▪ Xanthine oxidase pathway ▪ Release of mitochondrial intermediates	▪ Neutrophil infiltration
Dysregulated nitric oxide (NO) metabolism	▪ Loss of nitric oxide (an endothelial-derived relaxing factor)	▪ Accumulation of reactive peroxynitrite
Endothelial dysfunction	▪ Cytokine and chemokine signaling ▪ Expression of cellular adhesion markers	▪ Impaired vasodilation
Platelets aggregation and microembolization	▪ Platelets aggregation	▪ Microembolization
Immune system activation	▪ Innate immunity (e.g. complement activation and expression of toll-like receptors) ▪ Neutrophil accumulation	▪ Cell-mediated damage (macrophages and T cells)

PATHOGEN-INDUCED CELL INJURY

Induction of host cell death either directly or indirectly has been demonstrated in several cases by invading pathogens such as bacterial, viral, parasitic and fungal infections, which induce diverse responses in the host that include activation of innate immune response, inflammation and cell death.

- The death of an infected cell can promote efficient pathogen clearance. Destruction of infected tissues may also eliminate a pathogenic niche thereby inhibiting pathogen replication and dissemination.
- *Mycobacterium tuberculosis* thrive within immature phagosome of the infected cells by inhibiting phagosome-lysosome fusion. Phagocytosis of the apoptotic bodies sequestering pathogens permits more efficient fusion of phagosome with lysosome resulting in the degradation of the pathogen.
- Apoptosis of alveolar macrophages in *Streptococcus pneumoniae* infection results in pathogen elimination rather than evasion of the immune system.
- *Chlamydia* species are a group of obligatory bacteria that replicate inside of cytoplasm and protect infected cells from death during invasive stages of disease, presumably by blocking cytochrome c release from mitochondria.
- Rickettsia continues to replicate within cells through stimulating NF-κB signaling that prevents cell death.
- Pathogen-induced cell death is manifested by various distinct morphologic and biochemical characteristics indicative of oncotic necrosis, apoptosis, pyroptosis or autophagic cell death. Oncotic necrosis is highly inflammatory process that favors pathogen dissemination.Apoptosis and autophagy do not induce inflammation that are beneficial to the host.

Oncotic Necrosis

Necrosis is the term currently used for nonapoptotic, accidental cell death. It is generally regarded as a severe form of injury to cell membrane integrity. Oncotic necrosis can be induced by pathogens. The term 'oncotic necrosis' is defined as cell death with swelling (oncosis), that can be caused by ischemia, and toxic agents that interfere with ATP generation by mitochondria and plasma membrane ($Na^+/K^+/Cl^-/Ca^{++}$) pumps failure. Oncotic necrosis is characterized by cell swelling (oncosis), cell organelles swelling, plasma cell blebbing, loss of plasma membrane integrity, increased membrane permeability, chromatin fragmentation (pyknosis, karyorrhexis, karyolysis), release of lysosomal enzymes into extracellular spaces, eosinophilic cytoplasm, inflammation, vascularization and tissue repair.

Apoptosis

Several pathogens have been reported to trigger **apoptosis** through several mechanisms including bacterial toxins and virulence factors that interact with cell machinery.

- Apoptosis is a programed cell death mediated by initiator caspase enzymes (2, 8, 9, 10)/executioner caspases (3, 6, 7), which are cysteinyl aspartate-specific proteases.
- During apoptosis, pathogens are contained within apoptotic bodies and digested in the lysosomes of macrophages and surrounding cells, that phagocytose apoptotic bodies. It is worth mentioning, infected epithelial cells and lymphocytes undergo apoptosis.

Heterophagy

Heterophagy is the process by lysosomes digest material from the extracellular environment. Extracellular pathogens are taken up by the cells through the general process of endocytosis, which eventually fuse with lysosomes to form phagolysosomes, where engulfed material is digested in neutrophils and macrophages. Examples of heterophagocytosis include uptake and degradation of bacteria by neutrophils and removal of apoptotic cells by macrophages.

Pyroptosis

Pyroptosis is a process of cell death mediated by the activation of caspase 1, a protease that detects pathogen in the cytosol and also activates the inflammatory cytokines, IL-1β and IL-18. Pyroptosis also features cell lysis and release of cellular contents. It is worth mentioning that macrophages and dendritic cells die primarily by pyroptosis.

PHYSICAL AGENTS INDUCED CELL INJURY

Physical agents capable of causing cell injury include mechanical trauma, extremes of temperature (burns and severe cold), electric shock, radiation and sudden changes in atmospheric pressure.

Physical Trauma

The type of tissue injury is incurred with the type and severity of trauma (blunt injury, crush injury and gunshot wound) and the type of structures involved. Several broad categories of tissue injury are recognized: abrasion, contusion (bruise) and laceration.

- **Skin abrasion:** Skin abrasion is minor form of injury of superficial layers of epidermis. Healing of skin abrasion is rapidly achieved by regeneration of epidermal cells from the remaining deeper basal epidermal layers, and there is no scarring.

- **Contusion (bruise):** Contusion (bruise) in skin, myocardium and brain usually results from blunt trauma. Blunt trauma injures blood vessels leading to extravasation of blood into the tissue. The bleeding is usually rapidly controlled by hemostatic mechanisms.
  - The red blood cells present in the injured tissue slowly undergo degradation. The various pigments derived from the breakdown of hemoglobin are responsible for the change in the color from red through purple, black, green and brown in contusion site.
  - Histologic examination of such contusion in tissue demonstrates hemosiderin-laden macrophages that signify evidence of hemorrhage. In more severe injuries, collection of sufficient blood in the tissues produces a distinct swelling called hematoma.
  - Contusions are dangerous in patients with hemophilia as a result of massive bleeding in the soft tissues, skeletal muscles and joints.
  - Myocardial contusion may cause cardiac arrhythmias and acute cardiac failure.
  - In head injuries, contusions are usually formed in the inferior frontal lobe due to movement of the brain against the irregular bony surface of base of the skull in the anterior cranial fossa. Cerebral lesions represent foci for possible development of epileptic seizures.
- **Gunshot wound (GSW):** A gunshot wound is an example of physical trauma caused by a bullet from a firearm. A gunshot wound can produce penetrating wound, in which the bullet enters the body but does not exit. In re-entry wounds, the bullet passes through a body segment, exits and re-enters the body. Such physical trauma can cause bleeding, bone fracture, organ damage, infection of the wound or loss of the movements of the affected body part.

Extremes of Temperature

Extreme of temperature can adversely affect cellular functions. Temperatures between 46° and 60°C induce irreversible cellular injury proportional to the exposure time. Between 60° and 100°C, protein coagulation occurs instantly with irreversible cell injury of key cytosolic and mitochondrial enzymes and nucleic acid–histone complexes. Frostbite and extreme hypothermia can also cause impairment of cellular functions.

- **Frostbite:** Frostbite is a dire condition in which skin and tissues freeze after being exposed to extreme cold for a prolonged time. Frostbite most commonly occurs on the fingers, toes, nose and ears, that can lead to severe and permanent damage to blood vessels and tissues.
- **Extreme hypothermia:** Extreme hypothermia can cause heart, nervous system and other organs to enter a state of shock that may increase risk of myocardial infarction, respiratory failure and possibly fatal outcome. Symptoms of hypothermia include shivering, slow breathing, lack of coordination and confusion, cold air can cause wheezing and shortness of breath.
- **Heat stress:** Heat stress (higher than 50°C) is a physical stressor that stimulates excessive production of reactive oxygen species (ROS) in the mitochondria, triggering protein and DNA oxidative damage as well as lipid peroxidation of membranes and cell death. Excessive production of ROS has been associated with the number of pathologic disorders, including infertility, ischemic-perfusion injury, bone disorders, neurological disorders and aging by means of lipid peroxidation.

CHEMICAL AGENTS INDUCED CELL INJURY

Exposure to chemical agents or drugs do not result in overt tissue injury, but may adversely affect cellular functions in a more subtle way, thereby increasing the susceptibility to other forms of cell damage.

Acute Cellular Toxicity

Some inert gases and chemical agents cause oxygen deprivation and induce cellular toxicity in central nervous system.

- **Carbon monoxide (CO):** Carbon monoxide competes with oxygen for binding to hemoglobin and therefore causes oxygen deprivation to tissues for cellular metabolism resulting in cell death.
- **Cyanide poisoning:** Cyanide poison damages to mitochondrial cytochrome oxidase and thus inhibits oxidative phosphorylation resulting in impairment of cellular metabolism and utilization for energy. Methemoglobin has strong affinity to cyanide ion and keeps cyanide ions away from the target cells, hence it forms the basis for antidotal therapy.
- **Solvents used for sedation/anesthesia:** Some solvents used for sedation/anesthesia interact with the membranes of cells in the nervous system, which impair transmission of electrical and chemical signals. Strong acids and alkaline chemical agents can cause irritation, chemical burns and possible scarring.
- **Skin sensitization with 2,4-dinitrochlorobenzene:** Skin sensitization occurs, when 2,4-dinitrochlorobenzene binds with natural proteins in the skin resulting in formation of altered protein-bound complex. Subsequent exposure to 2,4-dinitrochlorobenzene triggers immune system response, which recognizes

and eliminates the altered protein-bound complex by releasing cytokines resulting in dermatitis.

- **Lung injury with toluene diisocyanate (TDI):** Toluene diisocyanate is easily absorbed by lungs. Lung sensitization occurs in workers on exposure to toluene diisocyanate. Subsequent prolonged exposure to toluene diisocyanate causes acute lung injury characterized by pulmonary edema, bronchial constriction and impaired breathing. Patient presents with progressive breathlessness, dry cough, arthralgia and headache.

Long-term Chronic Cellular Toxicity

On frequent exposures or acute exposure, chronic toxicity can produce irreversible cellular adverse effects. An example of chronic toxicity related to cigarette smoking and lung cancer. The majority of toxic effects are due to specific biochemical interactions without causing recognizable damage to a cell or its organelles.

- Toxic effects to cell and its organelles include: (a) interference with a chemical agent that transmits a message across a neural synapse such as the inhibition of the enzyme cholinesterase by organophosphorus pesticides, and (b) when one toxic chemical agent inhibits or replaces another essential chemical agent such as the replacement of oxygen on the hemoglobin molecule with carbon monoxide.
- Toxic damage to cells and tissues can be transient and nonlethal or lethal. There are four main endpoints to the cellular or biochemical toxicity.
  - Cells and tissues may be completely repaired and return to normal.
  - Cells and tissues may be incompletely repaired but are capable of sustaining its function and reduced capacity.
  - Complete loss of tissue organ can cause death of organism. In certain instances, the organism can continue to survive with the aid of medical treatment or organ transplantation.
  - Toxic agents can induce malignant transformation associated with fatal outcome. Some patients can be treated by medical and/or surgical treatment.

Acetylcholinesterase Enzyme Inhibitors

Acetylcholinesterase enzyme is involved in the termination of impulse transmission by rapid hydrolysis of the neurotransmitter acetylcholine in many cholinergic pathways in the central and peripheral nervous systems.

- Any pesticide such as organophosphates (OPs) and the carbamates (CMs) bind and inhibit acetylcholinesterase enzyme, making it unable to induce hydrolysis of acetylcholine, that leads to accumulation of acetylcholine, hyperstimulation of nicotine and muscarinic receptors and disruption of neurotransmission resulting in various symptoms, including respiratory arrest and death.
- The primary treatment of pesticide is the administration of atropine, which blocks the adverse effects of acetylcholine, and the administration of pralidoxime chloride, which reactivates acetylcholinesterase enzyme.

Metabolic Activation of Chemical Agents

Many chemical agents such as carbon tetrachloride (CCl_4), chloroform, 2-acetylaminofluorene, nitrosamines and paraquat are metabolically activated to form oxygen-derived free radicals or reactive intermediates, which inhibit and interfere with normal cellular function.

- Carbon tetrachloride (CCl_4) can induce hepatocellular injury through its metabolic products, that are generated by a cytochrome P450 enzyme dependent step. Immediately within minutes of exposure to carbon tetrachloride, it leads to modest accumulation of malondialdehyde formation, a fall in cellular glutathione and substantial injury in isolated hepatocytes.
- Subsequent exposure to carbon tetrachloride leads to increased malondialdehyde formation, oxygen-derived free radicals induced lipid peroxidation, a fall in cellular glutathione and substantial hepatocellular damage.

Malignant Transformation in Injured Cells

The fundamental abnormality resulting in development of cancer is the continued and unregulated proliferation of injured cells. Cancer can involve in any tissue of the body and have many different forms in each body area. The vast majority of cancer cases are due to environmental risk factors. Alterations in DNA involving critical genes can cause increased susceptibility to malignant lesions. DNA damage can be induced by exposure to natural and synthetic chemical agents and physical agents (ultraviolet radiation, radon from the soil, γ-radiation from medical procedures). Genetic syndromes predispose to development of cancer.

DRUG-INDUCED CELL INJURY

The mitochondria are a frequent subcellular target that is involved in the toxicity of therapeutic drugs. Following drug administration, inhibition of mitochondrial complex 1 activates pathway of cell death.

- The mitochondrial complex 1 inhibition causes ATP depletion, reactive oxygen species (ROS) production, reactive nitrogen species (RNS) production from the metabolism of L-arginine amino acid, and sirtuin 3 inhibition.

- Peroxynitrite production is a major mechanism of mitochondrial-mediated cell injury. The antiviral drug efavirenz (EVF) induces mitochondrial complex 1 inhibition. Iron porphyrins provide protection to hepatocytes against antiviral EVF-induced lethal injury.

PATHOPHYSIOLOGY OF CELL INJURY, MORPHOLOGIC PATTERNS AND OUTCOME OF NECROSIS

Depending on severity of cell injury, various cellular changes occur: (a) reversible cell injury, in which cells remain in hemodynamic equilibrium, (b) irreversible cell injury results in cell death (necrosis) and tissue damage, (c) ischemic/reperfusion injury.

- **Reversible cell injury** is usually the result of the initial stages of lack of oxygen supply (hypoxia) or lack of blood flow to the cells. Inadequate ATP supply impaired membrane $Na^+/K^+/Ca^{++}$ pumps resulting in cellular swelling, lipid accumulation, and cellular 'blebbing' on the plasma membrane.
  - Decreased pH causes swelling of endoplasmic reticulum, detachment of ribosomes from the endoplasmic reticulum leading to decreased protein synthesis. Low ATP production or ATP depletion disrupts key cellular functions.
  - Bleb formation in plasma membrane results from outpouching from the cell membrane to accommodate more water.
  - Myelin figures are aggregates of damaged cellular membranes in cell injury, which are demonstrated as intracellular whorls of laminated phospholipid material resembling myelin sheath of nerves. Presence of membrane bound structures containing lysosomal enzymes are known as myelin or myelinoid bodies.
- In irreversible cell injury, sustained hypoxia results in ATP depletion that impairs membrane calcium pump leading to increased cytosolic calcium.
  - Cells switch from aerobic glycolysis to anaerobic respiration to produce ATP, which results in accumulation of lactic acid, which decreases the cellular pH leading to dispersion of ribosomes. Mitochondrial permeabilization results in increased cytosolic calcium that activates phospholipases, proteases, endonucleases and DNAases, which damage the cell.
  - Nuclear fragmentation occurs via pyknosis, karyorrhexis, and karyolysis. Membrane permeabilization leads to release of lysosomal enzymes into extracellular spaces, leakage of proteins (creatine kinase, troponins, myoglobin, cellular enzymes) into blood, inflammation, vascularization and tissue repair. Schematic representation of pathophysiology of reversible and irreversible cell injury is shown in Fig. 1.15. Ultrastructural differences between reversible cell injury and irreversible cell injury are given in Table 1.21.

Pathology Pearls: Reversible Cell Injury versus Irreversible Cell Injury

- The hallmark of reversible injury is cellular swelling, swelling of endoplasmic reticulum and loss of microvilli.
- The hallmark of irreversible injury (cell death) is damage to membranes and genetic apparatus via nuclear condensation (pyknosis), fragmentation (karyorrhexis), and dissolution (karyolysis). The death of large group of cells is followed by acute inflammation.
- Reversible cell injury can usually be reversed by withdrawal of the noxious stimuli; while irreversible cell injury has progressed past a point of 'no return' to normal state.
  - Reversible cell injury is usually the result of the initial stages of lack of oxygen supply or lack of blood flow to the cells; while irreversible cell injury involves more injurious agents such as viruses, immunological responses, or genetic damage.
  - Reversible injury results in cellular swelling, lipid accumulation, and cellular 'blebbing' on the plasma membrane.
- Irreversible cell injury results in acidosis of the cellular microenvironment, damage to plasma membrane and organelles membranes (mitochondria, and lysosomes).
  - Mitochondrial membrane damage results in loss of the electron transport system located in inner mitochondrial membrane; and leakage of 'cytochrome c' into the cytosol; that activates apoptosis.
  - Lysosomal membrane damage results in leakage of hydrolytic enzymes into the cytoplasm, which in turn, are activated by the high cytosolic calcium. The end result of irreversible injury is cell death.
- Reversible cell injury is pharmacologically treatable leading to recovery; while irreversible cell injury results in permanent cell loss and death.

REVERSIBLE CELL INJURY

Hypoxia is most important cause of cell injury, which can result from inadequate blood supply of oxygen, obstruction of airways, inadequate oxygenation of

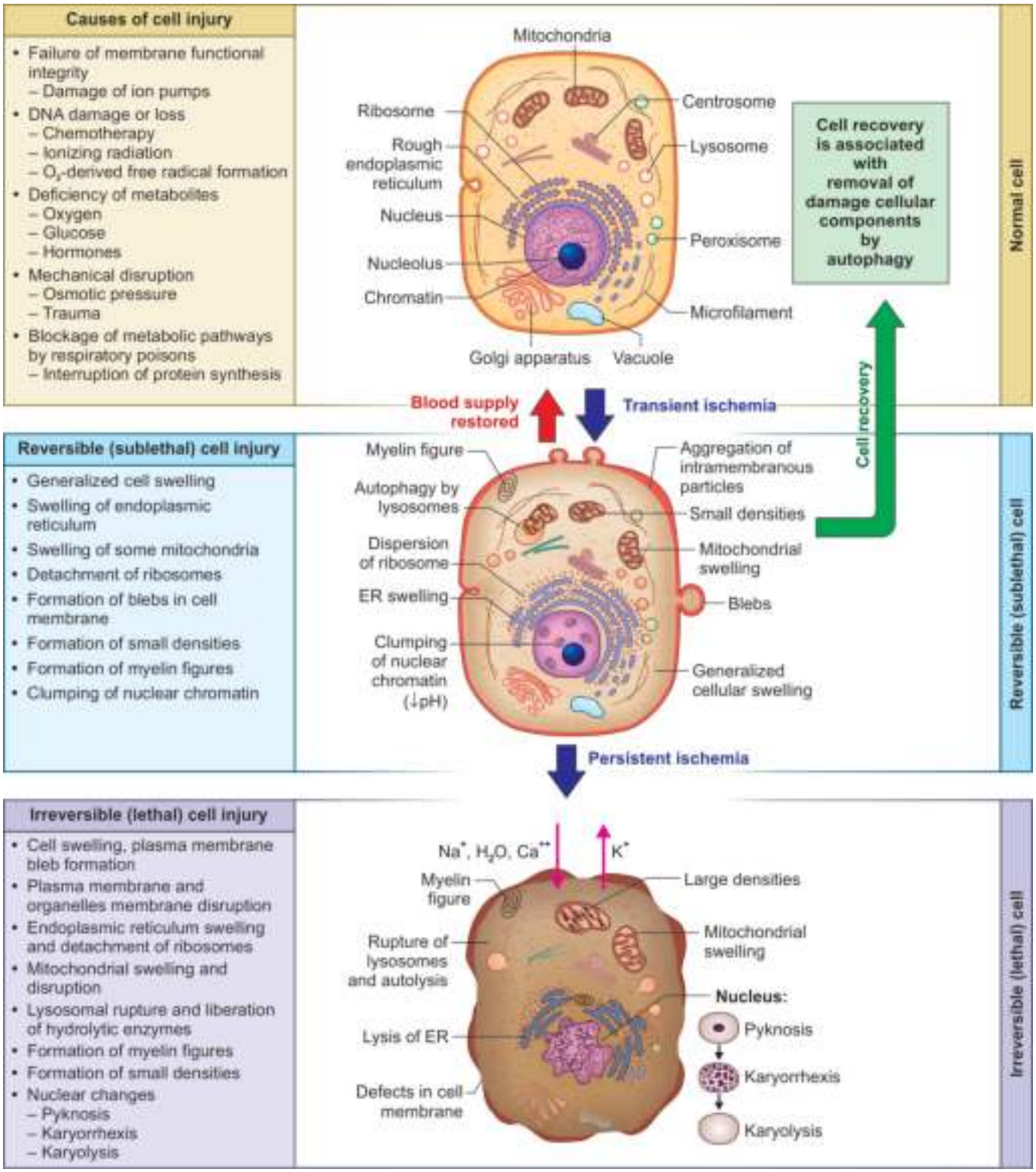


Fig. 1.15: Schematic representation of pathophysiology of reversible and irreversible cell injury. The main difference between reversible and irreversible cell injury is that the reversible cell injury can return to the normal state by altering homeostasis of the cells, whereas the irreversible cell injury cannot return to the viable state as the cell has passed the point of no return.

blood in the lungs, inadequate oxygen transport, inadequate perfusion of blood in the tissues and inhibition of cellular respiration. One global response of hypoxia in reversible cell injury is cellular swelling, that is a result of misfunctioning ion channels (Na^+/K^+ pump and calcium pump) in the plasma membrane.

Table 1.21 Ultrastructural differences between reversible cell injury and irreversible cell injury

Cellular Changes	Reversible Cell Injury	Irreversible Cell Injury
Adaptative response from cell	Adaptative response from cell is intact and in balance or better than cell injuries	Adaptative response from cell has been exceeded by cell injury
Cell membrane function	Sodium/potassium pump malfunction resulting in loss of homeostasis of fluids and ions; blebbing and loss of villi	Degradation of plasma membrane and organelle membrane occurs with lease of lysosomal enzymes
Global cellular response	Moderate cell swelling, increased lipid accumulation (fat storage)	Marked cell swelling, loss of cellular permeability, chemical absorption and toxin formation
Mitochondrial changes	Moderate mitochondrial swelling, rarefaction and accumulation of amorphous densities (precipitated calcium)	Marked mitochondrial swelling, leakage of cytochrome c into cell cytoplasm and accumulation of large amorphous densities (precipitated calcium) and aggregates of fluffy material (denatured proteins)
Endoplasmic reticulum	Swelling of endoplasmic reticulum with detachment of ribosomes	Swelling of endoplasmic reticulum with dissolution
Myelin figures	Myelin fibers (laminated structures derived from damaged plasma membrane and organelles) less prominent	Myelin fibers (laminated structures derived from damaged plasma membrane and organelles) more prominent
Lysosomal changes	Autophagy by lysosomes	Autolysis by rupture of lysosomes
Nucleus/chromatin changes	Aggregation of fibrillar elements in the nucleus	Dissolution of chromatin (pyknosis, karyorrhexis and karyolysis of nuclei)
Examples of type of cell injury	Hydropic change, fatty change in liver	Myocardial and cerebral infarction

DECREASED ATP PRODUCTION IN THE MITOCHONDRIA

Hypoxia impairs oxidative phosphorylation resulting in decreased adenosine triphosphate (ATP) production in mitochondria. Normally, ATP is required for the activity of Na^+/K^+/calcium pumps. Cell injury is induced, when ATP production in mitochondria reduces to 5–10% of normal levels.

- Decreased ATP supply causes impaired activity of Na^+/K^+/calcium pumps. It leads to influx of sodium and water in cells and efflux of potassium resulting in cell swelling, bleb formation on plasma membrane caused by disorderly function of the cellular cytoskeleton, and formation of myelin figures derived from damaged membranes of mitochondria, or rough endoplasmic reticulum and the plasma membrane. These cellular changes are demonstrated in cytoplasm on electron microscopy.
- Mild and transient injury results in reversible cell injury, in which cells can recover to their normal function. These cellular changes can be reversed if injurious noxious stimulus is removed.
- Reversible cell injury is acute sublethal injury to cytoplasmic organelles sparing nucleus in metabolically active cells of liver, heart and kidneys. In coronary artery disease, impaired myocardial contractility is reversed to normal on quick restoration of blood circulation in coronary arteries.
- Examples of reversible cell injury are hyaline change, hydropic change, and fatty change in the metabolically active cells of liver, heart and kidneys.

PLASMA MEMBRANE NA^+/K^+ PUMP FAILURE

Normally, adequate supply of ATP plays important role in regulating membrane. Ouabain sensitive Na^+/K^+-ATPase ion pump plays key role in maintenance of relatively high potassium and low levels of sodium concentration within cells.

- Cell membrane permeability is increased due to decreased ATP production. Decreased availability of ATP results in failure of the Na^+/K^+-ATPase pump, which causes influx of sodium ions along with water into cell. Potassium diffuses out of cell.
- These cellular changes cause swelling of endoplasmic reticulum as well as hydropic change in cytoplasm of cells. When intracellular acidosis threatens due to anaerobic glycolysis, hydrogen ions (H^+) are pumped out of the cell in exchange of Na^+ ion to maintain proper intracellular pH.

PLASMA MEMBRANE CALCIUM PUMP FAILURE

Under physiologic state, excess intracellular Ca^{++} is extruded by a calcium pump that is ATP-dependent. Increased Na^+ ion inhibits efflux of calcium due to activation of sodium/calcium exchanger. It leads to excessive accumulation of calcium in the cell resulting

in mitochondrial dysfunction and decreased oxidative phosphorylation.

PLASMA MEMBRANE BLEB FORMATION

Plasma membrane bleb formation on the surface of cell has been well observed in liver, brain, heart and kidney tissue during cell injury.

- Plasma membrane blebs are most likely caused by disorderly function of the cellular cytoskeleton. The formation of plasma membrane blebs may have unique injurious consequences specific for the organ in which they develop.
- For example, plasma membrane bleb formation during ischemic injury to proximal renal tubule cells may be shed into the tubules, promoting formation of tubular casts and obstruction of tubules. By phase contrast microscopy, plasma membrane blebs appear as bubble-like projections extending from the cell surface.

MYELIN FIGURES

Myelin figures are long, thin cylindrical structures derived from damaged membranes of mitochondria, rough endoplasmic reticulum and the plasma membrane; demonstrated in the cytoplasm on electron microscopy. Myelin figures are long, thin cylindrical structures.

Pathology Pearls: Examples of Reversible Cell Injury

Another global response in reversible cell injury is hyaline change, hydropic change, and fatty change (large lipid vacuoles in cytoplasm) in the metabolically active cells of liver, heart and kidneys.

Hyaline Change

This term hyaline change denotes a characteristic glassy, homogeneous, and eosinophilic staining of tissues demonstrated in hematoxylin and eosin-stained sections. Intracellular hyaline material is demonstrated as Russell bodies in plasma cells, reabsorption plasma protein droplets in proximal tubules, tumoral hyaline globules and Mallory alcoholic hyaline in liver.

Hydropic Change in Organs

- Hydropic change is acute sublethal reversible cell injury characterized by accumulation of fluid in cytoplasm resulting in pale and swollen cytoplasm.
- Hypoxia, chemical agents and pathogen toxins cause hydropic change by three mechanisms: (a) increasing the permeability of the plasma membrane to sodium, (b) damaging the membrane sodium potassium ATPase (pump), and (c) interfering with the synthesis of ATP, thereby depriving the pump of its fuel.
- Accumulation of sodium in the cell leads to an increase in water content to maintain isosmotic conditions, and the cell then swells.

Fatty Change in Organs

- Fatty change in organs is also known as fatty metamorphosis or steatosis. It is acute sublethal reversible cell injury characterized by accumulation of lipid droplets as a result of disturbance of ribosomal function and uncoupling of lipid from protein metabolism.
- Moderate degree of fatty change is in organs reversible, but severe fatty change may not be reversible. Fatty change is observed most frequently in the liver, heart, and kidney.
- Fatty change may be secondary to alcoholism, diabetes mellitus, protein malnutrition, obesity, acute fatty liver of pregnancy, congestive cardiac failure, methotrexate, steroid carbon and tetrachloride poisoning. It results from an imbalance between the uptake, utilization, and mobilization of fat from liver cells.
- Fatty change in liver can result from any of the mechanisms: (a) increased transport of triglycerides in hepatocytes, (b) decreased mobilization of fat from liver due to decreased synthesis of transporting apolipoproteins, (c) decreased utilization of fat by the cells, and (d) increased concentration of lipoproteins in cells.
- Fatty change is thus linked to the disaggregation of ribosomes and consequent decreased protein synthesis caused by failure of ATP production in CCl_4 induced injured cells.
- Fat accumulation in hepatocytes may disrupt the normal blood flow in the hepatic sinusoids. Fat accumulation may cause hepatocytes to become resistant to insulin. Oxidation of excessive fat stores in hepatocytes may generate free radical, that induces fibrosis resulting in liver damage. Mechanism of fatty change liver is shown in Fig. 1.16.

IRREVERSIBLE CELL INJURY (CELL DEATH)

Persistent severe cell injury transforms reversible into irreversible cell injury. **Necrosis** is defined as the cell death in living tissue, which results in the unregulated digestion of cell components. Lipid peroxidation and nuclear changes such as pyknosis, karyorrhexis, and karyolysis are signs of irreversible cell injury.

- **Causes of irreversible cell injury:** Irreversible cell injury is caused by environmental factors such as infections, toxins, mechanical trauma, ischemia, thermal damage from high or low temperature. Hypoxia is one of the severe cellular stressors, that can cause cell injury and even cell death. Cell injury results from functional and biochemical abnormalities in one or more of several essential cellular components such as mitochondria, plasma membranes, $Na^+/K^+/Ca^{++}$ ionic channels in cell membranes, rough endoplasmic reticulum, cytoskeleton and genetic appratus.
- **Pathophysiology of irreversible cell injury (cell necrosis):** Cell necrosis features membrane disruption, plasma membrane blebbing due to disorderly function of the cellular cytoskeleton, mitochondrial

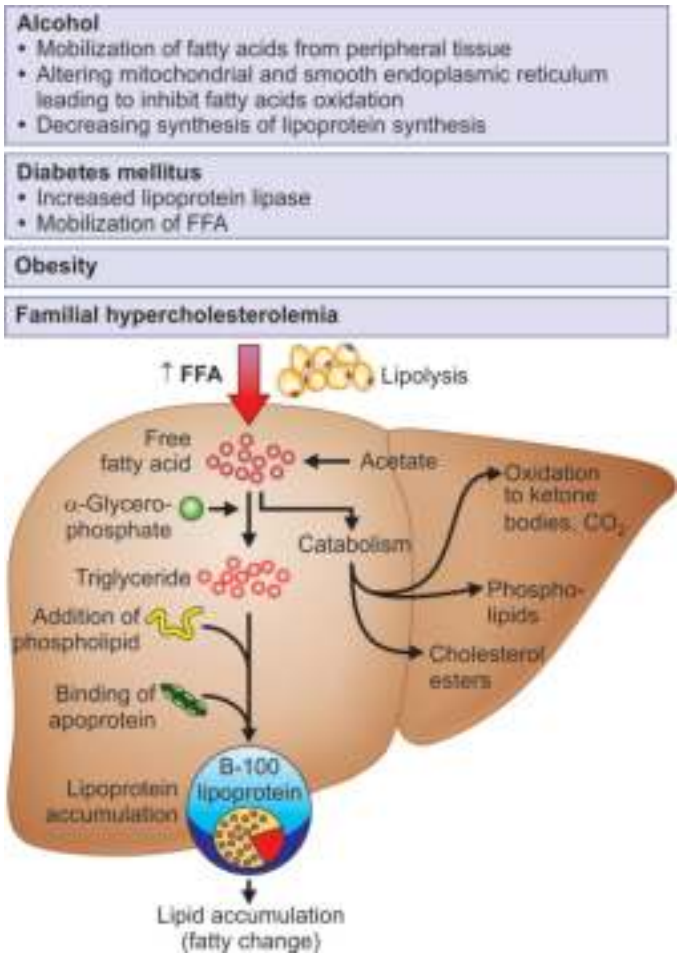


Fig. 1.16: Schematic representation of mechanism of fatty change in liver. Potential pathophysiologic mechanisms of fatty change in liver include the following: decreased mitochondrial fatty acid β-oxidation, increased endogenous fatty acid synthesis or enhanced delivery of fatty acids to liver, and deficient incorporation or export of triglycerides as very low-density lipoprotein (VLDL).

swelling, and cytoplasmic swelling, adenosine triphosphate (ATP) depletion, generation of reactive oxygen species, influx of calcium, random nuclear DNA fragmentation by different nucleases (pyknosis, karyorrhexis followed by karyolysis), formation of myelin figures derived from damaged membranes of mitochondria, or rough endoplasmic reticulum and the plasma membrane; demonstrated in cytoplasm on electron microscopy, uncontrolled release of cellular contents into the extracellular space resulting in initiation of inflammatory response attracting leukocytes.

- The leukocytes along with nearby phagocytic cells eliminate the necrotic cells and necrotic debris by phagocytosis. Initially, the tissue is infiltrated by neutrophils followed by accumulation of monocytes/macrophages. The sequence of events helps in establishing the date and time of tissue injury.
- On light microscopy, cellular outlines in irreversible cell injury are maintained with loss of structural details. Hematoxylin and eosin-stained section shows intense cytoplasmic eosinophilia of dead cell due to loss of cytoplasmic RNA and denaturation (coagulation) of proteins. Loss of basophilia is a sign of irreversible cell injury, indicating a cessation of protein synthesis.
- In irreversible cell injury (cell death), intracellular enzymes and proteins released from the dead cells into the blood circulation are used as diagnostic parameters. Creatine kinase-MB fraction enzyme and cardiac troponins are estimated in patients with myocardial infarction. Liver cell necrosis releases transaminases, alkaline phosphatase, glutamyltransferase and their levels are increased in blood circulation. Creatine kinase levels are increased in cardiac and skeletal muscle injury.

Pathology Pearls: Mechanism of Cell Injury

- Depletion of ATP
- Mitochondrial damage
- Influx of calcium and loss of calcium homeostasis
- Accumulation of oxygen-derived free radicals
- Defects in plasma membrane permeability
- Damage to endoplasmic reticulum
- Damage to genomic proteins

ATP DEPLETION IN MITOCHONDRIA

Mitochondria are surrounded by double-membrane system, consisting of inner and outer mitochondrial membranes separated by an intermembrane space. Inner membrane forms numerous folds which extend into the interior (matrix) of the organelle.

- In normal cells, adenosine triphosphate (ATP) synthesized by mitochondria oxidative phosphorylation is required for synthetic and degradation processes. Nutrients, such as glucose and fatty acids, as well as oxygen, enter the cell across the cell membrane.
  - Mitochondria play a critical role in the generation of metabolic energy derived from the breakdown of carbohydrates and fatty acids, which are converted to ATP by the process of oxidative phosphorylation in the interior folds.
  - The mitochondrial permeability transition pore (MPTP) opens and closes sporadically. Prolonged mitochondrial injury leads to sustained opening of MPTP and release of mitochondrial 'cytochrome c' from electron transport chain into cytosol. This process further diminishes ATP production. Under certain circumstances, it may also trigger apoptotic pathway resulting in cell death.

- Mitochondria are the earliest organelles affected in cell injury. Persistent hypoxia and cyanide poisoning cause injury to mitochondria leading to decreased oxidative phosphorylation and reduction in ATP production to 5–10% of normal levels.
- Depletion of ATP production in irreversible cell injury causes: (a) failure of oxidative phosphorylation in mitochondria decreases the delivery of O_2 and glucose to the cells, (b) failure of Na^+/K^+ pump results in influx of sodium, water and calcium, efflux of potassium ions, swelling of endoplasmic reticulum and loss of microvilli resulting in bleb formation, (c) failure of calcium pump results in excessive accumulation of calcium in the cell and thus mitochondrial dysfunction; because increased intracellular concentration of sodium inhibits calcium efflux, (d) increased anaerobic glycolysis results in overproduction of lactic acid by increased activities of phosphofructokinase and phosphorylase; hence mitochondria fail to oxidize lactic acid to pyruvate. Instead, pyruvate is reduced to lactate in cytosol, and its accumulation in the cytosol lowers the intracellular pH resulting in clumping of nuclear chromatin (pyknosis), karyorrhexis and karyolysis, (e) detachment of ribosomes results in decreased protein synthesis, and (f) lipid peroxidation is the oxidative degradation of lipids in the cell membrane resulting in defects in cell membrane.

Biochemical Alterations

In the initial stage, glucose metabolism (glycolysis) occurs in the cytosol, where glucose is converted to pyruvate. Pyruvate is then transported into mitochondria and oxidized to CoA, where its complete oxidation to CO_2 occurs via the citric acid cycle, yields bulk of usable energy ATP obtained from glucose metabolism. The oxidation of fatty acids also yields acetyl-CoA, which is similarly metabolized by the citric acid cycle in the mitochondria. The enzymes of the citric acid cycle are located in the matrix of mitochondria, which participate in the oxidative breakdown of both carbohydrates and fatty acids. Nutrients such as glucose and fatty acids, as well as oxygen, enter the cell across the cell membrane.

- Hypoxic injury results in an inadequate flow of glucose, fatty acids and oxygen to the cell. Persistent hypoxia and inadequate tissue perfusion lead to decreased anaerobic metabolic pathways for energy production. Consequences of mitochondrial damage are depletion of ATP production, formation of reactive oxygen species (ROS), formation of mitochondrial permeability pore and release of '**cytochrome c**'. Release of cytochrome c activates apoptotic pathway of cell death.
- Cellular ATP stores are rapidly used up. ATP production in mitochondria reduces to 5–10% of normal levels. In hypoxia, fall in cellular oxygen level results in rapid shift from aerobic to anerobic glycogen metabolism. Anaerobic glycolysis leads to the accumulation of lactic acid, that further causes reduction in ATP production in mitochondria for cellular function. Accumulation of excess of lactic acid in cell and blood results in lowering the intracellular pH, which interferes with cellular enzymes.
- Lactic acidosis reduces myocardial contractility, arteriolar responsiveness to further adrenaline and noradrenaline release, potentiating vasomotor collapse and stimulating the disseminated intravascular coagulation. However, low pH (acidemia due to accumulation of lactic acid) is beneficial in facilitating the release of oxygen from hemoglobin.
- Eventually, a large number of vasoactive, vasodilator and cytotoxic substances are released from injured cell into the blood circulation, resulting in progressive vasodilation, increased capillary permeability, myocardial contractility and eventually disseminated intravascular coagulation.

MITOCHONDRIAL DAMAGE

Damage to mitochondria is one of the hallmarks of irreversible cell injury. It is usually a common feature of all injurious pathways that follow cell injury.

- Two features that characterize irreversible cell injury (cell death) from reversible cell injury: (a) irreversible mitochondrial damage occurs that results in ATP depletion and lack of oxidative phosphorylation despite removal of the injurious stimulus; and (b) profound disturbances in cell membrane function occurs, that affect internal ionic concentrations and lysosomal enzyme activity.
- Mitochondria are damaged by increase in cytosolic calcium coupled with inorganic phosphate and fatty acids, reactive oxygen species, oxygen deprivation by ischemia/hypoxia; and defective turnover of mitochondrial proteins.
- It is worth mentioning that high inorganic phosphate and fatty acids alone cannot cause mitochondrial damage, but when coupled with high calcium are extremely damaging to a cell. High cytosolic calcium alone can still cause mitochondrial damage. If mitochondria are indeed damaged, then there are three major consequences discussed as under.

Mitochondrial Permeability Transition

Mitochondrial permeability transition (MPT) is defined as an increase in the permeability of the mitochondrial membranes to freely permit entry of molecules <1500 daltons molecular weight (MW).

- Outer membrane of the mitochondria contains proteins, that permit movement of molecules into the mitochondria. Because all molecules below 1500 daltons MW cannot be regulated due to presence of mitochondrial permeability transition brought about by a high conductance channel, the mitochondrial permeability transition pore (MPTP). All solutes <1500 daltons MW rush into and out of the cell.
- The mitochondria slowly swell as the MPTP channel opens. The opening of MPTP channel is triggered by increase in cytosolic calcium concentration. Thus, calcium interacts with calcium receptors on the matrix side of the MPTP.
- MPTP channel opening is triggered by abundance of reactive oxygen species. MPTP channel is closed, if there is high concentration of NADH, ATP, ADP and magnesium ions in the cell. MPTP contains cyclophilin D, that is crucial for the proper opening of MPTP.
- In clinical practice, it is essential to limit cellular injury to target cyclophilin D protein present in MPTP by an immunosuppressive drug, cyclosporine. In cases of ischemia/hypoxia, cyclosporine can act on cyclophilin D to reduce cellular damage.

Increase in Mitochondrial Oxidative Stress

Normally, antioxidant molecules such as glutathione are stored in the mitochondria to combat reactive oxygen species. As a result of opening of the MPTP channel, stored antioxidant molecules such as glutathione are removed leading to accumulation of reactive oxygen species within the cell. Further, improper oxidative phosphorylation by the compromised electron chain generates oxygen-derived free radicals resulting in cell necrosis.

Induction of Apoptosis

Apoptosis is a process of genetically programmed cell death that occurs in multicellular organisms. It is characterized by nuclear shrinkage, DNA fragmentation by endonucleases, cytoskeleton breakdown by proteases, membrane blebbing; and membrane-bound apoptotic bodies, which are phagocytosed by macrophages and adjacent cells. The mitochondrial damage results in sequestration of proapoptotic proteins such as 'cytochrome c' and caspases between the inner and outer membrane of mitochondria. As the cell permeability increases, these proteins and proapoptotic proteins leak into the cytosol and trigger cell death by apoptosis.

CALCIUM INFLUX AND IMPAIRED CALCIUM HOMEOSTASIS

Calcium pump ion transporter is present in the plasma cell membrane. It is responsible for the active transport of calcium out of cell for the maintenance of the steep calcium electrochemical gradient across the cell membrane.

- Normally, cytosolic calcium is maintained at extremely low levels. Excess intracellular Ca^{++} is extruded by a ATP-dependent calcium pump. Decreased ATP production causes failure of calcium pump across the plasma membrane. Increased cytosolic sodium inhibits efflux of calcium due to activation of sodium/calcium exchanger. Influx of calcium to the cytosol comes from the extracellular fluid and stores in mitochondria and smooth endoplasmic reticulum.
- Failure of the calcium pump leads to influx of Ca^{++} into the cell, activation of various enzymes such as phospholipase, protease, ATPase and endonuclease causing cell death. Influx of excess calcium in cytosol and activation of cellular enzymes is given in Table 1.22. Schematic representation of role of influx of excess calcium in cytosol and activation of cellular enzymes inducing irreversible cell injury is shown in Fig. 1.17.
- Activation of phospholipase degrades the membrane phospholipids releasing free fatty acids, which are potent mediators of inflammation. These free fatty acids also act as detergent that solubilize cell membrane. Hence, phospholipase results in decreased synthesis of phospholipids of all cellular membranes including cell organelles.
- Activated proteases attack the cytoskeleton and its attachments to the cell membrane resulting in disruption of interactions between cytoskeleton proteins and the plasma membrane; and formation of membrane plasma blebs, and alteration of shape of the cell. High cytosolic calcium activates ATPase, resulting in degradation of ATP.

Table 1.22 Influx of excess calcium in cytosol and activation of cellular enzymes

Phospholipase Cellular Enzyme
Membrane phospholipids degradation
Potent mediators of inflammation
Decreased synthesis of phospholipids in plasma cell membrane including mitochondrial membrane
Protease Cellular Enzyme
Disruption in interaction of cytoskeleton proteins to plasma cell membrane resulting in formation of membrane blebs and alterations of shape
ATPase Cellular Enzyme
ATP degradation
Endonuclease Cellular Enzyme
DNA fragmentation and dissolution

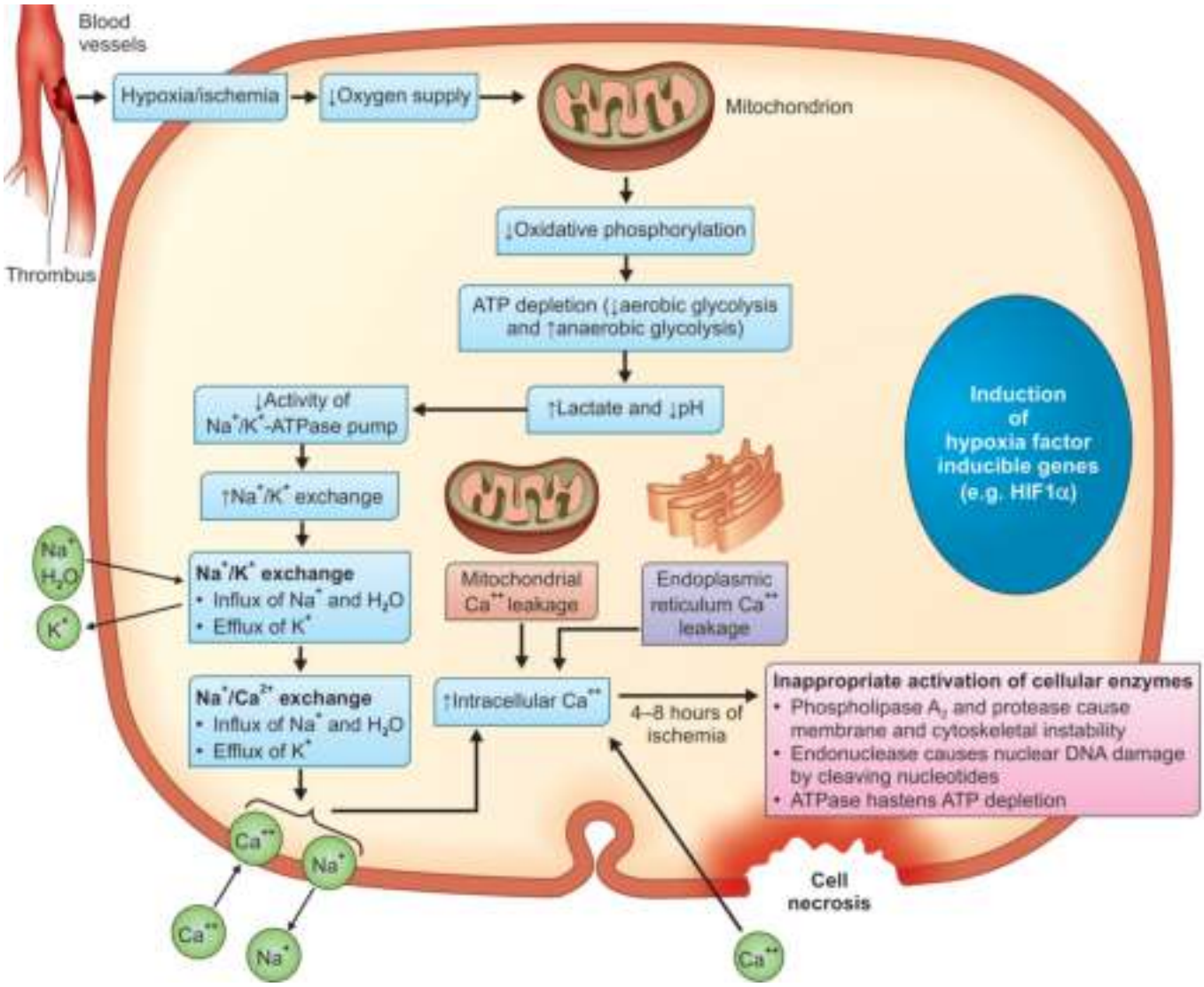


Fig. 1.17: Schematic representation of role of influx of excess calcium in cytosol and activation of cellular enzymes inducing irreversible cell injury. Increased cytosolic calcium concentration is responsible for cytoskeleton modification which alter cell shape, activation of cellular enzymes (phospholipases, endonucleases and ATPase) which results in perpetuation of membrane damage and finally mitochondrial calcification.

- Activation of endonuclease causes fragmentation and lysis of DNA. Myelin figures are derived from damaged membranes of mitochondria, or rough endoplasmic reticulum and plasma membrane.

REACTIVE OXYGEN SPECIES PRODUCTION INDUCED BY CELL STRESSORS

Under normal conditions, O_2 has emerged as an important signaling molecule, which regulates specific biochemical reactions and metabolic processes. A free radical is a molecule with an unpaired electron in its outer orbit. Another term for oxygen-derived free radicals is reactive oxygen species (ROS).

- Physiologic generation of reactive oxygen species occurs during oxidative phosphorylation in the mitochondria, when oxygen is reduced along the electron transport chain.
- Reactive oxygen species occur in the pathologic settings of hypoxia (oxidative stress), ischemia-reperfusion injury, inflammation, ultraviolet light, X-rays, ionizing radiation, inflammation, transitive metals (copper and iron), drugs and chemical agents (e.g. carbon tetrachloride). The excessive generation of highly reactive oxygen-derived free radicals induce oxidative stress in human beings and cause damage to cellular DNA, proteins and lipids and thus cell death. Most reactive oxygen species are generated as chemical intermediates in a variety of enzymatic reactions.
- Reactive oxygen species are formed in number of ways. During normal cellular respiration, about 3% of oxygen enters the mitochondrial electron transport chain. The sequential reduction of oxygen through the addition of electrons results in the formation of a

number of oxygen-derived free radicals collectively called as reactive oxygen species such as superoxide anionic radical (O_2^-), hydroxyl radical (–OH), hydrogen peroxide (H_2O_2), nitric oxide, peroxynitrite ($ONOO^-$), lipid peroxide radical (RCOO), and hypochlorous acid (HClO). Normally, nitric oxide is a potent vasodilator and regulator of blood flow.

- O_2-derived free radicals interact with DNA, biomolecules (proteins, lipids, carbohydrates, nucleic acids) and cause oxidative damage by lipid peroxidation of cell membranes, DNA fragmentation, and protein cross-linking (e.g. sulfhydryl groups) leading to increased degradation of biomolecules and decreased cell activity. Schematic representation of role of reactive oxygen species in cell injury is shown in Fig. 1.18.
- Nitric oxide can accumulate in high concentrations and can react with other oxygen-derived free radicals thereby setting up two mechanisms of cell death: oxidative stress injury and ATP depletion. The mitochondria can lose their membrane potential in high concentrations of nitric oxide and halt ATP production all together. This process can cause endothelial damage resulting in stimulation of the inflammatory response.
- Oxygen-derived free radicals can be prevented by various mechanisms: (a) catalase degrades hydrogen peroxide, (b) superoxide dismutase converts superoxide to hydrogen peroxide, (c) glutathione catalyzes breakdown of hydroxyl radicals, and (d) vitamins A, C and E have an antioxidant property.

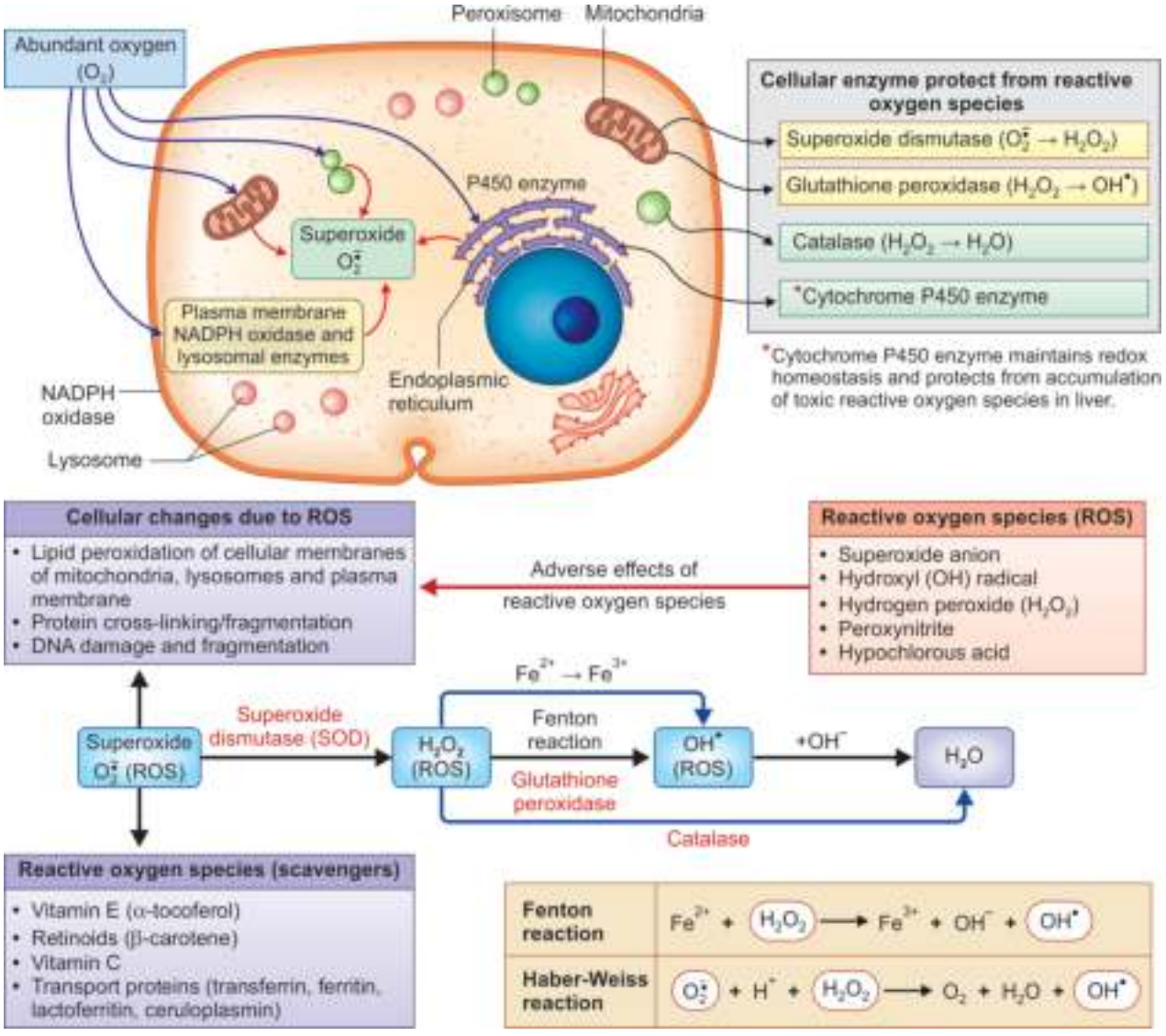


Fig. 1.18: Schematic representation of role of reactive oxygen species (ROS) in cell injury. Oxidative enzymes generating reactive oxygen species are located in mitochondria (sodium dismutase, glutathione peroxidase), peroxisome (catalase) and endoplasmic reticulum (cytochrome P450 enzyme). Reactive oxygen species (hydroxyl ions, hydrogen peroxide and superoxide, peroxynitrite, hypochlorous acid) react with cellular fatty acids, proteins and DNA leading to irreversible cell injury.

Table 1.23 Conditions generating reactive oxygen species

Reduction–oxidation reactions during normal metabolism
Transit metals (copper and iron)
Radiation (ionizing radiation hydrolyzes H_2O, which generate reactive oxygen species
Rapid burst in polymorphonuclear cells and macrophages during inflammation
Polymorphonuclear cells xanthine oxidase (reperfusion injury after ischemia)
Carbon tetrachloride (CCl_4) and barbiturates toxicity (inducing P450 oxidase system of smooth endoplasmic reticulum of hepatocytes)
Mutagens (chemical carcinogens)
Mitochondrial metabolism (biological aging)

- Protective molecules against accumulated toxic oxygen-derived free radicals in cells include superoxide dismutase, glutathione peroxidase, vitamin E, vitamin C and catalase.

Generation of Reactive Oxygen Species

Oxygen-derived free radicals also known as ractive oxygen species are derived from both endogenous sources (mitochondria, peroxisomes, endoplasmic reticulum, phagocytic cells) and exogenous sources (pollutants, tobacco smoke, transition metals such as copper and iron, industrial solvents, peptides, radiation, drugs). Conditions generating reactive oxygen species are given in Table 1.23.

Reactive Oxygen Species (ROS) Generation during Reduction–Oxidation Reaction during Normal Metabolic Process

Reactive oxygen species are formed during reduction-oxidation reaction during normal metabolism within the cell and within organelles such as mitochondria, peroxisomes, and endoplasmic reticulum. The reduction of molecular oxygen (O_2) produces superoxide, which is the precursor of most other reactive oxygen species. Dismutation of superoxide by superoxide dismutase produces hydrogen peroxide. The product hydrogen peroxide in turn may be partially reduced to form hydroxyl radical or fully reduced to water. Reactive oxygen species play important role in cell signaling and homeostasis. Generation of reactive oxygen species increases dramatically during environmental stress such as ultraviolet B or heat.

Reactive Oxygen Species (ROS) Generation during Absorption of Ionizing Radiation

The absorption of ionizing radiation by living cells/tissues can directly disrupt atomic structures, which produce biochemical changes in the cells/tissues. Ionizing radiation can act indirectly through radiolysis of water, thereby generating toxic reactive oxygen species such as superoxide, hydroxyl radical and hydrogen peroxide. These toxic chemical species interact with DNA, proteins and lipids and cause oxidative damage and cell death.

Reactive Oxygen Species (ROS) Generation by Transit Metals (Copper and Iron)

Transit metals, such as copper and iron ions, are common catalysts of lipid oxidation. Copper and iron promote the generation of oxygen-derived free radicals such as hydroxyl radicals, which cause damage to DNA, proteins, lipids and carbohydrates.

- Copper is more efficient catalyst. In the presence of both hydrogen peroxide and ascorbate, copper is reduced to Cu^+, that then reacts with hydrogen peroxide (H_2O_2) to generate hydroxyl radical (–OH) in the Fenton reaction.
- In healthy normal cells, iron is tightly controlled to promote the growth of the cell as well as to protect of the cell against toxicity.
- Accumulation of iron within tissues can generate toxic reactive oxygen species via Fenton reaction and Haber-Weiss reaction: (a) **Fenton reaction** results in the formation of hydroxyl radical from hydrogen peroxide (H_2O_2), a mitochondrial oxidative respiration and an iron catalyst. Highly toxic hydroxyl ions react with DNA, proteins; and lipids of cell membranes resulting in irreversible cell injury. (b) **Haber-Weiss reaction** generates hydroxyl radical (OH) from hydrogen peroxide (H_2O_2) and superoxide catalyzed by iron ions.

Respiratory or Oxidative Burst of Reactive Oxygen Species (ROS) in Activated Phagocytes during Inflammation

Phagocytic cells such as neutrophils, eosinophils, monocytes and macrophages phagocytose and kill internalized pathogens by respiratory or oxidative burst.

- During respiratory burst, phagocytes require energy and oxygen for the generation of a variety of cytotoxic reactive oxygen species. These phagocytes contain NADPH-dependent oxidase enzyme, which transfers one electron to an oxygen molecule to generate superoxide anion.
- The superoxide ion dismutases spontaneously in the presence of superoxide dismutase to hydrogen peroxide. The product hydrogen peroxide then serves as the parent product for a variety of other highly reactive metabolites such as hydroxyl radical and hypochlorous acid; which enter phagosome and together with enzymes participate in the killing of phagocytosed pathogens.

Reactive Oxygen Species (ROS) Generation due to Carbon Tetrachloride-induced Hepatocellular Injury

The liver plays an essential role in various metabolic processes. Carbon tetrachloride (CCl_4) belongs to the class of hepatotoxins, which acts after metabolic activation. CCl_4 is metabolized in the endoplasmic reticulum by cytochrome P450 enzymes (mostly CYP2E1) to the highly reactive trichloromethyl radical (CCl_3).

- CCl_3 rapidly reacts with oxygen to form the highly reactive trichloromethylperoxyl radical (CCl_3OO), that rapidly reacts with DNA, proteins; and lipids of all cell membranes resulting in formation of lipid peroxidation products.
- Polyunsaturated fatty acids of the endoplasmic reticulum are more susceptible to oxidation by the O_2-derived free radicals. The free radical mediated lipid peroxidation of cell membranes is one of the main mechanisms of hepatocellular injury by CCl_4.

Reactive Oxygen Species (ROS) and their Actions

In living organisms, there are two major reactive oxygen species, superoxide radical and hydroxyl radical that are being continuously formed in a process of reduction of oxygen to water.

- In the Haber-Weiss reaction hydroxyl radicals are generated in the presence of hydrogen peroxide and iron ions.
  - The first step involves reduction of ferric into ferrous ion: $Fe^{3} + {}^{\bullet}O_2^- \rightarrow Fe^{2++} + O_2$.
  - The second step is the Fenton reaction $Fe^{2+} + H_2O_2^- \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$.
- Reactions involving reactive oxygen species and their actions are given in Table 1.24.

Superoxide Anion Radical (O_2^-)

Superoxide anion (O_2^-) is a highly reactive radical generated when oxygen is reduced by a single electron, and may be generated during the normal catalytic function of a number of enzymes. It is then converted to H_2O_2 eventually to other reactive oxgen species (ROS).

- Normally, cells are able to maintain low levels of superoxide radical with the help of superoxide dismutase (SOD) enzyme. It does not readily diffuse far from its origin.
- Superoxide radical becomes toxic due to its capability to inactivate iron-sulfur cluster containing enzymes critical in various metabolic pathways, thereby releasing highly reactive free iron radical in the cell via Fenton reaction.

Hydroxyl Radical

The hydroxyl radical, the three-electron reduction product of molecular oxygen, is the most reactive oxygen-derived free radical also called reactive oxygen species.

- Normally, hydroxyl radicals are formed by the hydrolysis of water, the reaction of H_2O_2 with ferrous ions (Fenton reaction), and the reaction of O_2^- with H_2O_2 (Haber-Weiss reaction).
- Excessive intracellular accumulation of hydroxyl radical can damage virtually all types of macromolecules: carbohydrates, lipids (lipid peroxidation). DNA nucleic acids (single DNA strand breaks, modification of bases, and cross-links between strands) and cross-linking of amino acids (sulfur-containing amino acids—cysteine and methionine, as well as arginine, histidine, and proline).

Table 1.24 Reactions involving reactive oxygen species (ROS) and their actions

Reactive Oxygen Species	ROS Generation by Reactions	Actions
Superoxide anion (O_2^-)	Reduction of molecular oxygen by leaks in the electron transport chain	Not diffusing far from its origin
Hydroxyl radical (OH)	During physiologic state, hydroxyl radicals are formed by the hydrolysis of water, the reaction of H_2O_2 with ferrous ions (Fenton reaction), and the reaction of O_2^- with H_2O_2 (Haber-Weiss reaction)	Hydroxyl radical attacking cellular macromolecules
Hydrogen peroxide (H_2O_2)	Superoxide dismutase by Fenton reaction	Diffusing within cell
Peroxynitrite (ONOO)	NO + O_2^- (ONOO.)	Damaging macromolecules
Lipid peroxide radical (RCOO.)	Generated during lipid peroxidation	Initiating chain breakage, destroying unsaturated fatty acids resulting in a loss of membrane integrity
Hypochlorous acid (HOCl)	Generated by neutrophils (myeloperoxidase) and macrophages during respiratory burst that accompanies phagocytosis	Dissociating to yield hypochlorite radical

- During lipid peroxidation, hydroxyl radicals remove a hydrogen atom from bonds of unsaturated fatty acids of membrane phospholipids resulting in loss of membrane integrity.

Hydrogen Peroxide (H_2O_2)

Normally, superoxide dismutases (SOD1 in cytoplasm, SOD2 in mitochondria and SOD3 in extracellular region) are group of enzymes that catalyzes the dismutation of superoxide radicals to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) in small concentration.

- Hydrogen peroxide is a product of metabolism, that can do some nasty things. It can break apart to yield highly reactive hydroxyl radical in Fe^{++} catalyzed Fenton reaction, that can attack all variety of macromolecules such as lipid, carbohydrate, proteins and DNA resulting in cell damage.
- To protect itself from cell damage by hydrogen peroxide, the cells make catalase (within peroxisomes) and glutathione peroxidase (within cytosol and the mitochondria), that decompose hydrogen peroxide to oxygen and water before it can form hydroxyl radicals. In neutrophils, myeloperoxidase transforms hydrogen peroxide (H_2O_2) to the potent radical hypochlorite, which is lethal for microorganisms and cells.

Peroxynitrite ($ONOO^-$)

Peroxynitrite is an oxidant and nitrating agent formed by the interaction of superoxide with nitric oxide (NO). Peroxynitrite is a powerful oxidant, that exhibits a wide array of tissue damaging effects, including lipid peroxidation, inactivation of enzymes and dysfunctional ion channels via protein oxidation and nitration, and inhibition of mitochondrial respiration.

Hypochlorous Acid

Hypochlorous acid (HClO) is a weak acid that forms when chlorine dissolves in water, which partially dissociates to form hypochlorite (ClO^-). Hypochlorous acid and hypochlorite are oxidizers and primary disinfectant agents of chlorine solution. In biology, hypochlorous acid is generated in activated neutrophils by myeloperoxidase-mediated peroxidation of chloride ions, and contributes to the destruction of bacteria during respiratory burst that accompanies phagocytosis.

Nitric Oxide

Nitric oxide (NO) is a highly reactive gas that is involved in many biological processes. It is potent oxidant, that takes part in termination of lipid peroxidation reactions. Nitric oxide is synthesized by the NO synthase in vascular endothelium, that acts as vasodilator. Nitric oxide can also act as an oxidant in indirect reactions with oxygen molecules and superoxide ion.

Lipid Peroxides

Lipid peroxides are oxidative degradation products of lipids, generated by a free radical chain reaction. Ferrous iron can react with a lipid peroxide to generate the corresponding alkoxy radical that can propagate new peroxidation reactions, i.e. extensive peroxidation of polyunsaturated fatty acids of cell membrane, which compromise the integrity and function of cell membrane.

- The aldehyde degradation products of lipid peroxides exert toxic effects on cells by cross-linking of DNA and proteins.
- Lipid peroxides are divided into two classes: lipid hydroperoxide and lipid endoperoxide.
  - Lipid hydroperoxide is a key mediator of cell injury and cell death.
  - Lipid endoperoxide is a key intermediate in the synthesis of prostaglandins, that has importance in inflammation and disease.
- Additionally, ferroptosis is a form of regulated non-apoptotic cell death involving overwhelming iron-dependent peroxidation.

Reactive Oxygen Species and their Pathologic Effects

Reactive oxygen species (ROS) react with fatty acids, proteins and DNA resulting in peroxidation of cell membranes, oxidation of amino acids, abnormal folding of proteins (protein-protein cross linkages) and DNA mutations. Proteosomes act on altered proteins leading to further cell damage. Role of cellular enzymes in generation and removal of reactive oxygen species are given in Table 1.25.

- **Disruption of cell membrane:** Reactive oxygen species react with fatty acids present in plasma membrane and membranes of organelles resulting in disruption of membranes. During peroxidation, hydroxyl radicals remove a hydrogen atom from bonds of unsaturated fatty acids of membrane phospholipids leading to loss of membrane integrity.
- **Abnormal folding of proteins:** Reactive oxygen species adversely affect rough endoplasmic reticulum leading to decreased synthesis of proteins. Protein fragmentation occurs as a result of oxidation of amino acids residue side chain by reactive oxygen species. Oxidation of proteins leads to loss of enzymatic activity and abnormal folding of proteins.
- **DNA damage:** Reactive oxygen species react and cause oxidation of DNA leading to mutations and DNA breaks.

Reactive Oxygen Species Degradation

Fortunately, reactive oxygen species are inherently unstable and generally decay spontaneously. Antioxidants either block the initiation of oxygen-derived

Table 1.25 Role of cellular enzymes in generation and removal of reactive oxygen species

Generation Reactive Oxygen Species by Oxidative Enzymes	Removal of Reactive Oxygen Species by Antioxidant Mechanism
Mitochondria cell organelle	
Respiratory oxidase enzymes generate reactive oxygen species	▪ Superoxide dismutase (SOD) in mitochondria converts oxygen to hydrogen peroxide ▪ Glutathione converts hydroxyl ion to hydrogen peroxide
Cytoplasm	
Peroxisome oxidase enzymes generate reactive oxygen species	Catalase in peroxisome converts hydrogen peroxide (H_2O_2) to water and oxygen
Endoplasmic reticulum cytochrome P450 generates reactive oxygen species	Not applicable

Table 1.26 Mechanism of inactivation of oxygen-derived free radicals

Categories	Mechanism of Action
Intracellular oxidative enzymes	
Superoxide dismutase (SOD)	▪ First-line of defense against O_2^-, converting it to H_2O_2 and O_2 in cytosol and mitochondria ▪ Endoplasmic reticulum also contains P450 oxidase
Catalase	▪ Catalase located in peroxisomes ▪ Catalase eliminating H_2O_2 to O_2 ▪ Completing the dissolution of O_2^- by eliminating H_2O_2 and, therefore, its potential conversion to OH^-
Glutathione peroxidase (GPX)	Catalyzing the reduction of H_2O_2 and lipid peroxides in mitochondria and the cytosol
Exogenous antioxidants (either inhibit synthesis or scavenge free radicals)	
Vitamin E (α-tocopherol)	Fat-soluble vitamin protecting cell membranes against peroxidation by blocking free radical chain reaction
Retinoids	▪ Precursor of fat-soluble vitamin A ▪ Retinoids function as chain breaking antioxidants
Vitamin C (ascorbate)	Water-soluble and reacting directly with O_2, hydroxyl (OH^-) and some products of lipid peroxidation serving to regenerate the reduced form of vitamin E
Transport proteins	
Transferrin, ferritin, lactoferritin, ceruloplasmin	Endogenous antioxidants minimizing the generation of reactive forms by transition metals

free radical formation or scavenge oxygen-derived free radicals. In addition, several systems, i.e. detoxifying enzymes, antioxidants and transporting proteins contribute to free radical inactivation. Mechanism of inactivation of reactive oxygen species is shown in Table 1.26.

DEFECTS IN PERMEABILITY OF PLASMA MEMBRANE AND CELL ORGANELLES

Ionic and osmotic homeostasis of the cellular plasma membrane and its organelles membrane depend on cell membranes. Cell injury induced by hypoxia, mechanical trauma, chemical agents, radiation and pathogens adversely affects plasma membrane, cytoskeleton and membranes of mitochondria, lysosomes.

- Oxygen deprivation is accompanied by cellular plasma membrane disruption resulting in electrolyte disturbance. Without sufficient ATP supply to cell, the plasma membrane can no longer maintain normal ionic gradient across the cell membranes; and ATP-dependent Na^+/K^+ pump and calcium pump can no longer function, which leads to change in ionic concentration of sodium and potassium concentration. Potassium ions leak out of cell to the extracellular space and influx of sodium ions followed by water move into the cell resulting in cellular edema and an increased intracellular osmotic pressure.
- Oxidative stress leads to accumulation of oxygen-derived free radicals. DNA damage caused by ionizing radiation, chemotherapy and oxygen-derived free radicals adversely affect endoplasmic reticulum to synthesize proteins. Failure of the calcium pump leads to influx of Ca^{++} into the cell, activate various enzymes such as phospholipase, protease, ATPase and endonuclease causing cell death. The cells may eventually burst.

- Normally, adequate supply of ATP is essential to maintain high potassium and low sodium levels in the cellular cytosol with functional membrane Na^+/K^+ ATP-dependent pump.
- Reduced Na^+/K^+ ATP-dependent pump results in influx of sodium and water; and efflux of potassium. Cellular swelling may interfere with organelle function. The cytoplasmic membrane of the cells becomes more permeable to high molecular weight proteins due to the systemic intracellular energy debt. NADPH oxidase (phagocytic oxidase) is present in the plasma membrane and the membranes of the phagosomes of leukocytes. NADPH oxidase generates superoxide radical.

Lipid Peroxidation of Cell Membranes

Lipids, the main component of cellular membranes, maintain the structural integrity of the cells. Excessive oxidation of lipids alters the physical properties of cell membranes and can cause selective alterations in cell signaling, covalent modification of proteins and nucleic acids, and cytotoxicity.

- Reactive oxygen species (ROS) are common oxidants in the cells. Reactive oxygen species are formed by the partial reduction molecular oxygen to superoxide ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), lipid oxides (ROOH) or the corresponding hydroxyl ($OH^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$).
- Lipid peroxidation degrades cell membrane phospholipids results in loss of cell membrane integrity due to accumulation of unesterified free fatty acids, acylcarnitine, and lysophospholipids, catabolic products in the lipid bilayer of cell membrane and inside cell.

Pathology Pearls: Lipid Breakdown Products and Membrane Damage

Lipid Peroxidation Products

- During lipid peroxidation, hydroxyl radicals remove a hydrogen atom from the unsaturated fatty acids of membrane phospholipids. The lipid radicals so formed react with molecular oxygen and form a lipid peroxide radical.
- A chain reaction is initiated. Lipid peroxides are unstable and break down into smaller molecules. The destruction of the unsaturated fatty acids of phospholipids results in a loss of membrane integrity.
- Unesterified free fatty acids, acylcarnitine, and lysophospholipids, catabolic products accumulate in the injured cells as a result of phospholipid degradation. They have a detergent effect on membranes. They also either insert into the lipid bilayer of the membrane or exchange with membrane phospholipids, potentially causing changes in permeability and electrophysiological alterations. The important sites of membrane damage are as follows.

Lipid Peroxidation-induced Membrane Damage

- Plasma membrane damage leads to loss of osmotic balance and influx of fluids and ions, as well as loss of cellular contents. The cells may also leak metabolites that are vital for the reconstitution of ATP, thus further depleting energy stores.
- Damage to mitochondrial membrane results in decreased ATP production, culminating in necrosis, and release of proteins that trigger apoptotic death.
- Injury to lysosomal membrane results in leakage of their enzymes into the cytoplasm and activation of the acid hydrolases in the acidic intracellular pH the injured (e.g. ischemic) cell. Lysosomes contain RNases, DNases, proteases, glucosidases, and other enzymes. Activation of these enzymes leads to enzymatic digestion of cell components, and the cells die by necrosis.

ROUGH ENDOPLASMIC RETICULUM DISRUPTION

Rough endoplasmic reticulum (ER) plays a pivotal role in the folding and processing of newly synthesized proteins, which are calcium-dependent.

- Endoplasmic reticulum calcium homeostasis plays an important role in maintaining the physiologic state in cells. Irreversible cell injury to rough endoplasmic reticulum leads to decreased synthesis of protein due to detachment of ribosomes. Increased intracellular calcium and reactive oxygen species adversely affect rough endoplasmic reticulum leading to decreased protein synthesis.
- Misfolded proteins lead to the unfolded protein response which may further injure the cell. Intense eosinophilia of dead cell occurs due to loss of RNA and coagulation of proteins.

CYTOSKELETON DISRUPTION

Cytoskeleton filaments serve as anchors connecting the plasma membrane to the cell interior. It is a network of protein fibers and composed of three main structural components: (a) microtubules formed by tubulins, (b) microfilaments formed by actins; and (c) intermediate filaments (keratin filaments, neurofilaments, desmin filaments, vimentin filaments and glial filaments).

- All three components of cytoskeleton interact with each other noncovalently. Cytoskeleton helps cells in maintaining their shape and internal organization, and also provides mechanical support that enables cells to perform essential functions like cell division and movement.
- Hypoxia-induced cell injury leads to decreased ATP production resulting in increased cytosolic calcium, that activates series of proteases.
- Activated proteases attack the cytoskeleton and its attachments to the cell membrane. As the interactions between cytoskeleton proteins and the

plasma membrane are disrupted, it leads to formation of membrane blebs, and alteration of shape of the cell.

GENETIC APPARATUS DISRUPTION

The cell nucleus is a membrane-bound structure that contains a cell's hereditary information and controls its growth and reproduction. The nucleus involves regulating expression of gene, initiating cellular material essential for all these tasks. The cell nucleus regulates the synthesis of proteins through the messenger RNA (mRNA) resulting in protein synthesis and then travelling to cytoplasm through nuclear pores of the nuclear envelope. Once in the cytoplasm, ribosomes and another RNA molecule called transfer RNA work together to regulate mRNA in order to synthesize proteins.

- Hypoxic injury may cause damage to DNA resulting from increased intracellular calcium and reactive oxygen species.
- When the cell can no longer maintain itself as a metabolic unit, necrotic cell death occurs. Disruption of electron transport system and sustained opening of mitochondrial permeability transition pore (MPTP) indicate irreversible cell injury.
- Nuclear changes include condensation of chromatin (pyknosis), fragmentation of the chromatin (karyorrhexis) and lysis of chromatin due to the action of endonuclease (karyolysis) leaving a shrunken cell devoid of nucleus.
- Hematoxylin and eosin-stained section shows intense cytoplasmic eosinophilia of dead cell due to loss of cytoplasmic RNA and denaturation (coagulation) of proteins. Loss of basophilia is a sign of irreversible cell injury, indicating a cessation of protein synthesis.

MORPHOLOGIC PATTERNS OF NECROSIS

Necrosis is a morphologic sign of cell death in a living tissue. It is the result of denaturation of intracellular proteins and enzymatic digestion of the lethally injured cell.

- Necrotic cells are unable to maintain cell membrane integrity. Necrotic cells may ultimately become calcified in the settings of coagulative necrosis, liquefactive necrosis, caseous necrosis, fat necrosis, gangrenous necrosis and fibrinoid necrosis.
- An infarct is a localized area of coagulative necrosis in an organ. Coagulative necrosis involves multiple tissue planes in gangrene.
- Liquefactive necrosis occurs due to enzymatic dissolution of necrotic cells that results in transformation of the tissue into a liquid viscous mass. It most often seen in cerebrum and in abscesses.
- Fat necrosis in mesentery results from release of activated pancreatic lipases that cause fat destruction in a localized area.
- Caseous necrosis in tuberculosis is a collection of lysed cells and amorphous granular debris enclosed within a distinctive inflammatory border.

COAGULATIVE NECROSIS

Coagulative necrosis is the type of necrosis in which protein denaturation is more prominent than enzymatic breakdown. It most often occurs when sudden interruption in blood supply to an organ supplied by end arteries with limited collateral circulation (e.g. heart, kidney, spleen, adrenal gland except brain) results in cell ischemia and cell death. Widespread tissue necrosis, is called as an infarction. In brain with a high fat content, coagulative necrosis is rapidly followed by liquefactive necrosis.

- Coagulative necrosis occurs in myocardial tissue following a myocardial infarction, which takes several hours to develop coagulative necrosis. However, the loss of integrity of plasma membrane in coagulative necrosis allows the leaking of cardiac enzymes and troponins into the blood stream, making them useful as biochemical markers to aid in the diagnosis of myocardial infarction.
- Microscopically, cellular outlines of the affected tissue are maintained but structural details are lost. The necrotic tissue appears homogenous, glassy eosinophilic cells due to loss of cytoplasmic RNA (basophilic) and glycogen (granular) within preserved tissue architecture. Nucleus may show pyknosis, karyorrhexis or karyolysis. Brain with high lipid content does not contain protein content, hence shows liquefactive necrosis due to ischemia.
- Exposure of cells to heavy metals (e.g. lead and mercury) and ionizing radiation lead to intracellular accumulation of lactate anions (intracellular acidosis); which cause denaturation of structural proteins. Renal cortical coagulative necrosis is seen in mercurial poisoning.

Pathogenesis

Normally, calcium concentration in interstitial fluids is 10,000 times higher than that inside the cell. Lack of O_2 impairs mitochondrial electron transport results in depletion of ATP, which facilitates the generation of oxygen-derived free radicals.

- Following damage to plasma membrane, massive influx of calcium ion into the cell. Ischemia inhibits activity of lysosomal hydrolytic enzymes in the cells, so there is no dissolution of dead cells.
- Mitochondrial damage promotes the release of '**cytochrome c**' into the cytosol, and the cell dies.

Hydrolytic enzymes released by lysosomes start to dissolve cellular components, and trigger an acute inflammatory reaction in which white blood cells migrate to the necrotic area and begin to digest the dead cells.

Pathology Pearls: Pale and Hemorrhagic Infarcts

Pale (Ischemic) Infarct in Solid Organs

- Pale/white infarct is secondary to the sudden occlusion of a vessel. It is commonly seen in solid organs such as heart, kidney, and spleen. Gross morphology of pale/white infarct in spleen is shown in Fig. 1.19.
- Increased density of solid tissue prevents RBCs from diffusing through necrotic tissue. It produces wedge-shaped infarct with apex pointing to the source of obstruction, and the base of infarct at the periphery of the organ.
- Myocardial infarct is most often caused by ischemia related to coronary artery occlusion by atheromatous plaque.

Hemorrhagic (Red) Infarct in Loose-textured Organs

- Hemorrhagic infarct is present in loose-textured tissue such as that found in the organs (e.g. lung and small intestine, ovaries, testis) allowing RBCs to diffuse through necrotic tissue.
- Hemorrhagic infarct is wedge-shaped area of hemorrhage extending to the pleural surface due to embolus in one of the pulmonary artery tributaries.
- Venous occlusion also causes hemorrhagic infarcts (e.g. splenic vein thrombosis).
- Arterial infarcts typically appear as wedge-shaped lesions that are white in appearance (blood poor).
- Venous infarcts typically appear as irregularly shaped lesions that are red in appearance (blood-rich). Infarction is gross manifestation of coagulative necrosis.

Fig. 1.19: Gross morphology of pale/white infarct in spleen. Infarct is a localized area of tissue necrosis caused by arterial occlusion and is usually seen in the spleen, kidney and heart. Pale/white infarct lacks hemorrhage and limited red blood cells accumulation, when compared to hemorrhagic infarct. (*Courtesy*: Department of Pathology, Sapthagiri Institute of Medical Sciences, Bengaluru).

Surgical Pathology: Coagulative Necrosis

Gross Morphology

Infarction is gross manifestation of coagulative necrosis. Infarcts are of two types, i.e. pale infarct and hemorrhagic infarct depending on the consistency of the tissue.

- Pale infarct appears as wedge-shaped, pale/white and firm (blood poor) and retains its normal shape due to lack of enzymatic lysis as cellular proteins including enzymes have undergone coagulation.
- Hemorrhagic infarct appears as irregularly-shaped lesions that are red in appearance (blood-rich). Gross morphology of renal infarct due to occlusion of arterial segment is given in Table 1.27.

Light Microscopy

- Hematoxylin and eosin-stained section shows intense cytoplasmic eosinophilia with dead cell is due to loss of cytoplasmic RNA and denaturation (coagulation) of proteins.
- Loss of basophilia is a sign of cell injury, indicating a cessation of protein synthesis.
- Nuclei undergo phases of condensation of chromatin along the nuclear membrane (pyknosis), fragmentation of the chromatin (karyorrhexis) and lysis of chromatin due to the action of endonuclease (karyolysis) leaving a shrunken cell devoid of nucleus.

LIQUEFACTIVE NECROSIS

Liquefactive necrosis, also known as colliquative necrosis, occurs in situations in which enzymatic breakdown is more prominent than protein denaturation in organs that lack a substantial protein-rich matrix (e.g. lipid-rich organs such as brain).

- Liquefactive is typically found in brain due to prolonged ischemia or in suppurative bacterial or fungal infections in lung (lung abscess). Examples of liquefactive necrosis include brain infarct, abscess and wet gangrene.
- The necrotic tissue is transformed into soft, circumscribed and creamy liquid viscous through the action of hydrolytic enzymes liberated from brain cells or in the case of an abscess from neutrophils and macrophages.

Liquefactive Necrosis in Brain

Hypoxia causes liquefactive necrosis by digestive enzymes (hydrolases) in brain. The necrotic area of brain becomes soft (encephalomalacia) with liquefied center containing necrotic debris by digestive enzymes.

- Necrotic tissue is phagocytosed by macrophages. Later, a cyst is formed in the necrotic tissue of brain, which is filled by diffusion of fluid from the surrounding interstitial spaces of the brain.

Table 1.27 Gross morphology of renal infarct due to occlusion of arterial segment

Branch of Renal Artery	Size of Renal Infarct	Relationship to the Renal Lobes
Segmental artery	4–5 cm	Entire lobe and portion of adjacent lobe
Interlobar artery	2–3 cm	Columns of Bertin and portion of adjacent lobe
Arcuate artery	0.5–1.0 cm	One sixth to one eighth of a lobe extending to the middle of the lobe
Intralobular artery	0.1–0.2 cm	Small portion of a lobe

- Microscopically, the cystic space contains necrotic cell debris, macrophages filled with phagocytosed material. The cyst wall is formed by proliferating capillaries, inflammatory cells, and gliosis (proliferating glial cells) in brain.
- It is worth mentioning that hypoxia causes liquefactive necrosis in central nervous system, that lacks proteins. On the other hand, hypoxia causes coagulative necrosis in solid organs rich in proteins (e.g. kidney, spleen and heart).
- Gross morphology of liquefactive necrosis in the frontal lobe of brain with formation of cystic spaces is shown in Fig. 1.20. Gross morphologic and histologic features of cerebral infarct are given in Table 1.28.

Fig. 1.20: Gross morphology of liquefactive necrosis in the frontal lobe of brain with formation of cystic spaces (arrow). Liquefactive necrosis is type of necrosis which results in a transformation of the tissue into liquid viscous mass as a result of lysosomal release of digestive enzymes.

Liquefactive Necrosis in Organ's Abscess

An abscess is a collection of pus that has built up within the tissue of the body due to bacterial infections. Typically, it presents as a cavity filled with pus—that is, liquefied tissue of the affected organ permeated with dead or dying bacteria and neutrophils.

- Abscess can form throughout the body, both in locations that are visible and internally, where it may go unnoticed and cause serious problems including organ damage.
- The most common sites of abscesses are skin and dental region. Less common sites of abscess are abdomen, liver (amoebic or pyogenic), brain, epidural region, Bartholin gland, anorectal region, peritonsillar region, spinal cord.

Partial Liquefactive Necrosis in Wet Gangrene of Extremities

Wet gangrene is a clinical term for ischemic necrosis accompanied by superadded bacterial decomposition, which results in partial liquefaction of the tissues. It occurs due to lytic activity of bacteria and inflammatory cells in the affected tissue. Swelling, blistering and wet appearance are common features of wet gangrene. It may develop after severe burns, frostbite and diabetes mellitus in foot or toes. Wet gangrene needs to treated immediately, because it spreads quickly and can be fatal.

Table 1.28 Gross morphologic and histologic features of cerebral infarct

Feature	Duration	Gross Features	Histologic Features
Acute cerebral infarct	Hours to few days	Poorly defined soft and edematous infarct	Liquefactive necrosis of neurons, mild neutrophilic infiltrate and rarefaction of white matter
Subacute cerebral infarct	Hours to weeks	Partially liquefied infarct	Liquefactive necrosis of neurons and glial components drop out, marked macrophage infiltrate, reactive capillaries and gliosis at periphery
Chronic cerebral infarct	Months to years	Cystic cavity with collapse of surrounding tissue	Cystic spaces lined by 'gliotic scar' at periphery without collagen, loss of parenchyma, sparing of subpial region as opposed to old contusion; with or without hemosiderin and macrophages

CASEOUS NECROSIS

Caseous necrosis is hallmark of tuberculosis, which appears as amorphous, coarsely granular, eosinophilic debris. Certain fungal infections can also exhibit caseous necrosis. Caseous necrosis is a combination of coagulative and liquefactive necrosis seen in tuberculosis, in which cellular outlines as well as structural details are lost.

- Mycobacterium tubercle bacillus is a nonmotile acid-fast, 2–4 μm in length with very slow generation time between 15 and 20 hours. The cell envelope of Mycobacterium tubercle bacillus is composed of peptidoglycan (40%) and of lipids (60%). The lipid fraction of mycobacterial tubercle bacillus cell wall is composed of three major components, mycolic acids, cord factor and wax-D. It is partially resistant to digestion and phagocytosis by tissue macrophages.
- Exposure to Mycobacterium tubercle bacillus leads to activation and recruitment of macrophages to form epithelioid cell granulomas and Langhans type of giant cells. Caseous necrosis is derived from mycolic and other lipid constituent of the lipid-rich envelope of Mycobacterium tubercle bacillus, hence the descriptive term 'caseous necrosis'. Differences between coagulative and caseous necroses are shown in Table 1.29.

Pathogenesis

Lungs are the most common site of tuberculosis in children and adults. Most common organs involved in children include lungs, lymph nodes, spleen and meninges. Lungs, adrenal glands, kidneys, liver, spleen, bone, meninges, serous membranes, fallopian tubes, epididymis and lymph nodes are involved in adults. Tissues rarely involved include cardiac muscle, skeletal muscle, stomach, thyroid gland, and pancreas.

Pathogenesis of Tuberculosis in <3 Weeks Duration

Mycobacterium tubercle bacilli's mannose capped glycolipid (lipoarabinomannan) binds to mannose receptors expressed on alveolar macrophages leading to engulfment of AFB within three weeks of infection. Cord factor synthesized by AFB prevent fusion of phagosomes with lysosomes. Hence, unchecked proliferation of tubercle bacilli continues inside alveolar macrophages.

Pathogenesis of Tuberculosis in >3 Weeks Duration

Macrophages are antigen presenting cells, which present Mycobacterium tubercle bacilli antigen to CD4+ helper T cells. Macrophages synthesize IL-12, which takes part in differentiation of T-helper cells in lungs and lymph nodes in >3 weeks duration of infection.

- Bactericidal activity of immune system occurs due to synthesis of interferon-γ by T cells. Interferon-γ stimulates nitrogen synthase enzyme, which causes synthesis of nitrogen intermediates (NO, NO_2 and HNO_3) and O_2-derived free radicals resulting in oxidative destruction of AFB.
- Macrophages synthesize tumor necrosis factor and chemokines, which participate in recruitment of monocytes and sensitization of CD4+ helper T cells.
- This process of recruitment of monocytes and sensitization of T cells leads to formation of epithelioid granulomas composed of macrophages and surrounded by lymphocytes. Epithelioid cell granulomas prevent spread of infection by confining bacteria within a compact collection of activated macrophages and T cells.

Table 1.29 Differences between coagulative and caseous necroses

Coagulative Necrosis	Caseous Necrosis
Etiopathogenesis	
Hypoxic injury to heart, kidney and spleen	Mycobacterium tubercle bacillus
Due to intracellular acidosis (due to hypoxia) structural as well as enzymatic denaturation of proteins occurs. Dead cells are removed by fragmentation and phagocytosis	Reaction between Mycobacterium tubercle bacillus antigens and inflammatory cells. Cellular outlines are completely obscured
Gross morphology	
Firm texture of organs	Cheesy white appearance
Light microscopy	
Cellular outlines are maintained and structural details are lost. Cells are acidophilic, coagulated with loss of nuclear details due to blockage of cytoplasmic hydrolytic enzymes. There is no dissolution of tissue.	Cellular outline as well as structural details are lost. It is amorphous granular debris (fragmented, coagulated cells and granular debris) surrounded by chronic inflammatory cells. Epithelioid granulomas and Langhans giant cells are present

Surgical Pathology: Caseous Necrosis

Gross Morphology

- Caseous material resembles clumpy cheese-like consistency, hence the name caseous necrosis. It is formed by the release of lipid from the cell walls of Mycobacterium tubercle bacilli after destruction by macrophages.
- This type of caseous necrosis appearance also occurs in systemic fungal infection by *Histoplasma capsulatum*. Gross morphology of tubercular lymphadenitis is shown in Fig. 1.21.

Light Microscopy

- The tubercular lesion is composed of epithelioid granulomas (activated macrophages, CD4+ helper T cells), caseous necrosis and Langhans' type giant cells.
- Tissue architecture is distorted. Caseous necrotic tissue lack cellular outlines as well as cellular details, which appears amorphous, finely granular, eosinophilic appearance with increased affinity for acidophilic dye. Histology of tuberculous lymphadenitis is shown in Fig. 1.22.

Histochemical Stain

- Ziehl-Neelsen staining is a type of acid-fast bacteriological stain used to identify Mycobacteria tuberculosis.
- Ziehl-Neelsen stain contains carbol fuchsin, acid alcohol and methylene blue.
- Mycobacterium tubercle bacilli appear as slightly curved or straight under oil immersion. Ziehl-Neelsen staining for demonstration of acid-fast bacilli is shown in Fig. 1.23.

FAT NECROSIS

Fat necrosis refers to the destruction and death of fat cells causing scar to form in breast, pancreas and other tissues. It often occurs in breast tissue and anywhere in the body that contains fat tissue.

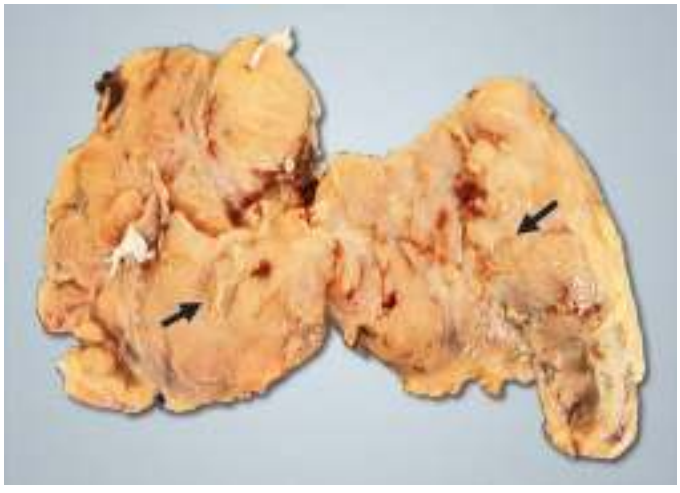


Fig. 1.21: Gross morphology of tubercular lymphadenitis. Cut surface of tubercular lymphadenitis shows cheesy appearance on cut surface. (*Courtesy*: Department of Pathology, Dr. DY Patil Medical College, Pune, Maharashtra.)

- Fat necrosis occurring in acute pancreatitis is mediated by pancreatic enzymes.
- Traumatic fat necrosis, unlike enzymatic fat necrosis, is not enzyme-mediated but is secondary to direct severe trauma to organs rich in fat, i.e. female breast, surgical trauma to organs rich in adipose tissue such as abdominal wall and omentum. Fat necrosis in breasts occurs due to severe traumatic injury or breast augmentation surgery.

Fat Necrosis in Female Breast

Damage to the adipose tissue can occur following severe traumatic injury, breast biopsy, radiotherapy to the breast, breast surgery including breast reconstruction or augmentation or breast reduction or lipomodelling (fat taken from another region and injected into the breast).

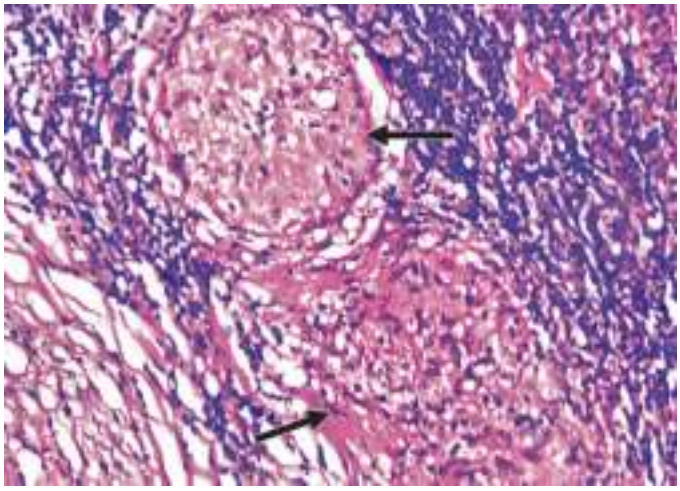


Fig. 1.22: Histology of tuberculous lymphadenitis. It shows epithelioid granulomas (arrows) caseous necrosis and Langhans' giant cells (400X).

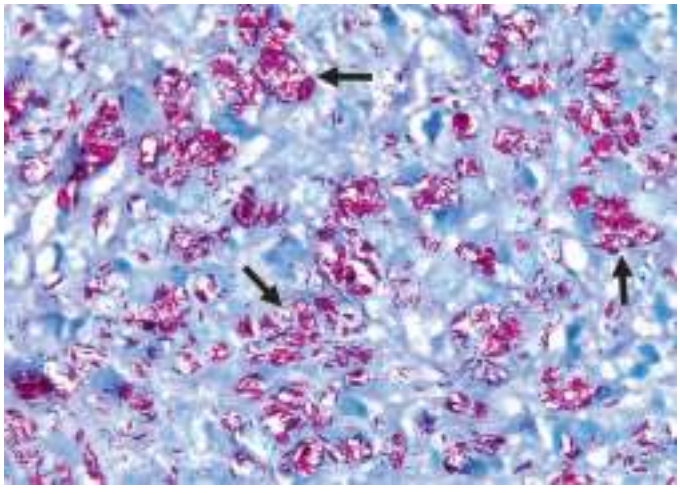


Fig. 1.23: Ziehl-Neelsen staining for demonstration of acid-fast bacilli. Microscopic examination of clinical samples for acid-fast bacilli using Ziehl-Neelsen stain has been a standard diagnostic tool and is used globally for rapid tuberculosis diagnosis (arrows) (400X).

- Lipase enzyme liberated from traumatized adipose tissue breaks down intracellular triglycerides into free fatty acids. Free fatty acids released, via hydrolysis react with calcium, sodium, magnesium to form chalky white areas known as 'saponification'.
- The tissue becomes opaque and chalky white demonstrated as calcified lesion on radiograph.
- Patient presents with unilateral painful localized superficial irregular breast mass in acute stage.
- On clinical examination, affected breast shows firm, erythema of the overlying skin, dimpling and nipple retraction mimicking breast cancer. If the mammogram or ultrasound scan clearly shows fat necrosis, fine needle aspiration cytology, core biopsy or vacuum assisted biopsy may not be needed.
- Fat necrosis in breast is harmless, that may resolve in few months. It does not increase risk for breast cancer. Patients do not require any surgical or medical treatment.

Surgical Pathology: Fat Necrosis in Breast

Gross Morphology

- Fat necrosis of breast shows chalky white areas of fat saponification.
- Variegated color and areas of hemorrhage are demonstrated on the cut surface of this lump.
- The lesion is gritty to cut because of the presence of spotty calcification.

Light Microscopy

- Initially, acute inflammatory reaction consists of necrosis of adipocytes and hemorrhage.
- Chronic inflammatory lesion is composed of plasma cells, macrophages, multinucleated giant cells, foam cells termed as lipophages.
- There is presence of foreign body giant cells and dystrophic calcification demonstrated by imaging techniques.
- Fibroblastic proliferation leads to fibrosis. As a result, an irregular, fixed, hard mass may ensue and clinically resemble breast cancer. Thus, the lesions often require biopsy to establish their benign character.

Fat Necrosis in Pancreas

Enzymatic fat necrosis in pancreas represents autodigestion by pancreatic enzymes such as lipases and proteases released from pancreatic cells.

- The lipase and protease enzymes damage peripancreatic fat cells laden with triglycerides leading to liberation of free fatty acids, which combine calcium ions, sodium, or magnesium to form calcium soaps (saponification). Hemorrhage occurs as a result of vessel erosion.
- Predisposing factors of acute pancreatitis include alcoholism, hypercalcemia, hyperlipemia and autoimmune etiology. Serum calcium level is decreased in patients, who had a recent bout of acute pancreatitis.
- Patient experiences sudden-onset abdominal pain, distention, and vomiting. The precipitated calcium in the soaps can be visualized by radiological imaging.

Surgical Pathology: Fat Necrosis in Pancreas

Gross Morphology

Macroscopically, fat necrosis looks like opaque chalky-white nodules composed of calcium soaps in the fat surrounding the pancreas or in the abdominal cavity.

Light Microscopy

- Microscopic examination reveals irregular islands of necrotic fat cells, which lose their outlines and become indistinct.
- There is presence of inflammatory cells, macrophages filled with fat and calcium deposits.
- Deposition of calcium around the periphery of necrotic fat cells gives a basophilic tinge.
- Giant cell reaction around these foam cells may occur. Histology of fat necrosis in pancreas is shown in Fig. 1.24.

GANGRENE

The terms 'gangrene' and 'necrosis' are often confusing. Necrosis refers to irreversible cell injury and cell death without superadded bacterial infection in a living organism. Gangrene involves the cellular and morphological changes that can lead to scarring and permanent tissue/organ loss of function. Gangrenous necrosis is most often seen on extremities usually due to trauma.

- Gangrene refers to the death of body tissue/organ due to either a lack of blood flow or a serious superadded bacterial infection or traumatic wounds (gunshot or crush injuries) affecting extremities including toes/fingers, muscles and internal organs in living organism.

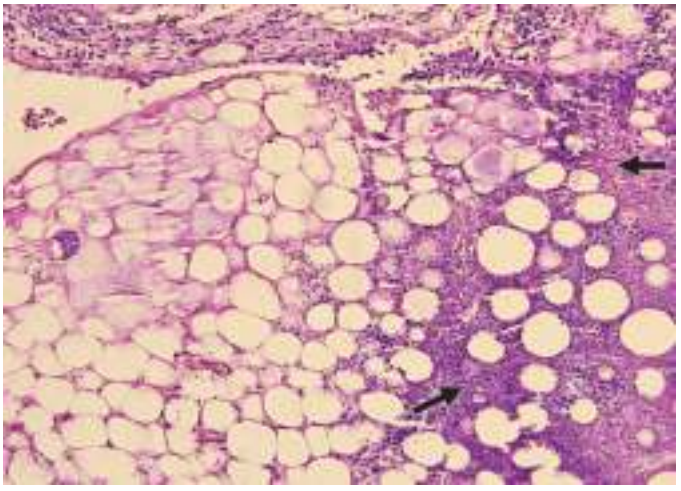


Fig. 1.24: Histology of fat necrosis in pancreas. It shows irregular islands of necrotic fat cells, which have lost their outlines. There is presence of chronic inflammatory cells. Macrophages are filled with fat and calcium (arrows) (100X).

Table 1.30 Differences between dry gangrene and wet gangrene

Dry Gangrene	Wet Gangrene
Etiopathogenesis	
Slow occlusion of arteries	Sudden interruption of blood flow in both artery and vein
Atheromatous plaques in medium-sized arteries	Emboli, ligation, crush injury
Organs involved	
Limbs (small area involved due to presence of collaterals)	Bowel (large area involved due to absence of collaterals)
Odor absent	Odor present
Line of demarcation of dry gangrene presents at the junction of healthy and gangrenous part	Line of demarcation of wet gangrene absent
Superadded infection absent	Superadded infection present
Gross morphology	
Dry, decreased in size with black discoloration	Wet, swollen, edematous and soft in consistency
Prognosis	
Prognosis is most often better due to little septicemia	Prognosis is most often poor due to profound toxemia
Treatment	
Conservative amputation	Major surgical resection essential

- There are two main categories of gangrene, i.e. dry and wet. Dry gangrene refers to death of tissue/organ without superadded bacterial infection. Wet gangrene refers to death of tissue/organ with superadded bacterial infection, multiplication and release of toxins and pus.
- Several risk factors for gangrene include diabetes mellitus, atherosclerosis (tibial and popliteal arteries), severe traumatic injury, tobacco smoking and obesity. Differences between dry gangrene and wet gangrene are given in Table 1.30.

Dry Gangrene

Dry gangrene refers to ischemic necrosis without superadded bacterial infection accompanied by drying of the organs/tissues. It occurs most commonly in persons, who have arterial blood vessel disease of extremities (atherosclerosis) or diabetes mellitus. Dry gangrene is characterized by dry, brown to purplish blue or black skin due to the deposition of iron sulphide from degraded hemoglobin.

Wet Gangrene

Wet gangrene is a clinical term for ischemic necrosis accompanied by superadded bacterial infection, which results in partial liquefaction of the tissues due to lytic activity of bacteria and inflammatory cells in the affected tissue. Swelling, blistering and wet appearance are common features of wet gangrene. It may develop after severe burns, frostbite and diabetes mellitus in foot or toes. Wet gangrene needs to be treated immediately, because it spreads quickly and can be fatal. Gross morphology of ischemic bowel disease (gangrene intestine) is shown in Fig.1.25.

Fig. 1.25: Gross morphology of ischemic bowel disease (gangrene intestine). Gangrene of the bowel occurs due to sudden occlusion of arterial blood supply by a thrombus leading to ischemia involving short or long segments of bowel. External surface shows dusky blackish discoloration. Gangrene bowel represents an irreversible cell injury.

Clinical Pearls: Gas Gangrene

- Gas gangrene is synonymous with myonecrosis, which occurs in an injured deep muscle tissue or surgical wound with infection by *Clostridium* species, that is depleted of blood supply.

- Gas gangrene is highly lethal infection of deep soft tissue caused by *Clostridium perfringens* (80–90%), *Clostridium novyi* (40%), *Clostridium septicum* (20%), *Clostridium bifermentans* (10%), and *Clostridium fallax* (10%). These organisms are present in soil and organic wastes especially contaminated with fecal matter.
- Bacterial infection releases toxin produces toxin, that releases gas bubbles in the injured deep tissues. Like wet gangrene, gas gangrene can be life-threatening. Lecithinase released by *Clostridium perfringens* breaks down cell membrane resulting in cell death and tissue necrosis. Lecithinase causes hemolysis and adverse effect on heart.

FIBRINOID NECROSIS

Fibrinoid necrosis is a descriptive term for irreversible cell necrosis of arterioles and muscular arteries. Deposition of antigen–antibody complex leads to activation of complement cascade and subsequent chemotactic influx of neutrophils and monocytes associated with type 3 hypersensitivity reaction.

- Deposits of these immune-complex, together with fibrin that has leaked out of blood vessel, results in a bright eosinophilic (pink) amorphous necrotic debris that appears brightly eosinophilic (i.e. fibrin-like) in hematoxylin and eosin-stained tissue sections, called 'fibrinoid necrosis' (fibrin-like) by histopathologist.
- If necrotic cells and cellular necrotic debris are not promptly destroyed and reabsorbed, they tend to attract calcium salts and often minerals and to become calcified. In small blood vessel vasculitis, fibrin plugs frequently occur into the blood vessel lumen, but the term 'fibrinoid necrosis' usually refers to material outside the lumen of a blood vessel.
- Fibrinoid necrosis also occurs in the walls of arterioles in malignant hypertension (blood pressure more than 200/130 mm Hg), immune-mediated vasculitis (e.g. eclampsia, immune-mediated vasculitis (Henoch-Schönlein purpura, lupus erythematosus and polyarteritis nodosa), infection and hyperacute organ transplant rejection.
- Fibrinoid necrosis has no distinct grow morphologic features. Histologic examination shows glassy, eosinophilic fibrin-like material deposit within vascular walls.
- Symptoms related to fibrinoid necrosis involves bleeding gums, muscle bruises, hematuria, blood in stool and hemorrhage of spleen.
- Treatment of fibrinoid necrosis involves treating the underlying cause.

GUMMATOUS NECROSIS

Gummatous necrosis is a granulomatous destructive rubbery soft lesion seen in the tertiary syphilis, which most often occurs in the liver (gumma hepatitis). It may also be demonstrated in brain, heart, testes and bone leading to numerous potential problems such as neurological and cardiac valve disorders.

- A gumma has a soft, necrotic center surrounded by inflamed tissue. On gross examination, gumma has firm necrotic amorphous proteinaceous mass that may be partly hyalinized.
- Histologic examination of gumma shows central area of necrosis with partly retaining structural details of previously normal tissue. There is presence of epithelioid cell granulomas, multinucleated giant cells in the central region of gumma. Peripheral zone of gumma shows fibroblasts, capillaries, lymphocytes and plasma cells.

SUBCUTANEOUS NECROSIS IN NEWBORN

Subcutaneous necrosis in newborn is self-limited disease affecting term neonates and young infants. It is characterized by circumscribed, indurated, nodular areas of fat necrosis. It is related to trauma on bony prominences during delivery or maternal diabetes mellitus, which resolves spontaneously by 2–4 weeks without fibrosis. Subcutaneous necrosis in newborn is also called adiponecrosis neonatorum.

ZENKER'S NECROSIS IN SKELETAL MUSCLE

Zenker's necrosis refers to glassy waxy necrosis of the striated muscle such as rectus abdominis and diaphragm in acute severe infectious diseases such as typhoid fever and burns. It has been named by Friedrich Albert von Zenker. Zenker's necrosis in skeletal muscle appears pale and friable. Histologic examination shows pale and friable swollen skeletal muscles with loss of striations.

POSTMORTEM AUTOLYSIS

Postmortem autolysis occurs secondary to the release of endogenously derived intracellular enzymes moments after death. There is no inflammatory infiltrate, since an inflammatory response occurs only in living tissue.

OUTCOME OF NECROSIS

Cell injury can be reversible or irreversible. Depending on the extent of injury, cellular response may be adaptative and where possible, homeostasis is restored. Cell death occurs when the severity of the injury exceeds the cell's ability to repair itself. It is essential to remove necrotic tissue and cell debris at or near the site of the cell death surgically in a case of gangrene, a procedure known as debridement.

COMPLETE RESOLUTION

Macrophages phagocytose necrotic cellular debris and apoptotic neutrophils leading to removal from injured site. Edema fluid and proteins are finally drained into the lymphatics. Injured cells with intact supporting stroma undergo regeneration of labile and stable cells by mitosis with restoration of normal structure and function.

- Labile cells multiply constantly throughout life. These are present in bone marrow epidermis, epithelial lining gastrointestinal tract, bronchi and vagina; and epithelial lining excretory ducts (salivary glands, pancreas, and biliary tract).
- Stable cells spend most of the time in the quiescent G0 phase of the cell cycle, but can be stimulated to enter the cell cycle when needed. These cells are present in proximal tubules of kidney, liver, endocrine glands, fibroblasts, vascular endothelium, cartilage and bone. Liver is composed of stable cells, which have limited proliferative capacity. Following partial hepatectomy or chemical induced liver injury, liver is able to regenerate by proliferation of remaining hepatocytes. It is well known that as little as 25% of the original liver tissue can regenerate back to its full-size organ.
- Permanent cells are terminally differentiated and incapable of regeneration. Examples are brain cells, neurons, cardiac myocytes, skeletal muscle, renal glomeruli and red blood cells.

INJURED TISSUE REPAIR BY FIBROSIS

Injured tissue repair by fibrosis involves removal of injured tissue by inflammation, angiogenesis, migration and proliferation of fibroblasts, scar formation and remodeling of connective tissue.

- Organization is defined as the replacement of necrosed tissue (infarct), thrombi, fibrinous exudates, hematoma (wound, bone fracture) by granulation tissue.
- Myofibroblasts, which are hybrids of fibroblasts and smooth muscle cells, contain both myofilaments and rough endoplasmic reticulum and thus have purple-staining cytoplasm. These cells play central role in wound healing and fibrosis in various organs (liver, lung, kidney) by synthesizing extracellular matrix (ECM), which consists of structural fibrous glycoproteins (collagen fibers, fibronectin, laminin, and elastin) and amorphous ground substance (gel-like material that absorbs water).
- Myofibroblasts are derived from epithelial cells of epithelial–mesenchymal transition (EMT), activation of stellate cells (e.g. hepatic Ito cells or pancreatic stellate cells), direct myofibroblastic differentiation of a progenitor cell resident in a stromal tissue.

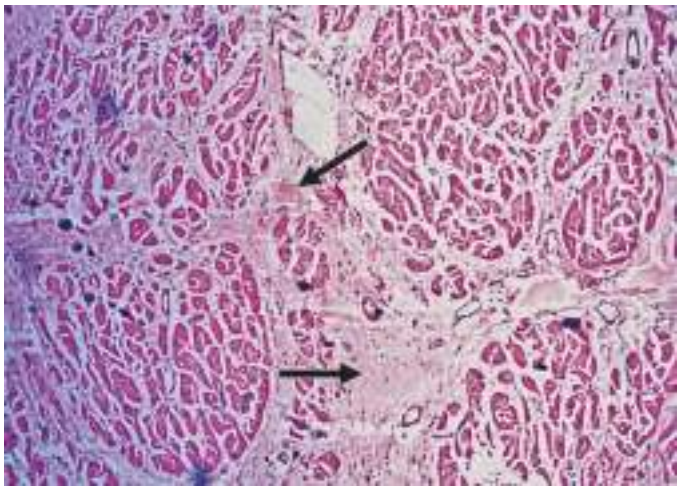


Fig. 1.26: Histology of healed myocardium after infarction. Myocardial infarct shows healing by fibrosis (arrows) (100X).

Healing in Myocardial Infarct

In myocardial infarction, the dead myocardial cells are removed by inflammatory cells and replaced by a fibrous scar. Healing starts with granulation tissue formation by first week. By second week, granulation tissue is replaced by yellow necrotic myocardium. By third week, yellow necrotic myocardium is replaced by fibrous scar. Phagocytosis of necrotic debris by macrophages is complete. By sixth week, infarct tissue is replaced by white, patchy and noncontractile scar tissue. Histology of healed myocardium after infarction is shown in Fig. 1.26.

DYSTROPHIC CALCIFICATION

Dystrophic calcification is deposition of calcium salt in the injured tissues in the presence of normal calcium and phosphorous metabolism. It is different from systemic mineral imbalance causing metastatic calcification, in which calcium deposition occurs in normal tissues. Calcium is demonstrated by alizarin stain and von Kossa stain. Dystrophic calcification occurs by two processes: initiation and propagation. Calcium has strong affinity for cellular phospholipids.

- Membrane bound vesicles containing calcium are formed inside cell. Now tiny crystals of calcium are released from membrane bound vesicles into the extracellular compartment. Phosphatase participates in deposition of phosphates.
- Further deposition of calcium is known as propagation, that depends on the presence of calcium and phosphate in the extracellular compartment; and presence of collagen fibers and other proteins.

Dystrophic calcification occurs in caseous necrosis, liquefactive necrosis, fat necrosis (acute pancreatitis, traumatic fat necrosis in breasts) and hematomas in the vicinity of the bone fracture.

RESORPTION OF NECROTIC TISSUE

Liquefactive necrosis (or colliquative necrosis) results in transformation of the tissue into a liquid viscous mass. It most often occurs in the lungs, especially in lung abscess and certain infections.

- After removal of cell debris by hydrolytic enzymes of neutrophils and macrophages, a fluid-filled soft circumscribed pseudocyst consisting of remains of necrosed tissue and pus is left. Dead leukocytes will remain as a creamy yellow pus. The pseudocyst wall is formed by proliferating capillaries, inflammatory cells and proliferating fibroblasts in abscess cavities.
- Hypoxia causes liquefactive necrosis of central nervous system by brain digestive enzymes (hydrolases). The affected area of brain becomes soft with liquefied center containing necrotic debris by brain digestive enzymes. Later, a cyst is formed in the affected area of brain. Microscopically, the cystic space contains necrotic cell debris, macrophages filled with phagocytosed material. The cyst wall is formed by proliferating capillaries, inflammatory cells, and gliosis (proliferating glial cells) in brain.

REGULATED CELL DEATH (RCD)

Nomenclature Committee on Cell Death (NCCD) 2018 has formulated guidelines for highlighted nomenclature of cell death on morphologic, molecular, pharmacologic, and functional basis; and identification of dead cells with irreversible plasma membrane disruption and complete cellular fragmentation.

- Cell death manifests with macroscopic and morphologic alterations. Dead cells and their fragments are disposed by three mechanisms: necrosis, apoptosis, and autophagy. Necrosis occurs when cells are irreversibly damaged by an external stimulus. In contrast, apoptosis is considered to be physiologic form of programmed cell death, a cell provokes its own demise in response to a stimulus. Autophagy manifests with extensive cytoplasmic vacuolization and similarly culminating with phagocytic uptake and consequent degradation.
- Oncotic necrosis is the term currently used for nonapoptotic, accidental cell death. It is generally regarded as a severe lethal injury to cell membrane integrity. The term 'oncotic necrosis' is defined as cell death with swelling (oncosis), that can be caused by ischemia, and toxic agents that interfere with ATP generation by mitochondria and plasma membrane ($Na^+/K^+/Cl^-/Ca^{++}$) pumps failure. Oncotic necrosis is characterized by cell swelling (oncosis), organelles swelling, plasma cell blebbing, loss of plasma membrane integrity, increased membrane permeability, chromatin fragmentation (pyknosis, karyorrhexis, karyolysis), release of lysosomal enzymes into extracellular spaces, eosinophilic cytoplasm, inflammation, vascularization and tissue repair.
- Morphologically, necrosis occurs in several forms such as coagulative necrosis, liquefactive necrosis, caseous necrosis, fibrinoid necrosis, gummatous necrosis, subcutaneous necrosis and autolysis in newborn.
- Biochemically, necrosis of cell has been demonstrated to represent a number of genetically determined signaling pathways. Novel ancillary tests are performed to detect abnormal signaling pathways in cell necrosis.

- Regulated cell death functions to eliminate potentially harmful cells to maintain tissue homeostasis. Multiple cell death pathways have been defined according to the morphologic, biochemical and functional aspects.
  - Examples of regulated cell death include apoptosis (type 1 cell death), autophagic cell death (type 2 cell death), necroptosis, pyroptosis, ferroptosis, entotic cell death, NETotic cell death, lysosomal dependent cell death, antibody-dependent cell death, and immunogenic cell death. The morphologic alteration is focused on cell size, membrane integrity, chromatin density, organelle arrangement and presence of vacuoles. Features of regulated cell death (RCD) are given in Table 1.31.
  - The clinical implications of necroptosis, pyroptosis, ferroptosis and many other emerging forms of programmed necrosis extend across a range of pathologists and disease models, but play an obvious and prominent role in cancer, the ultimate imbalance between cellular life and mortality.

Table 1.31 Features of regulated cell death (RCD)

Apoptosis
▪ Apoptosis is mediated by extrinsic death receptor/intrinsic mitochondrial/granzyme and perforin pathways ▪ Apoptosis features cell shrinkage, plasma membrane blebbing, DNA chromatin condensation and karyorrhexis, cell breaks apart into several apoptotic bodies and phagocytosed by macrophages and surrounding epithelial cells and lack of inflammation
Autophagy-dependent Cell Death
▪ Starvation induced cell death ▪ Massive cytoplasmic vacuolization and loss of organelles ▪ Fusion of autophagosome and lysosome and forming phagolysosome ▪ Cell degradation
Pyroptosis
▪ Pyroptosis is cytokines-induced cell death ▪ PAMP/DAMP → CASP1/cytosolic lipopolysaccharide (LPS) → caspase 11 → proteolytic maturation of the pore-forming protein gasdermin D (GSDMD) → N-terminal fragments → plasma membrane pores and rupture ▪ DNA condensation and karyorrhexis
Necroptosis
Necroptosis is triggered by perturbations of extracellular or intracellular homeostasis that is critically depends on MLKL, RIPK3 or RIPK1. It leads to membrane rupture and random DNA degradation
NETosis
▪ Infection → NADPH oxidase activation → reactive oxygen species (ROS) → histone citrullination → NETotic cell death → release of neutrophil chromatin traps containing antimicrobial products ▪ Membrane rupture and DNA decondensation
Ferroptosis
▪ Ferroptosis is initiated by oxidative perturbances of the intracellular microenvironment, generation of reactive oxygen species causing lipid peroxidation that is under control of GPX4 and can be inhibited by iron chelators and lipophilic antioxidants ▪ Can be inhibited by iron chelators and lipophilic antioxidants
Entotic Cell Death
Entotic cell death originates from actomyosin-dependent cell-in-cell internalization (entosis) and is executed by lysosomes
Parthanatos (PARP-1-dependent cell death)
Parthanatos is initiated by PARP1 hyperactivation and precipitated by the consequent bioenergetic catastrophic coupled to AIF-dependent and MIF-dependent DNA degradation
Anoikis
Specific variant of intrinsic apoptosis initiated by loss of integrin-dependent anchorage
Immunologic Cell Death
Adaptive immune response in immunocompetent hosts induces immunologic cell death occurs
Autosis
A specific instance of autophagy-dependent cell death that critically relies on the plasma membrane Na/K- ATPase

MITOCHONDRIAL PERMEABILITY TRANSITION PORE DRIVEN NECROSIS

Mitochondria are surrounded by double-membrane system, consisting of inner and outer mitochondrial membranes separated by an intermembrane space. Inner membrane forms numerous folds which extend into the interior (matrix) of the organelle.

- Mitochondrial permeability transition pore (MPTP) is a transmembrane protein residing in the mitochondrial inner membrane.
- Normally, MPTP is closed. Mitochondria are key players in cell survival by providing ATP energy for vital cellular processes.
- Mitochondria participate in programmed cell death (apoptosis) *via* activation of caspase cascade, which

requires preserved ATP levels. When mitochondria cannot maintain adequate ATP levels, cell death occurs via necrosis.

MOLECULAR PATHOGENESIS

During ischemia (MPT priming phase), cytosolic Ca^{++} overload, long chain fatty acid accumulation, increased phosphate concentration, and severe oxidative stress (reactive oxygen species) and ATP depletion progressively increase mitochondrial susceptibility to mitochondrial permeability transition (MPT), increasing the likelihood that MPT will occur on reperfusion (MPT triggering phase) leading to matrix swelling, osmotic breakdown of both mitochondrial membrane, release of apoptotic signaling molecules such as 'cytochrome c' from the intermembrane space, and irreversible injury. Minimal mitochondrial permeability transition leads to full recovery. Localized mitochondrial permeability transition can result in recovery or apoptosis in 50% of cases. Generalized mitochondrial permeability transition leads to cell necrosis in 50–90% of cases.

APOPTOSIS

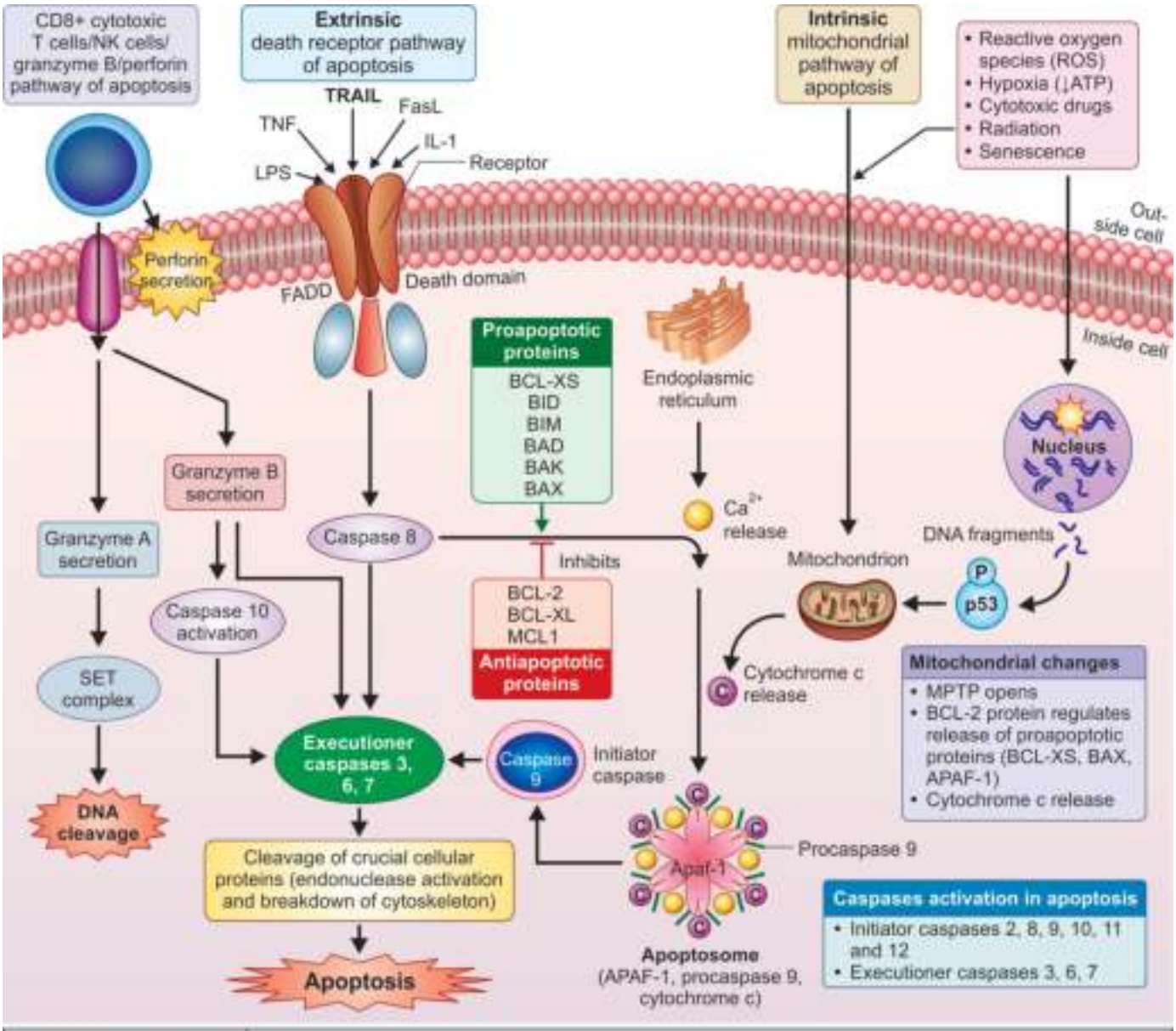
Apoptosis is an 'ATP-dependent' death receptor/mitochondrial pathway mediated programmed cell death that enables the removal of potentially harmful single or group of DNA damaged cancerous cells, virus infected cells, or otherwise unwanted cells in a controlled manner without releasing harmful contents into surrounding environment. Hence, apoptotic signaling pathways help to safeguard the 'genomic stability'. Kindly refer to apoptosis in details in this chapter.

- Apoptosis is characterized by cell shrinkage, plasma membrane blebbing, DNA chromatin condensation and karyorrhexis, cell breaks apart into several apoptotic bodies and phagocytosed by macrophages and surrounding epithelial cells and without inducing inflammation.
- Apoptosis is mediated by molecular pathways that culminate in the activation of family of cysteine proteases, known as the caspases in order to maintain tissue homeostasis during embryogenesis and postnatal life, which orchestrate the dismantling and clearance of the apoptotic cell.
- Apoptosis is initiated via three molecular pathways: (a) extrinsic (signal through cell membrane death receptors such as Fas or tumor necrosis factor) pathway, (b) intrinsic (release of 'cytochrome c' from mitochondria through an extracellular domain) pathway, and (c) CD8+ cytotoxic T cells/NK cells (granzyme B/perforin) mediated pathway. Schematic representation of extrinsic (death receptor) and intrinsic (mitochondrial) pathways of apoptosis is shown in Fig. 1.27.
- Apoptosis exhibits cytoplasmic shrinkage, condensation of nuclear chromatin (pyknosis), nuclear DNA fragmentation (karyorrhexis), plasma membrane blebbing, mitochondrial dysfunction, culminating with the formation of apparently intact small vesicles (known as apoptotic bodies), that are efficiently phagocytosed by macrophages and neighboring healthy cells and degraded within lysosomes. There is no inflammatory response in apoptosis, because harmful cellular contents of apoptotic cells are not released into the surrounding tissue.
- Techniques for detection of apoptotic cells include: cytomorphologic changes (light and electron microscopy), DNA discontinuous fragmentation ladder assay (conventional agarose gel electrophoresis), terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay, annexin V (immunohistochemistry), p53 (immunohistochemistry and RT-PCR, Western blot, enzyme-linked immunosorbent assay, flow cytometry), executioner 3 activation (immunohistochemistry, Western blot, enzyme-linked immunosorbent assay), Fas, TNF-α (tumor necrosis factor α), TRAIL: tumor necrosis-related apoptosis-inducing ligand (RT-PCR, Western blot, immunohistochemistry) and cytochrome c release (ELISA: enzyme-linked immunosorbent assay).

NECROPTOSIS

Necroptosis is an alternative mode of regulated caspase independent and receptor interacting protein (RIP1, RIP3) kinase mediated cell death mimicking features of apoptosis and necrosis. Formation of the necrosome is key event in the induction of necroptosis by inducing cell swelling, cell membrane disruption, inflammation and vascularization. Necroptosis is a strong trigger of innate and adaptive immune response. Apoptosis and necroptosis differ in several aspects.

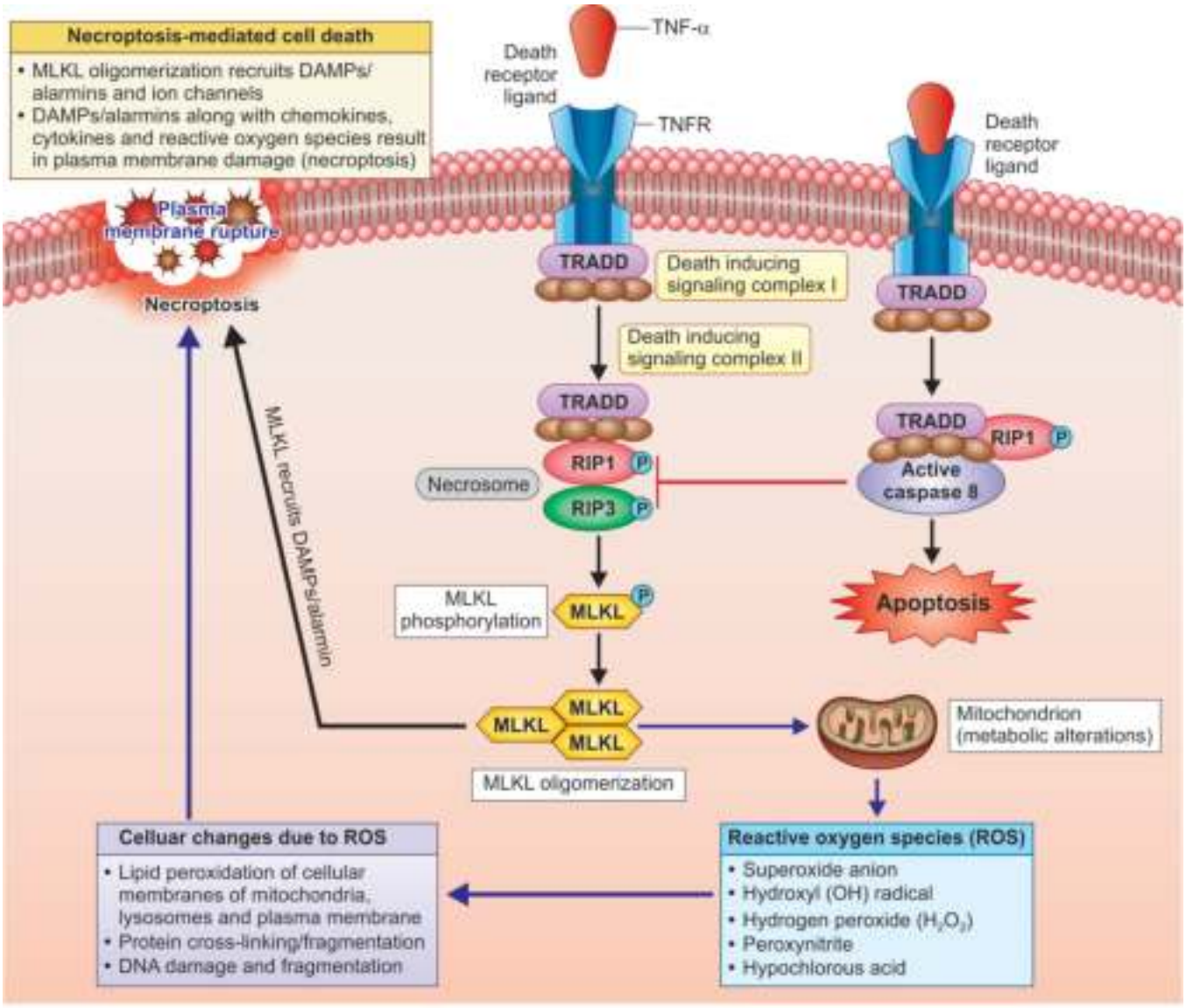
- Apoptosis mainly occurs during embryogenesis, while necrosis is more common during disease states. Apoptosis is mediated by caspases. Generally, in apoptosis, cells undergoing apoptosis maintain the integrity of their cell membranes and avoiding the exposure of intracellular contents to surrounding environment and there is no inflammatory response.
- Necroptosis may directly activate and modulate inflammatory responses by releasing intracellular contents through the disrupted plasma membrane



Morphologic changes	Apoptosis is characterized by cell shrinkage, chromatin condensation, nuclear fragmentation, plasma membrane blebbing without loss of cell integrity, organelles in the plasma membrane blebs, generation of apoptotic bodies.
Clearance	Degradation of apoptotic bodies by phagocytes (macrophages and surrounding cells).
Laboratory diagnosis	Techniques for detection of apoptotic cells include: (a) cytomorphologic changes, (b) DNA discontinuous fragmentation ladder assay on agarose gel electrophoresis, (c) TUNEL assay, executioner caspase 3 and annexin V (IHC), (d) p53, executioner caspase 3 activation, FasR, TNF-α, and TRAIL (IHC, RT-PCR, Western blot, ELISA), and (e) cytochrome c release (ELISA).

Fig. 1.27: Schematic representation of extrinsic (death receptor) pathway, intrinsic (mitochondrial) pathways of apoptosis. The extrinsic pathway is activated by signal such as Fas ligand (FasL), that on binding to the Fas receptor, forming a death-inducing complex by joining the Fas-associated death domain (FADD) to the death domain of the Fas receptor. Intrinsic pathway is activated by injurious stimuli such as reactive oxygen species and DNA damage that induce the release of mitochondrial cytochrome c into the cytoplasm. Both pathways of apoptosis differ in their induction and regulation. Both pathways culminate in the activation of 'executioner' caspases and activation of caspases by cytotoxic T cells.

into the surrounding environment. However, cells undergoing necroptosis are indistinguishable from that undergoing necrosis, using standard histologic techniques. Necroptosis requires that the function of caspase 8 either be disrupted or inhibited. Schematic representation of necroptosis is shown in Fig. 1.28.



Death receptor-dependent pathway of necroptosis	
• **Necroptosis** is an alternative mode of regulated kinase mediated cell death mimicking features of apoptosis and necrosis. It emerges as a backup mechanism, when apoptosis remains nonfunctional. It involves the release of intracellular 'danger signals' which results in considerable inflammation. • Necroptosis depends on receptor interacting protein kinase 3 (RIP3)-mediated phosphorylation of the pseudokinase mixed-lineage kinase domain-like (MLKL). • Necroptosis is demonstrated by immunohistochemistry and immunofluorescence employing antibodies to phosphorylated MLKL. Patients are treated with inhibitors of necroptosis such as necrostatins.	
Process	Mostly passive process, always pathological
Induction stimuli	Viruses, chemical exposure, radiation, endogenous or pathological factors
Morphologic changes	Swelling of cells and organelles, loss of plasma membrane integrity, receptor shedding, lysosomal exocytosis
Molecular changes	Acidosis, random degradation, release of cellular proteins
Clearance of necrotic cells	Necrotic cells phagocytosed by macrophages associated with significant inflammation

Fig. 1.28: Schematic representation of necroptosis. Necroptosis mimics features of necrosis and apoptosis. It involves the release of intracellular danger that results in considerable inflammation. Necroptosis requires protein RIPK3, a regulator of inflammation, cell survival and disease. Necroptosis is regulated by receptor-interacting protein kinase 1 and 3 (RIPK1, RIPK3) mediated phosphorylation of mixed-lineage kinase domain-like (MLKL). Necroptosis is demonstrated by immunohistochemistry and immunofluorescence microscopy by applying monoclonal antibody to phosphorylated MLKL.

MOLECULAR PATHOGENESIS

Necroptosis can be triggered by multiple stimuli such as toll-like receptors superfamily (TLR3, TLR4), death receptors (Fas/CD95, TNFR1), TNF-α, reactive oxygen species (ROS), interferon and some other mediators; but without activation of caspases (especially caspase 8); and regulated by receptor-interacting protein kinase 1 and 3 (RIPK1, RIPK3) mediated phosphorylation of mixed-lineage kinase domain-like (MLKL). Receptor-interacting protein kinase 1 and 3 (RIPK1, RIPK3) have been well recognized regulator of inflammation, cell survival and diseases. Key proteins involved in the necroptosis pathway are given in Table 1.32.

- Based on the driving forces, necroptosis is classified into three categories: (a) TNF-α mediated extrinsic necroptosis, (b) reactive oxygen species mediated intrinsic necroptosis, and (c) ischemia mediated necroptosis.
- All signaling components converge on RIP3 for the execution of necroptosis. TNF-α mediated extrinsic necroptosis occurs by activation of TNFR1 by engagement of TNF-α, which can trigger the formation of a prosurvival complex I containing receptor-interacting protein kinase 1 (RIPK1). When complex I undergoes ubiquitination, it leads to NF-κB mediated survival.
- Alternately, deubiquitination of RIPK1 due to ubiquitin carboxyl-terminal hydrolase or cellular targeted pharmacological inhibitors of apoptosis can activate complex IIa containing tumor necrosis factor receptor type 1 (TNFR1) associated death domain protein (TRADD), Fas-associated protein with death domain (FADD) and RIP1. In the process of caspase 8, complex II preferentially drives toward complex IIa resulting in apoptosis.
- However, in the presence of caspase 8 and presence of RIP3, complex II switches to pro-necroptotic complex IIb. The complex IIb induces necroptosis via the phosphorylation of mixed-lineage kinase domain-like (MLKL) by RIPK3 or complex IIb association of phosphoglycerate mutase family member 5 (PGAM-5), that results in opening of the mitochondrial permeability transition pore (MPTP) complexes.
- Necroptosis plays a role in the pathogenesis of biological processes involving central nervous system, cardiovascular system, respiratory system, urinary system, gastrointestinal system including liver and pancreas. Necroptosis may induce autoimmune disorders, organ transplant rejection and infections.

Table 1.32 Key proteins involved in the necroptosis pathway

Protein	Protein Role in Necroptosis
RIPK1	Protein kinase that recruits RIPK3 to necrosome, resulting in mutual phosphorylation of RIPK1 and RIPK3
RIPK3	Protein kinase that phosphorylates MLKL. RIPK3 is activated by phosphorylation by RIPK1 and subsequent oligomerization
MLKL	MLKL is kinase domain-like protein. Once phosphorylated by RIPK3, MLKL translocate to the cell membrane to mediate cell death.
Caspase 8	Caspase 8 inhibits necroptosis

Necroptosis disrupts caspase 8.

CLINICAL SIGNIFICANCE

Necroptosis can be both beneficial and deleterious during viral infections. HIV-1 can induce necroptosis of T cells. Hence, it is most important to block necroptosis in HIV infection. Hence, blockage of necroptosis is a primary concern for controlling certain viral diseases.

- **Necroptosis beneficial effects:** Necroptosis can be beneficial during certain viral infection. Cell death by the process of necroptosis occurs before completion of viral replication cycle, that can limit disease progression. For example, murine cytomegalovirus replication can be prevented by necroptosis.
  - Cancer is treated by radiotherapy and chemotherapy to induce apoptosis and kill malignant cells.
  - Resistance to these therapeutic modalities (i.e. radiotherapy and chemotherapy) occur due to the malignant cells resisting apoptotic death. In such cases, alternate mechanisms of cell death can be successfully induced by necroptosis.
- **Necroptosis harmful effects:** Necroptosis can induce various human disorders involving multiorgans. Patients are treated with inhibitors of necroptosis such as necrostatins. Necroptosis induced various human disorders are given in Table 1.33.

DIAGNOSTIC TECHNIQUES

Necroptosis is demonstrated by immunohistochemistry and immunofluorescence employing antibodies to phosphorylated mixed-lineage kinase domain-like (MLKL).

- Four types of immunohistochemical techniques include enzyme-linked immunosorbent assay, flow cytometry, immunoelectron microscopy and Western blotting.
- Necroptosis is diagnosed by detection of RIPK1, RIPK3; receptor interacting serine/threonine-protein kinase (immunofluorescent staining), MLKL (quantitative RT-PCR), RIPK1/RIPK3 complex, RIPK3, MLKL (Western blot), RIPK1, RIPK3, mixed-

Table 1.33 Necroptosis induced various human disorders

Organs/Systems	Disorders
Central nervous system	Neurodegenerative disorders (Alzheimer's disease, Huntington's disease, Parkinson's disease), cerebral stroke
Cardiovascular system	Coronary atherosclerosis, myocardial infarction, abdominal aortic aneurysm
Respiratory system	Chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS)
Gastrointestinal tract	Inflammatory bowel disease
Liver	Hepatocellular injury
Pancreas	Pancreatitis
Urinary system	Acute renal injury
Autoimmune disorders	Psoriasis, rheumatoid arthritis
Organ transplantation	Organ transplant rejection
Infections	Influenza, West Nile

lineage kinase domain-like (MLKL) pseudokinase, enzyme-linked immunosorbent assay (ELISA), annexin V/propidium iodide + or annexin V +/propidium iodide + (flow cytometry), RIPK1/RIPK3 complex (immunoprecipitation and electron microscopy), RIP1 (immunoblotting), membrane translocation (immunofluorescence microscopy) and TRF (microscopy).

PYROPTOSIS

Pyroptosis is a highly inflammatory form of programmed cell death that occurs most frequently upon infection with intracellular pathogens. Caspase 1 is the most important enzyme that mediates pyroptosis, which controls intracellular pathogens by immune system. Schematic representation of pyroptosis is shown in Fig. 1.29.

MOLECULAR PATHOGENESIS

The immune cells recognize foreign danger signals within themselves by toll-like receptors (TLRs) and nod-like receptors (NLRs).

- Toll-like receptors recognize pathogen-associated molecular patterns (PAMPs), which are located either on cell surface or within endosomes. The resulting recognition by TLRs initiate signaling pathway including activation of transcription factors NF-kB and MAPKs signaling pathway. This in turn will be responsible for synthesis of inflammatory cytokines (IFN-α/-β, TNF-α and IL-12); and proinflammatory cytokines (IL-1β, IL-6, IL-8, IL-18) processed by cysteine mediated caspase 1.
- Nod-like receptors are similar to toll-like receptors, which also perform similar functions. The released inflammatory cytokines attract other immune cells to fight infection and contribute to inflammation in the tissues leading to rapid clearance of intracellular pathogens by host defense system.
- Pyroptosis requires three key molecules: (a) inflammasome formed in the macrophage within minutes of infection, (b) caspase 1 activation by inflammasome, and (c) membrane-pore forming effective gasdermin-D protein activation by inflammasome.
- Pyroptosis features cell membrane pore formation, cytoplasmic swelling, rupture of plasma membrane and the release of cytosolic contents such as damage-associated molecular pattern (DAMP) molecules, IL-1β and IL-18 into the extracellular environment, amplifying the local or systemic inflammatory effects. At the same time, the nucleus is damaged and important intracellular substrates are cleaved including cytoskeleton, chaperones, glycolytic proteins and caspase 7, which contribute to pyroptotic cell death.

CLINICAL SIGNIFICANCE

Pyroptosis acts as a defense mechanism to control intracellular pathogens (Salmonella, Shigella). The formation of inflammasome and the activity of caspase 1 determine the balance between pathogen resolution (clearance of infectious agents) and disease.

- In contrast, persistent inflammation will attract excessive immune cells, which may cause metabolic disorders (obesity, type 2 diabetes mellitus), autoimmune disorders and hepatocellular injury associated inflammation. Subsequent excessive production of IL-1β and IL-18 cause destruction of β-cells of islets of Langerhans' of pancreas and impaired secretion of insulin resulting in type 2 diabetes mellitus.
- Mutation in the gene NRPR3 encoding inflammasome leads to gain of function of inflammasome

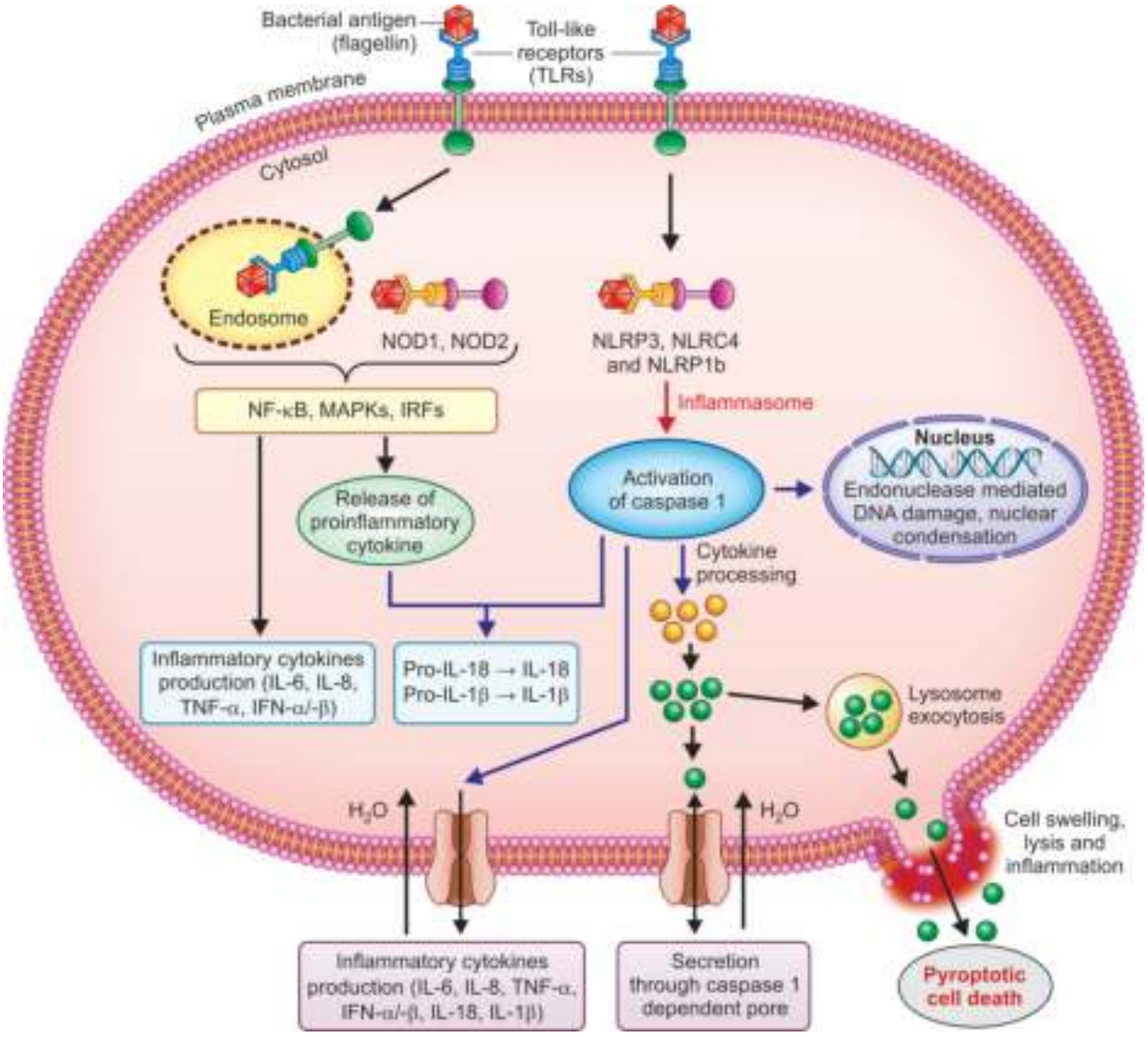


Fig. 1.29: Schematic representation of pyroptosis. Pyroptosis is recently identified caspase 1, interleukins (IL-6, IL-8, TNF-α, IFN-α/-β, IL-18, IL-1β) and inflammasome-dependent pathway of host cell death associated with inflammation. It is triggered by proinflammatory signals and a range of microbial infections. Necroptosis is seen primarily in inflammatory cells such as macrophages. Pyroptosis is stimulated by a range of microbial infections. Pyroptosis is characterized by cell swelling, plasma membrane lysis and endonuclease-mediated DNA damage, nuclear condensation.

resulting in autoinflammatory disorders called cryopyrinopathies such as Muckle-Wells syndrome, cold autoinflammatory syndrome and chronic infantile neurologic cutaneous and articular syndrome. All these autoinflammatory disorders present with sudden onset of fever and localized inflammation.

- Recent studies revealed that caspase 1 mediated pyroptosis drives CD4+ helper T cell depletion and inflammation by HIV. In pathogenic inflammation elicited by HIV infection leads to destruction of CD4+ helper T cells resulting in progression to AIDS. Caspase 1 inhibitors ('anti-AIDS') are safe therapeutic agents used to treat AIDS patients in clinical practice.
- Atherosclerotic plaques are formed by number of events: endothelial dysfunction, accumulation and oxidation of low-density lipoproteins (LDLs), recruitment monocytes and lymphocytes, migration and proliferation of smooth muscle cells; activation of proinflammatory cytokines; adherence of platelets.
  - Recent studies showed that risk factors of atherosclerosis could activate NLRP3 inflammasome in both endothelial cells and macrophages.
  - In addition, NLRP3 inflammasome-mediated pyroptosis plays an important role in the progression of atherosclerotic plaque.
  - Cholesterol crystals have been demonstrated in atherosclerotic plaque. Various studies revealed that

cholesterol crystals activate NLRP3 inflammasome that induces lysosomal damage resulting in release of IL-1β.

- Caspase 1 activation induced by NLRP3 inflammasome is a major mediator for pyroptosis, that takes part in the progression of atherosclerosis.
- NLRP3 inflammasome activation and related pyroptosis are potential novel therapeutic targets for myocardial ischemia–reperfusion injury (MIRI). Patients are treated with inhibitors of inflammasome.

DIAGNOSTIC TECHNIQUES

Pyroptosis can be analyzed by looking at caspase 1 activation by employed antibody, gasdermin-D cleavage, or by inhibiting key components of the pyroptotic pathway by immunohistochemistry and immunofluorescence employing specific antibodies against epitopes in gasdermin protein family members. Pyroptosis can be diagnosed by detection of bicinchoninic acid (BCA), caspase 1 (Western blot; RT-PCR), caspase 1, CD31 (TUNEL staining, terminal deoxynucleotidyl transferase and immunostaining), IL-1β, IL-18, pro-IL-1β, IL-1α, ELISA (enzyme-linked immunosorbent assay), FAM-FLICA-caspase 1 and Pl (flow cytometry), NLRP3 (NOD-like receptor protein 3), caspase 1, IL-1β, IL-18 (immunofluorescence microscopy), calcium (immunofluorescence microscopy), IL-1β, caspase 1, caspase 8 (immunostaining), determination of LDH (lactate dehydrogenase), phospholipid fatty acid (PLFA) (isotope labeling), DAB (3,3′-diaminobenzidine) staining and AEC chromogranic staining.

FERROPTOSIS

Ferroptosis is a regulated cell death dependent on iron that involves the depletion of intracellular antioxidants including reduced glutathione (GSH) and consequent accumulation of lethal lipid peroxides and reactive oxygen species (ROS) in cells, ultimately leading to oxidative cell death. Ferroptosis is inhibited by glutathione peroxidase 4 (GPX4), a major gatekeeper of intracellular redox homeostasis.

MOLECULAR PATHOGENESIS

Normally, glutathione (GSH) is an antioxidant capable of preventing damage to important cellular components caused by reactive oxygen species (ROS) such as oxygen-derived free radicals, lipid peroxides, and heavy metals. **Erastin** is a small molecule capable of initiating ferroptotic cell death.

- Glutathione peroxidase 4 (GPX4) converts glutathione into oxidized glutathione (GSSG) and reduces the cytosolic lipid peroxides to the corresponding alcohols. Inhibition of **glutathione peroxidase 4** activity can lead to the accumulation of lethal lipid peroxides, which is marker of ferroptosis.
- Lipid peroxides and iron are two major participants of ferroptosis. Thus, iron chelators (e.g. deferoxamine) and several lipophilic antioxidants (e.g. α-tocopherol) can rescue ferroptosis. Additionally, reactive oxygen species (ROS) produced through Fenton reaction catabolized by iron contributes to the initiation of ferroptosis.
- Ferroptosis diagnosed by detection of ATP5G3, PTGS2, IREB2 (iron responsive element binding protein 2), CS, RPLS (quantitative RT-PCR), JNK, ERK1/ERK2 (extracellular signal-regulated kinase 1), p38, LC3-I/LC3-II, NRF2, p62, Slc7a11 (Western blot), Fe^{++} release assay (flow cytometry), GPX4; glutathione peroxidase 4, ELISA (enzyme-linked immunosorbent assay), NADP/NADPH, LC3 (immunofluorescence microscopy).
- Recent studies revealed that ferroptosis is closely related to the pathophysiologic processes of many disorders, such as tumors, nervous system disorders, heart diseases, liver diseases, gastrointestinal diseases, pancreatic diseases, lung diseases and kidney diseases. Ferroptosis induced disorders are given in Table 1.34.

Table 1.34 Ferroptosis induced disorders

Organ/System	System-related Disorders
Nervous system	Neurodegenerative diseases, cerebral stroke, traumatic brain injury
Cardiovascular system	Ischemia-perfusion injury
Liver	Hepatocellular carcinoma, liver fibrosis, ischemia-related injury
Gastrointestinal tract	Gastric carcinoma, colorectal carcinoma
Lungs	Lung carcinoma
Kidney	Acute renal injury, ischemia-related injury, clear cell variant of renal cell carcinoma

AUTOPHAGY

Term autophagy, nonapoptotic cell death is derived from Greek words '*auto*' meaning self and '*phagy*' meaning eating, that maintains homeostasis.

- Autophagy is the process that delivers cytoplasmic material of endogenous (cell's own contents such as mitochondria, endoplasmic reticulum and peroxisomes) or exogenous material (misfolded proteins) to the lysosomes for degradation and recycle nutrients for new cell formation and survival.

- Cell features in autophagy include vacuolization, degradation of cytoplasmic contents, slight condensation of chromatin.

MOLECULAR PATHOGENESIS

Autophagy is initiated in response to extracellular or intracellular stress signals such as starvation, hypoxia, deprivation of nutrients and growth factors, accumulation of misfolded proteins, endoplasmic reticulum stress, pathogens, irradiation, chemotherapy and change in cell volume. Autophagy handles damaged cellular organelles and protein aggregates.

- Damage to mitochondria causes disruption of electron transport and dissipates the electrochemical gradient across the mitochondrial membrane.
  - Increased reactive oxygen species (ROS) result in oxidative damage to mitochondria leading to recruitment of cytosolic proteins, parkin and a ubiquitin-like (UBL) protein.
  - The complex of fragmented mitochondria-parkin-ubiquitin-like protein binds to p62. Proteins that have sustained oxidative damage are conjugated to UBL protein and form aggregates, which are then bound by p62.
  - The p62-bound complexes with damaged mitochondria and aggregated proteins are recognized by a specific receptor in the phagophore, thereby resulting in autophagy.
- Defective autophagy plays important role in the pathogenesis of cancer, infectious diseases and neurodegeneration.

TYPES OF AUTOPHAGY

Autophagic degradation is generally divided into three categories: microautophagy, macroautophagy, and chaperone-mediated autophagy. Schematic representation of macroautophagy is shown in Fig. 1.30. Schematic representation of chaperone-mediated autophagy is shown in Fig. 1.31. Comparison of macroautophagy, microautophagy and chaperone-mediated autophagy is given in Table 1.35.

Microautophagy

In microautophagy, cytosolic cargoes are phagocytosed by inward invagination of lysosomal membrane for delivery. The contents are then degraded by lysosomal enzymes. Insulin suppresses autophagy in the liver, while glucagon encourages it.

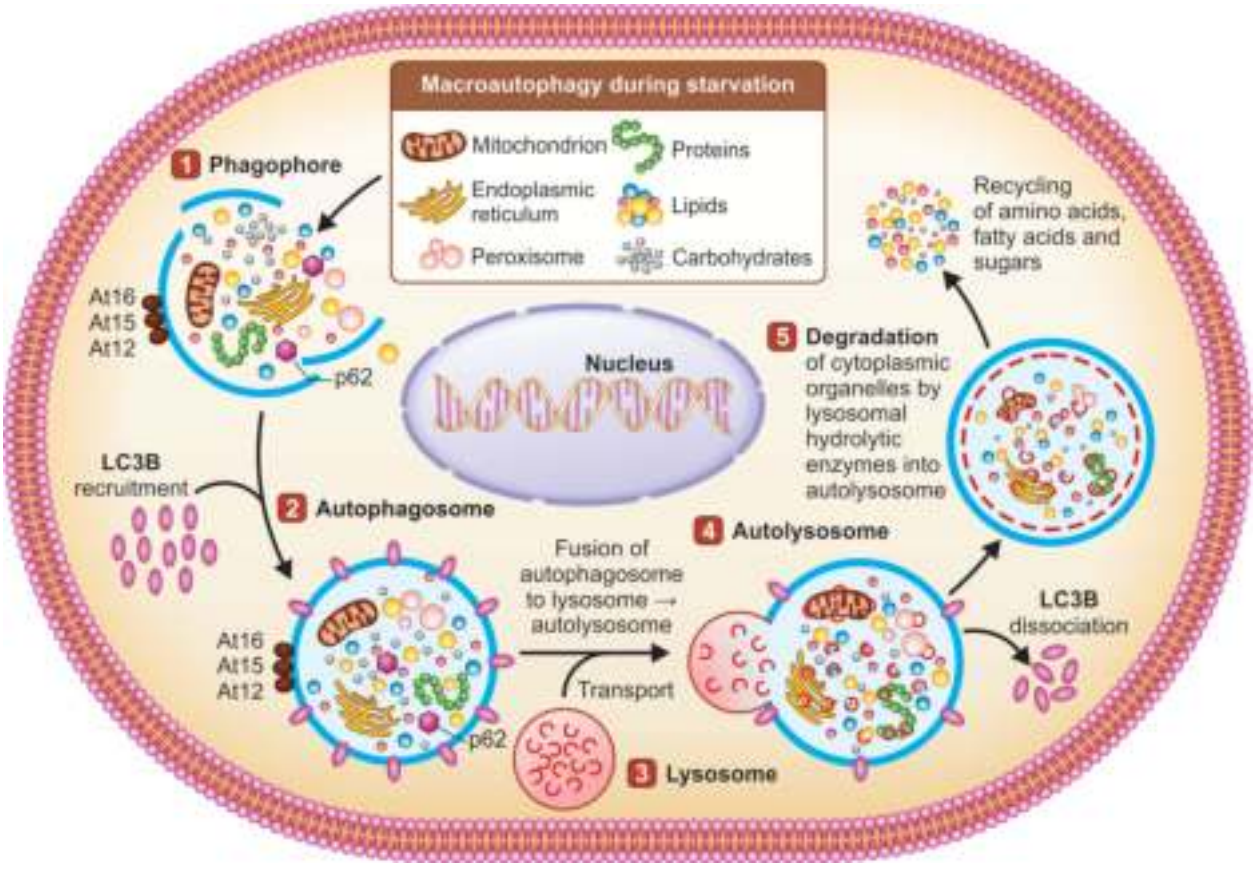


Fig. 1.30: Schematic representation of macroautophagy. Macroautophagy is a form of autophagy process substrates are sequestrated within cytosolic double-membrane vesicles termed autophagosomes before it fuses with the lysosomes. The substrates of macroautophagy include superfluous and damaged organelles, cytosolic proteins and invasive microbes.

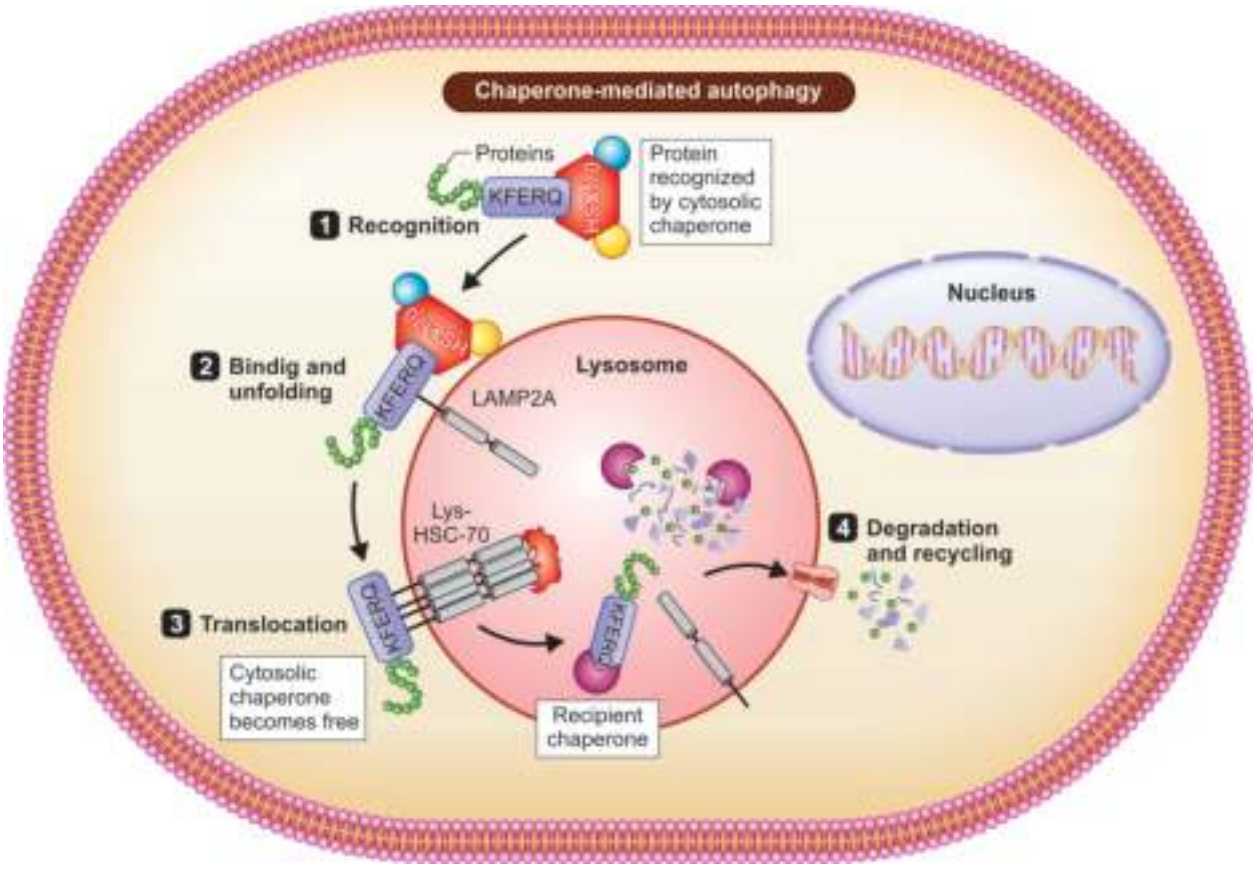


Fig. 1.31: Schematic representation of chaperone-mediated autophagy. It is a lysosomal pathway of proteolysis that degrades 30% of cytosolic proteins under conditions of prolonged nutrient deprivation. Molecular chaperones in the cytosol and in the lysosomes stimulate this proteolytic pathway.

Table 1.35 Comparison of microautophagy, macroautophagy and chaperone-mediated autophagy

Definition	Morphological Features	Laboratory Diagnosis
Microphagy (normal physiologic process digesting soluble cytosolic proteins)		
Transport of cytoplasmic contents within lysosomal membrane invagination or deformation and phagocytosis of organelles	Lysosomal membrane invagination or deformation	Transmission electron microscopy
Macrophagy (stress-related process digesting soluble cytosolic proteins)		
Cargo is transported to the lysosome by *de novo* formation of autophagosomes and phagocytosis of organelles	Membrane expansion and bend; phagophore nucleation and elongation and formation of autophagosome	▪ Electron microscopy ▪ Immune colloidal gold technique ▪ Immunofluorescence microscopy (GFP-LC3 or mRFP-GFP-LC3) ▪ Western blot (LC3-II/LC1, Beclin, ATG5, ATG7, p62 and phosphorylation status of ULK) ▪ Radiolabeling (LDH sequestration) ▪ MDC staining
Chaperone-mediated autophagy (stress-related and receptor-mediated process digesting only one letter code for amino acid sequence)		
KFERQ tagged, the one letter code for amino acid sequence Lys-Glu-Phe-Arg-Gln		
Unfolded proteins containing the KFERQ motif are transported directly across the lysosomal membrane through the action of cytosolic chaperones without phagocytosis of organelles	Multimerization of LMP2A binding to the luminal side of the lysosomal membrane by HSP90	▪ Immunofluorescence microscopy (Hsc with lysosomal markers) ▪ Western blot (LAMP2A) ▪ Radiolabeling (LAMP2A)

Macroautophagy

Macroautophagy is the major form of autophagy involving the sequestration and transportation of cytoplasmic organelles in the cytosol in a double membrane bound autophagic vacuoles. In macroautophagy, cytoplasmic organelles (mitochondria, endoplasmic reticulum, Golgi appratus, soluble protein, aggregated protein) are directly sequestrated by the lysosomes by an open membrane, called 'phagophore'.

- Upon closure by fusion, phagophore becomes autophagosome, which then delivers its content to a lysosome. Lysosomal enzymes degrade the contents to small molecules for reutilization.
- Macroautophagy is regulated by the mammalian target of rapamycin (mTOR) and AMP-activated kinase (AMPK) pathways. Macroautophagy inhibits mTOR and stimulates unc-51-like autophagy-activating kinase (ULK) and class III phosphatidylinositol-3 kinase complex.
- Further, the phagophore formation is concluded by light chain 3 (LC3) and autophagy-related gene 12 systems. Then the autophagosome can fuse with the lysosome to form an autolysosome for substrate degradation in the lysosome through autophagosomes.
- In selective macroautophagy, there are receptors like p62 that recognize the cargo proteins and target them to the autophagosome.

Chaperone-mediated Autophagy

Chaperone-mediated autophagy is a lysosomal pathway of proteolysis, in which target proteins with a KFERQ sequence are recognized by a cytosolic chaperone heat stroke cognate 70 (HSC-70) and form a chaperone complex.

- Subsequently, these chaperone-mediated autophagy substrates are recognized by lysosomal-receptor proteins (LAMPs) and translocated to the lysosomal interior through the action of LAMP-2A, where they are received by a second chaperone.
- Targeted intracellular proteins are degraded in the lysosome. The original, extralysosomal chaperone survives to work further.

LABORATORY DIAGNOSIS

Autophagy is diagnosed by detection of immune colloidal gold technique, GFP-LC3 (green fluorescent protein-light chain 3) or mRFP-GFP-LC3 (immunofluorescence microscopy), LC3-II/LC3-1, beclin, ATG5, ATG7 (autophagy-related protein 5/7), p62 and phosphorylation status of ULK (unc-51-like kinase) (Western blot), LDH (lactate dehydrogenase) sequestration, MDC staining, HSC-70 with lysosomal markers (immunofluorescence microscopy) and LMP2A assay (Western blot).

LYSOSOMAL-DEPENDENT CELL DEATH

Lysosomes contain numerous hydrolases that can degrade most cellular macromolecules. Lysosomal membrane permeabilization (LMP) and consequent leakage of the lysosomal hydrolases into the cytosol results in lysosomal-dependent cell death.

- **Reactive oxygen species (ROS):** Reactive oxygen species (ROS) and ischemia-reperfusion injury contribute to lysosomal membrane permeabilization. Various oxidative stimuli include drugs, metals, ionizing radiation, ischemia-perfusion injury, inflammation and neurodegenerative disorders.
  - Upon oxidative stress, excess H_2O_2 diffuses into lysosomes where it reacts with redox active iron, resulting in the production of hydroxyl radicals in Fenton-type reaction.
  - Hydroxyl radicals are highly reactive, that can destabilize the lysosomal membrane by causing lipid peroxidation and damaging lysosomal membrane proteins.
  - Additionally, reactive oxygen species (ROS) contribute lysosomal membrane permeabilization by activating Ca^{++} channels or altering the activity of lysosomal phospholipase A2 enzyme. Various antioxidants and redox regulators as well as iron-binding proteins confer protection against oxidative stress-induced lysosomal membrane permeabilization.
- **Viral proteins:** Virus infection requires the transmission of viral proteins into the cell, which mostly occurs by penetrating the endolysosomal membranes with viral entry proteins that become active in the acidic environment. Nonenveloped viruses (adenovirus, rhinovirus HRV14, poliovirus) contribute to lysosomal membrane permeabilization resulting in release of lysosomal contents into the cytosol.

Pathology Pearls: Cellular Senescence and Mitotic Catastrophe

The term 'cellular senescence' refers a pathophysiologic process by which the cells permanently lose their proliferative capacity (i.e. permanent state of cell cycle arrest) subjected to different stresses, while remaining viable and metabolically active. Senescence is therefore a cellular defense mechanism that prevents cells to acquire an unnecessary damage.

- Senescence takes place in several tissues during physiologic and pathologic processes such as tissue remodeling, embryogenesis, cell injury, cellular aging and cancer. In tissue remodeling and embryogenesis, senescent cells are required for tissue repair and proper development of the embryo.
- In cancer, senescent cells act as potent barrier to prevent tumorigenesis. Research is going on to induce senescence in cancer cells by pharmacological interventions in the light of the development of novel therapeutic targets.

- Mitotic catastrophe is a mechanism of delayed mitotic-linked cell death resulting from aberrant mitosis during the metaphase/anaphase transition, which occurs due to combination of deficient cell-cycle checkpoints (DNA structure checkpoints and the spindle assembly checkpoint) and cellular damage.
- In aberrant mitosis, cells fail to execute apoptotic program divide asymmetrically in the next round of cell division with subsequent degeneration of aneuploid cells.

APOPTOSIS

Apoptosis word is derived from Greek in 1972, that means dropping of the leaves and petals from flowers. Apoptosis is an 'ATP-dependent' programmed cell death ('cell suicide') that enables the removal of potentially harmful single or group of DNA damaged cancerous cells, virus infected cells, or otherwise unwanted cells in a regulated manner. Hence, apoptotic signaling pathways, help to safeguard the 'genomic stability'.

- Apoptosis can be induced by a broad range of injurious stimuli such as deprivation of growth factors, loss of hormone stimulation, DNA damage, ionizing radiation, chemotherapeutic drugs, accumulation of misfolded proteins (endoplasmic reticulum stress), reactive oxygen species (ROS), replication stress, increased cytosolic calcium, microtubular alterations or mitotic defects.
- Apoptosis is mediated by molecular pathways that culminate in the activation of a family of cysteine proteases, known as the caspases in order to maintain tissue homeostasis during embryogenesis and postnatal life, which orchestrate the dismantling and clearance of the apoptotic cell.
- Inducers of apoptosis are growth factor withdrawal, TNF family, calcium, nutrient deprivation, toxins, ultraviolet radiation, γ-radiation and oncogenes. A cell that has been treated with an apoptotic inducer can also initiate apoptosis that does not rely on caspase activation.
- Apoptosis does not induce inflammatory response. In contrast to apoptosis, necrosis is an energy-independent process that can cause inflammation.
- Apoptotic cells can be detected by hematoxylin and eosin-stained section examination by light microscopy, electronic microscopy, gel electrophoresis (showing typical DNA ladder pattern) or quantification using fluorescent dyes, flow cytometry, *in situ* end labeling (ISEL) method, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), and caspase 3 by immunohistochemistry.
- Dysregulation of apoptotic signaling pathways can cause cancerous growth or autoimmune disorders, while accelerated apoptosis is evident in degenerative disorders, immunodeficiency disorders and infertility. Comparison of necrosis and apoptosis is given in Table 1.36.

Table 1.36 Comparison of necrosis and apoptosis

Characteristics	Necrosis	Apoptosis
Major pathways involved		
Definition	Accidental cell death by injurious agents	Controlled (programmed cell death) results in nuclear fragmentation
Provoking stimuli	Usually associated with a pathologic process such as severe hypoxia, toxins, massive insult, conditions of ATP depletion, metabolic stress, absence of nutrients, changes in pH	Physiologic (embryology, thymus involution), and pathologic (removal of misfolded proteins, removal of neutrophils in acute inflammation) conditions without ATP depletion
Enzyme(s) activation	Phospholipase, protease and endonuclease activation	Initiator caspases (2, 8, 9, 10, 11, 12), executioner caspases (3, 6, 7) activation by intrinsic and extrinsic pathways
ATP level	ATP depletion and metabolic disruption	ATP and protein synthesis sustained

Contd...

Table 1.36 Comparison of necrosis and apoptosis (*Contd...*)

Characteristics	Necrosis	Apoptosis
Protein synthesis	Cessation of protein synthesis	Requires protein synthesis
Energy requirement	Energy independent	Energy dependent
Gross morphological changes		
Gross findings	Disruption of normal tissue architecture, i.e. infarct and later scarring	Minimal changes or atrophy without scarring
Histologic changes		
Extent of cell death	Contiguous regions of cell death in whole fields	Death of single cells separating from neighbor cells scattered throughout the affected tissue
Inflammatory response	Present	Absent
Cell size	Cell swelling	Cell shrinkage
Cell borders	▪ Plasmalemmal blebs without organelles (loss of cell borders with irregular fragmentation) ▪ Cell lysis with release of intracellular contents	▪ Zeiotic blebs containing large organelles (formation of round bodies, often within a clear halo) ▪ Cell fragmentation into apoptotic bodies
Nuclear changes	▪ Nuclear swelling and karyolysis ▪ Random DNA degradation (irregular chromatin clumping, pyknosis, karyorrhexis, karyolysis; rupture of nuclear envelope)	▪ Chromatin condensation and fragmentation (apoptotic bodies) ▪ Intranucleosomal DNA degradation (chromatin condensation into caps or crescents, within round nuclear bodies; preservation of nuclear envelope)
Ultrastructural changes		
Cell membrane	Swelling and loss of surface structures with blebbing and loss of apical portions of cytoplasm	Cell condensation with formation of membrane blebs or buds in degenerating cells
Cytoplasm	Rarefaction of cytoplasm followed by condensation after death	Condensation of cytoplasm followed by rarefaction after ingestion by phagocytes
Cell organelles	Swelling and loss of morphology organelles	Organelles preserved
Mitochondria	Mitochondrial swelling and dysfunction	Mitochondrial permeabilization
Lysosomes	Lysosomes abnormal, rupture with release of enzymes	Lysosomes unaffected and remain intact
Internal and external cell membranes	Rupture of cell membranes, bursting of cell and leakage of cell contents	Preservation of cell membranes and nuclear envelope
Intracellular calcification	Present	Absent
Molecular changes		
Gene involved	Gene activity none	BCL-2 (antiapoptotic), BAX (proapoptotic) and BAK (proapoptotic) genes involved
Chromosomal DNA	Random cleavage of chromosomal DNA	Cleaved at specific sites
Ion pump function	Ion pumps lost	Ion pumps continue to function
Intracellular calcium	Unaffected	Increased
Sequelae		
Lysosomal enzymes	Release of intracellular enzymes into extracellular compartment	Retention of intracellular enzymes within the apoptotic bodies
Neutrophils and macrophages	Tissue infiltrated by neutrophils followed by macrophages, which phagocytose dead cells	Ingestion of apoptotic cells by tissue macrophages or surrounding healthy parenchymal cells
Inflammation and scarring	Active inflammation with scarring of tissue	Atrophy with stromal collapse without scarring

THREE PATHWAYS OF APOPTOSIS

Apoptosis is initiated via three molecular pathways: (a) extrinsic (signal through cell membrane death receptors such as Fas or tumor necrosis factor) pathway, (b) intrinsic mitochondrial (release of cytochrome c from mitochondria through an extracellular domain) pathway, and (c) CD8+ cytotoxic T cells/NK cells (granzyme B/perforin) mediated pathway. Schematic representation of apoptosis mediated by extrinsic (death receptor), intrinsic (mitochondrial) and perforin/granzyme (CD8+ cytotoxic T cells and natural killer cells) pathways is shown in Fig. 1.32. Schematic representation of apoptosis mediated by extrinsic (death receptor) and intrinsic (mitochondrial) pathways is shown in Fig. 1.33. Schematic representation of intrinsic (mitochondrial) and extrinsic (death receptor) pathways of apoptosis is shown in Fig. 1.34.

- Apoptosis cascade can be categorized into three phases: (a) signal activation-induction of apoptosis, (b) regulation and execution, and (c) cellular structural alterations.
- The caspases trigger apoptosis by cleaving specific proteins present in cytoplasm and nucleus of all cells in the inactive precursors or procaspases, which are usually activated by cleavage by other caspases.
- Extrinsic death receptor pathway of apoptosis begins with a pro-death signal originating from outside the cell, most often by CD8+ cytotoxic T cells/natural killer cells, granzyme B/perforin-mediated pathway, when conditions in the extracellular environment determine that a cell must die.
- Intrinsic mitochondrial pathway of apoptosis begins when an injury occurs within the cells and depends on the release of cytochrome c and proapoptotic proteins from the intermembrane space of mitochondria.
- During intersection of the extrinsic and intrinsic pathways of apoptosis, a receptor-ligand interaction activate caspase 8, which may in turn cleave cytosolic BID to yield truncated derivative, BID. In turn, tBID translocates to mitochondria, thereby activating the intrinsic pathway of apoptosis. Sequence of events includes: caspase 8 → activates BID → cleavage to truncated derivative BID → activates intrinsic (mitochondrial) pathway.
- CD8+ cytotoxic T cell/NK cell (granzyme B/perforin) mediated pathway induces apoptosis releasing two types of preformed cytotoxic proteins such as granzyme B and perforin. Granzyme B induces apoptosis of target cell, and the pore forming perforin protein, which punches holes in the target cell membrane through which the granzyme B can enter the target cell to induce apoptosis.
- All three pathways of apoptosis can flow independently and converge until the last step of DNA degradation by executioner caspases (3, 6, 7). The executioner caspases disrupt cytoskeletal components or cell replication machinery, changes to cell surface molecules, which facilitate phagocytosis. Apoptotic cells are removed by macrophages and surrounding epithelial cells.
- Apoptosis inducible factor (AIF) is a caspase-independent pathway of apoptosis, which regulates intrinsic or mitochondrial pathway. AIF migrates to the nucleus, passes through the membrane, binds to DNA and triggers the characteristic condensation of chromatin, which is the hallmark of apoptosis. Role of caspases and mitochondrial proteins in apoptosis is given in Table 1.37.

Pathology Pearls: Regulation of Apoptosis by Gene Products

- Mitochondria play a pivotal role in apoptosis, which contain genes regulating proapoptotic and antiapoptotic proteins.
- Apoptosis is regulated by caspases and gene regulating proteins and p53 tumor suppressor proteins. Glucocorticoids induce apoptosis. Sex steroids inhibit apoptosis.
- Proapoptotic proteins facilitate apoptosis. When cells are deprived of survival signals or subjected to stress, BCL-2 and/or BCL-X are lost from the mitochondrial membrane and replaced by proapoptotic members of the family, such as BAX, BAK, and BIM.
- Antiapoptotic proteins normally reside in mitochondrial membranes and the cytoplasm. Growth factors and other survival signals stimulate the production of antiapoptotic members of the BCL-2 family of protein.
- Mitochondrial proteins, i.e. BAD, BID and PUMA that regulate balance between proapoptotic and antiapoptotic proteins.
- DNA is protected by p53 tumor suppressor gene. The p53 protein induces apoptosis by several mechanisms: (a) it downregulates transcription of the antiapoptotic protein: BCL-2, (b) it upregulates transcription of the proapoptotic genes: BAX, BAK and BIM, (c) it activates proteins that arrest the cell in G1 phase of cell cycle, permitting time for DNA repair to go ahead. If the DNA damage is irreparable, p53 tumor suppressor gene activates mechanisms that terminate in apoptosis.

EXTRINSIC DEATH RECEPTOR PATHWAY OF APOPTOSIS

In extrinsic death receptor pathway of apoptosis, ligand–receptor interactions lead to caspase activation. A number of ligands bind their corresponding death receptors, which include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5. The sequence of events that define the extrinsic pathway of apoptosis are best characterized with TNF-α/TNFR1 and FasL/FasR models.

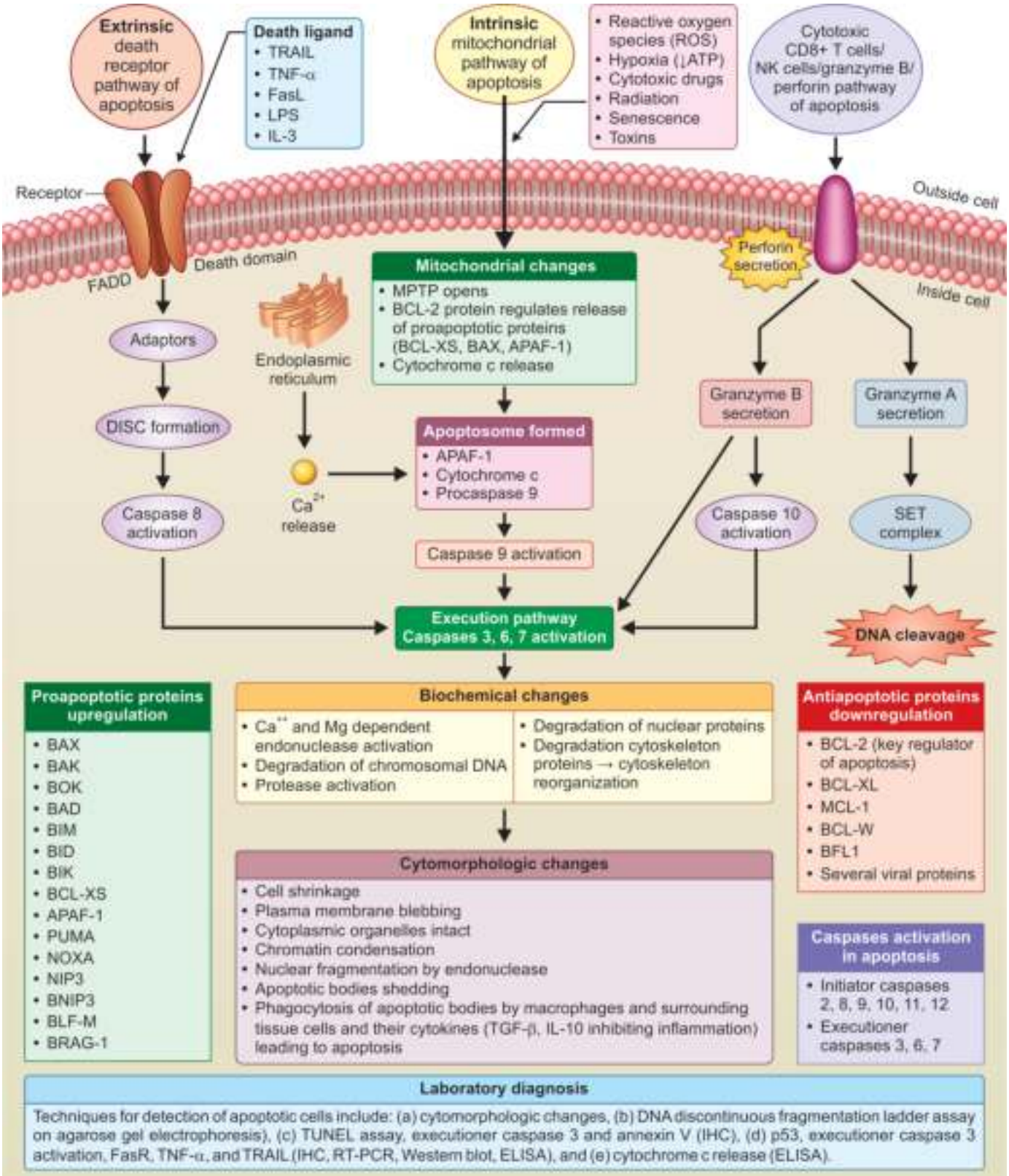
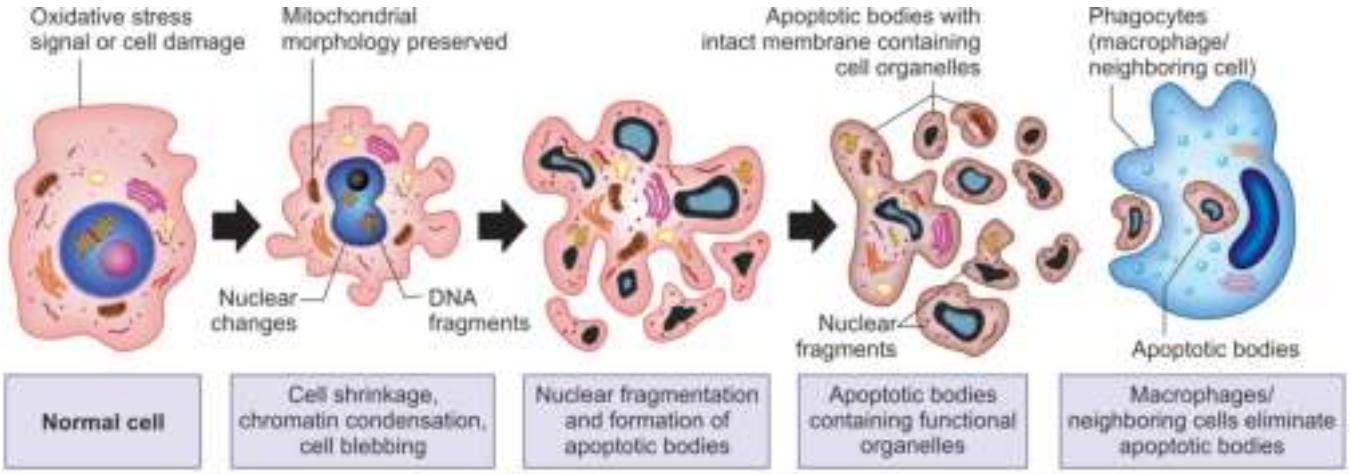


Fig. 1.32: Schematic representation of apoptosis mediated by extrinsic (death receptor), intrinsic (mitochondrial) and perforin/granzyme (cytotoxic T cells and natural killer cells) pathways. Apoptosis is characterized by cell shrinkage, plasma membrane blebbing, intact cytoplasmic organelles, chromatic condensation, nuclear fragmentation by endonuclease, apoptotic cell bodies shedding and phagocytosis of apoptotic bodies by macrophages and surrounding epithelial cells and lack of inflammation.



Apoptosis (programmed cell death)	
Induction stimuli	Oxidative stress, death receptor ligands and chemotherapy
Process	Active process executed by caspases (physiologic or pathologic processes)
Sequence of events to induce apoptosis in normal cell	***Role of TP53 (p53 protein) pathway to induce apoptosis in normal cell:*** Phosphorylated p53 protein → Activation of **BAX, PUMA** and **NOXA** → **Apoptosome** (APAF, cytochrome c, procaspase) → Activated **caspases 9 and 3** → **Apoptosis**
Sequence of events to induce apoptosis under pathologic state	***Extrinsic death receptor pathway of apoptosis:*** **FasL** → **Fas** → **FADD** (Fas activated death domain) → Activation of **caspase 8** → Activation of **caspases 3, 6, 7** → **Apoptosis** ***Intrinsic mitochondrial pathway of apoptosis:*** **BCL-2** protein → **BAX** proapoptotic protein → **Mitochondrial cytochrome c leakage/APAF-1** (apoptotic protease activating factor) → **Apoptosome** (cytochrome c + APAF-1 + caspase 9) → Activation of **caspase 9** → Activation of **caspases 3, 6, 7** → **Apoptosis** • Both extrinsic and intrinsic pathways can flow independently until the last step of DNA degradation by executioner caspases. The caspases cause proteolysis of specific substrates resulting in various morphological and biochemical characteristics in cells. • **Initiator caspases or upstream caspases like 2, 8, 9, 10, 11 and 12** • **Effector caspases or downstream caspases like 3, 6 and 7**
Cytomorphologic changes	Cell shrinkage, chromatin condensation, nucleus undergo fragmentation, plasma membrane blebbing without loss of cell integrity, organelles located in the plasma membrane blebs, and generation of apoptotic bodies.
Molecular changes	Cleavage of caspases, poly (ADP-ribose) polymerase (PARP involved in DNA repair) and nuclear fragmentation by endonucleases.
Clearance	Apoptotic bodies containing functional organelles are phagocytosed by neighboring cells and macrophages.
Laboratory diagnosis	Techniques for detection of apoptotic cells include: (a) cytomorphologic changes, (b) DNA discontinuous fragmentation ladder assay on agarose gel electrophoresis, (c) TUNEL assay, executioner caspase 3 and annexin V (IHC), (d) p53, executioner caspase 3 activation, FasR, TNF-α, and TRAIL (IHC, RT-PCR, Western blot, ELISA), and (e) cytochrome c release (ELISA).

Fig. 1.33: Schematic representation of apoptosis mediated by extrinsic (death receptor), intrinsic (mitochondrial) pathways. Both pathways can flow independently until the last step of DNA degradation by executioner caspase. Apoptosis is characterized by cell shrinkage, chromatin condensation, nuclear fragmentation by endonuclease, plasma membrane blebbing, generation and phagocytosis of apoptotic bodies by macrophages and surrounding epithelial cells and lack of inflammation.

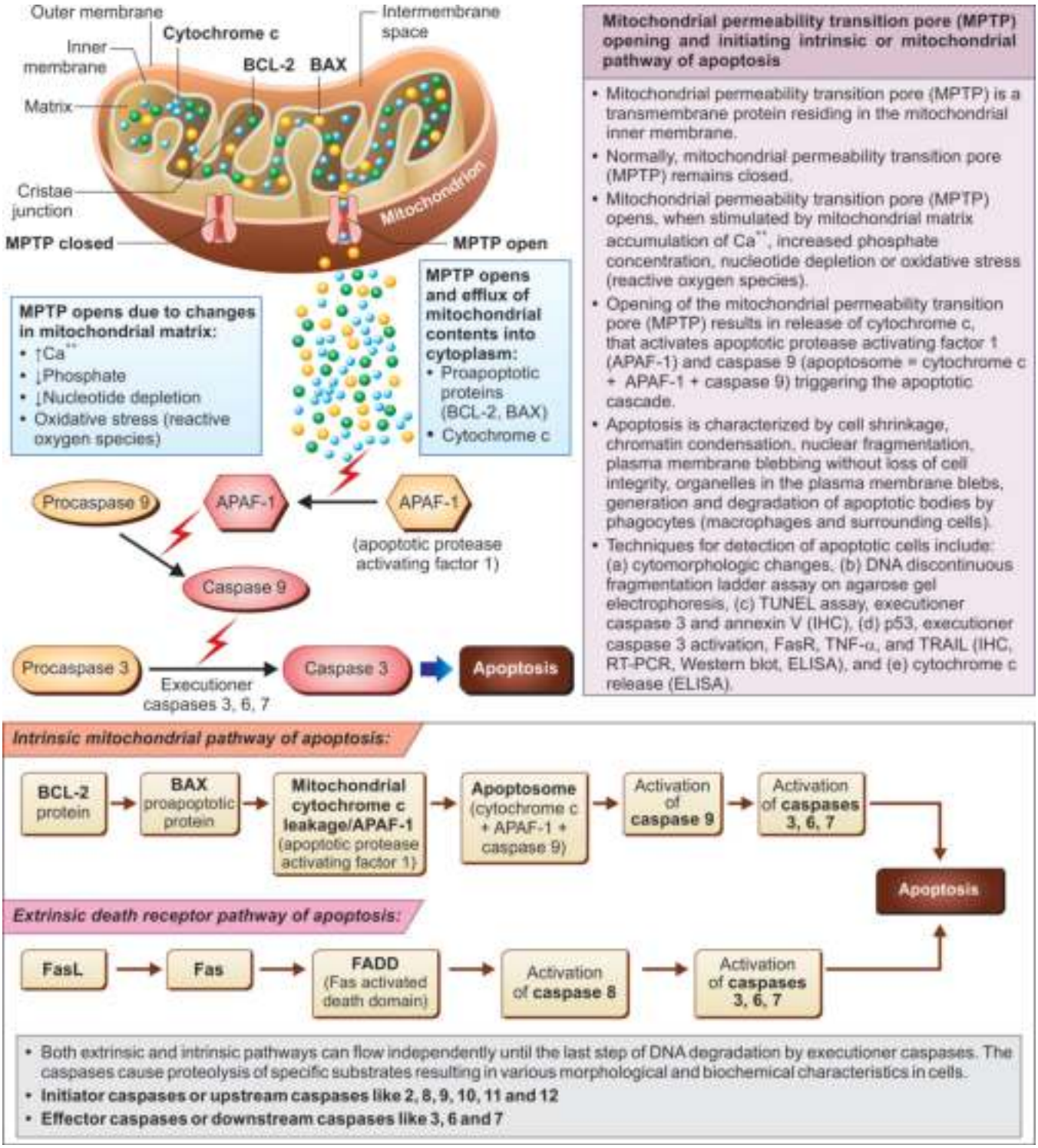


Fig. 1.34: Schematic representation of intrinsic (mitochondrial) and extrinsic (death receptor) pathways of apoptosis. Both pathways can flow independently until the last step of DNA degradation by executioner caspases, which cause proteolysis of specific substrates resulting in various cytomorphological and biochemical characteristics in cells.

- The intracellular portion of all these receptors contains a cytoplasmic region of about 80 amino acids called 'death domain', of docking proteins, to form a **death-inducing signaling complex (DISC)**. The death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways.
- Death domains possess the capacity to oligomerize with each other thereby promoting interactions between proteins such as the TNF-receptor-associated

Table 1.37 Role of caspases and mitochondrial proteins in apoptosis

Caspases Role in Apoptosis and Inflammation	
Initiator or inducer caspases	2, 8, 9, 10.
Executioner or effector caspases	3, 6, 7
Other caspases	11, 12, 13, 14
Inflammatory caspases	1, 4, 5
Mitochondrial Proteins Role in Apoptosis	
Proapoptotic proteins	BAX, BAK, BIM, BCLXs, APAF-1, BAD, BID, PUMA, NOXA, BOK (BCL-2 related protein), BIK (BCL-2 interacting killer), APAF-1 (apoptotic protease activating factor 1), NIP3, BNIP3, BLF-M, BRAG-1
Anti-apoptotic proteins	BCL-2 (key gene regulator of apoptosis) located on chromosome 18), BCL-XL, MCL-1, BFL1, BCL-W,

Mitochondrial proteins, i.e. BAD, BID and PUMA that regulate balance between proapoptotic and antiapoptotic proteins.

death domain (TRADD) or Fas-associated death domain (FADD) adaptor proteins, that are not present in unliganded receptor molecules.

- Binding of ligand to corresponding death domains lead to recruitment of cytoplasmic adaptor proteins. The binding of Fas ligand to Fas receptor results in the binding of the Fas-associated death domain adaptor protein and the binding of TNF ligand to TNF receptor results in the binding of TRADD with recruitment of FADD and receptor interacting protein (RIP).
- Fas-associated death domain adaptor protein then associates with procaspase 8 via dimerization of the death effector domain. At this point DISC is produced leading to autocatalytic activation of procaspase 8.
- Caspase 8 is the most upstream protease participating in the activation of cascade responsible for death receptor-induced cell death.
- The conversion of procaspase 8 to activated caspase 8 then converts precursor procaspases (3, 6, 7) to their active form. Caspases 3, 6 and 7, especially caspase 3, are executioners and cleave target proteins, which lead to apoptosis.
- Caspase 8 cleaves BID into tBID, which initiates the intrinsic mitochondrial pathway of apoptosis leading to release of cytochrome c and proapoptotic proteins, which induce apoptosis.
- Extrinsic death receptor pathway of apoptosis can be inhibited by a protein called cellular FLICE inhibitory protein (c-FLIP) which will bind to FADD adaptor protein and caspase 8 rendering them ineffective. Another point of potential apoptosis regulation involves a protein called 'Toso', also known as Fas apoptosis inhibitory molecule 3 (FAIM3), which has been shown to block Fas-induced apoptosis in T cells via inhibition of caspase 8 processing.
- The sequence of events in extrinsic death receptor pathway of apoptosis include: FasL-FasR → FADD (Fas-associated death domain) and TNF ligand-TNFR → TRADD (TNF-receptor-associated death domain) → activation of caspase 8 → activation of executioner caspases (3, 6, 7) → FADD and RIP recruitment → cleavage of cellular proteins → apoptosis.

INTRINSIC MITOCHONDRIAL PATHWAY OF APOPTOSIS

Intrinsic mitochondrial pathway of apoptosis plays a pivotal role in apoptosis. Mitochondria are central regulatory organelle involved in apoptosis, where antiapoptotic and proapoptotic proteins interact and determine the fate of the cell.

- Sequence of events in intrinsic mitochondrial pathway of apoptosis include: inhibition of antiapoptotic BCL-2 proteins → activation of proapoptotic proteins (e.g. BAK, BAX, BOK, BIM, BID, BAD) → mitochondrial cytochrome c leakage/APAF-1 (apoptotic protease activating factor 1) → apoptosome (cytochrome c + APAF-1 + caspase 9) → activation of caspase 9 → activation of caspases (3, 6, 7) → cleavage of cellular proteins → apoptosis. Due to DNA damage, activating p53 raises the level of BAX, which enhances cytochrome c release.
- In a healthy cell, the outer membrane of the mitochondria displays BCL-2 family of apoptosis-related proteins regulated by BCL-2 (B cell lymphoma/leukemia 2) gene mapped on chromosome 18. BCL-2 family of apoptosis related-proteins are given in Table 1.38.
- BCL-2 family of apoptosis-related proteins are key players of both antiapoptotic and proapoptotic processes.
- BCL-2 family can be divided into three groups, differentiated by structure and function. This division reflects the numbers of BCL-2 homology (BH) domains on the protein.
  - The presence of the BH4 domain characterizes the antiapoptotic family members.

Table 1.38 BCL-2 family of apoptosis-related proteins

Numbers of BCL-2 homology (BH) Domains	BH4 Domain	Examples
Multi-BH antiapoptotic proteins	BH4 domain present	BCL-2, BCL-XL, MCL-1 and other proteins
BH-to-BH3 proapoptotic proteins	BH4 domain absent	BAK, BAX, BOK
BH3 only proapoptotic proteins	BH4 domain absent	BIM, BID, BAD, BIK, BMF, NOXA, PUMA and HRK

- Multi-BH antiapoptotic proteins include BCL-2. BCL-XL, MCL-1 and others. Multi-BH antiapoptotic BCL-2 proteins prevent mitochondrial leakage of cytochrome c. On the other hand, proapoptotic BCL-2 family members lack the BH4 domain and may have BH1-to-BH3 or only BH3.
- BH1-to-BH3 proapoptotic proteins which lack BH4 domain, and include BAK, BAX and BOK. BH3 proapoptotic proteins lack BH4 domain, and include BIM, BID, BAD, BIK, BMF, NOXA, PUMA and HRK.

Pathology Pearls: BCL-2 (B Cell Lymphoma 2) Gene

- BCL-2 is human proto-oncogene located on chromosome 18. Its gene product is an integral outer mitochondrial membrane protein (called BCL-2), that works to prevent apoptosis by intrinsic apoptotic pathway.
- Overexpression of BCL-2 can contribute to metastasis in certain human cancers. In hematologic malignancies (follicular lymphoma, Burkitt's lymphoma, diffuse large cell lymphoma), reciprocal chromosomal translocation t(8;14) (q24;q32) involves the c-Myc gene (8q24) and the immunoglobulin heavy chain (IgH) locus (14q32) results in fusion of BCL-2 gene located near to IgH locus (14q32) resulting in overexpression of BCL-2 antiapoptotic protein.
- BCL-2 inhibitors are being developed to downregulate BCL-2. BCL-2 consists of four conserved domains (BH4, BH3, BH1 and BH2), which differentiate it from other BCL-2 family of members (e.g. BIM, BID, PUMA, NOVA, BAD, HRK, BMF and BIK). Among these homologies, motifs, BH3, BH1 and BH2 are the most commonly targeted therapy in clinical practice.

Intrinsic Mitochondrial Pathway of Apoptosis Activation

Intrinsic mitochondrial pathway of apoptosis is activated by numerous stimuli, including growth factor deprivation, oxidants, calcium overload, oncogene activation, DNA-damaging agents and microtubule targeting drugs.

- Mitochondrial permeability transition pore (MPTP) is a transmembrane protein residing in the mitochondrial inner membrane. Normally, MPTP remains closed.
- At equilibrium, '**cytochrome c**', DIABLO protein encoded by DIABLO gene mapped on chromosome 12 also referred to as second mitochondrial-derived activator of caspases (SMAC), and apoptosis inducing factor (AIF) either are attached to the mitochondrial inner membrane or float in the intramembranous space. The complex of oligomeric BAK/BAX with antiapoptotic BCL-2 family of proteins resides at the outer membrane of mitochondria. SMAC/DIABLO protein binds inhibitor of apoptosis proteins, thus freeing caspases to achieve apoptosis.
- When BH3-only members of BCL-2 family members are activated, they interpose themselves between their prosurvival relatives and BAK/BAX, thereby freeing BAK/BAK proteins.
- Later BAK protein forms mitochondrial permeability transition pore in the outer mitochondrial membrane.
- MPTP opens when stimulated by mitochondrial matrix accumulation of Ca^{++}, increased phosphate concentration, nucleotide depletion or oxidative stress by reactive oxygen species.
- Opening of the mitochondrial permeability transition pore results in release of proapoptotic proteins, SMAC/DIABLO, apoptosis inducing factor, 'cytochrome c', that activate apoptotic protease activating factor 1 (APAF-1) and caspase 9 (apoptosome = cytochrome c + APAF-1 + caspase 9) triggering the apoptotic cascade.
- Apoptosis is characterized by cell shrinkage, chromatin condensation, nuclear fragmentation, plasma membrane blebbing without loss of cell integrity, organelles in the plasma membrane blebs, generation and degradation of apoptotic bodies by macrophages and surrounding healthy cells.

CD8+ CYTOTOXIC T CELLS/NK CELLS (GRANZYME B/PERFORIN) MEDIATED PATHWAY OF APOPTOSIS

CD8+ cytotoxic T cells and natural killer cells carry out their killing function of virus-infected and/or transformed cells by releasing two types of preformed proteins: granzymes and pore-forming perforins. Synaptotagmin VII, Rab-27A and unc-13 homolog D (Munc12-4) regulate cytotoxic granules maturation and fusion with the plasma membrane leading to exocytosis. Schematic representation of perforin-granzyme pathway of apoptosis is shown in Fig. 1.35.

- **Perforin:** Perforin is a pore-forming protein that punches holes in the target cell-membrane through which the granzyme can enter into the cytosol, mitochondria and nucleus.

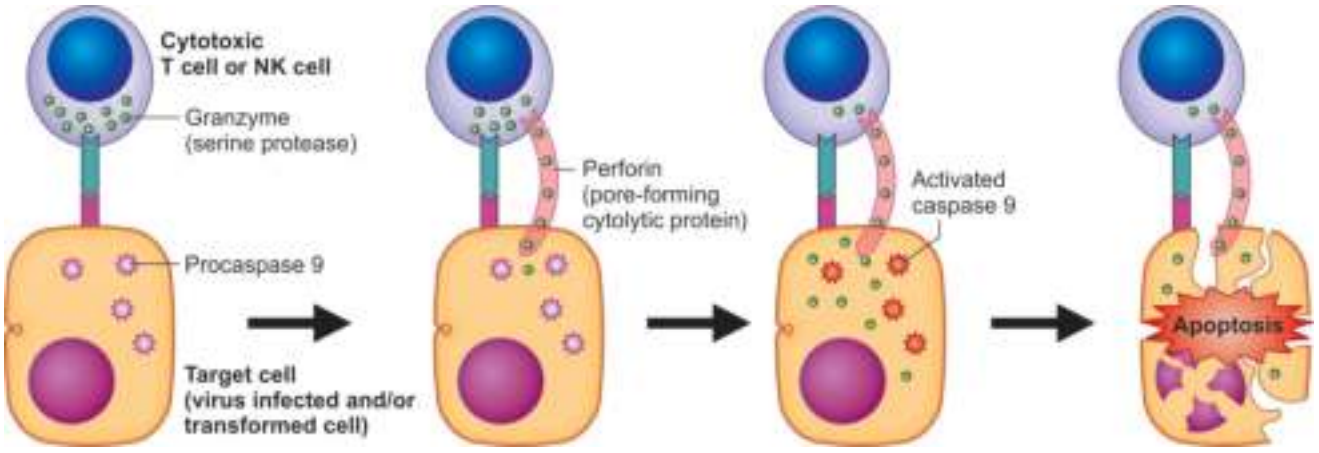


Fig. 1.35: Schematic representation of perforin-granzyme pathway of apoptosis. Perforin-granzyme induced is the main pathway used by cytotoxic T cells and natural killer cells (NK cells) to eliminate virus-infected or transformed cells. Perforin delivers granzyme to induce target cell apoptosis. At higher concentrations, perforin multimerizes in the plasma membrane to form pores. Granzyme is serine protease released by cytoplasmic granules within cytotoxic T cells and NK cells, that induces apoptosis by activating caspase 3, that cleaves many substrates, inducing caspase-activated DNase to execute apoptosis.

- **Granzyme B:** Granzyme B directly cause a rapid increase in superoxide, which is a key molecule of nuclear translocation of another granzyme A substrate, known as endoplasmic reticulum-associated SET complex.
  - Cleavage of SET complex liberates granzyme A activated DNase and NDPK-A that causes DNA damage. NDPK-A activates p53 tumor suppressor protein that can induce p53-dependent apoptosis.
  - Granzyme B cleaves cytoplasmic BID to its active forms, tBID, which translocates into mitochondria and triggers the intrinsic mitochondrial pathway of apoptosis. Granzyme B also activates procaspases 3, 6, 7 to executioner caspases 3, 6, 7, which leads to apoptosis. Granzyme B may also disrupt the complex between caspase activated DNase (CAD) and caspase activated DNase inhibitor (ICAD).
  - Disruption of CAD-ICAD releases the caspase-activated DNase, which elicits a caspase-independent form of apoptosis. The CAD-ICAD complex may also be cleaved by caspase 3.

Clinical Pearls: Role of Granzymes and Perforin in Acute Rejection of Solid Organ Allografts

- Granzymes/perforin act together to directly induce cell death of allograft parenchymal cells, thereby leading to organ failure.
- In heart allografts, the formation of plasma membrane pores by perforin alone may cause cardiac myocyte contractile dysfunction through the dysregulation of cytoplasmic ion concentrations.
- Granzyme B (GrB) and perforin act together to induce endothelial cell death within solid organ allografts. The destruction of the graft microvasculature in this manner leads to hemorrhage and thrombosis, thereby contributing to acute rejection by causing ischemic organ failure.

FAMILY OF CYSTEINE PROTEINS 'CASPASES'

Caspases are a family of endoproteases important for maintaining homeostasis through regulating apoptosis and inflammation. The caspases exist in all cells as inactive precursors or procaspases, which are usually activated by cleavage by other caspases, producing a proteolytic caspase cascade.

- Caspases cut off contact with surrounding cells, reorganize cytoskeleton, shut down DNA replication and repair, interrupt splicing, degrade DNA, disrupt nuclear structure, induce cell to display signals marking it for phagocytosis and disintegrate cells into apoptotic bodies.
- Within these proteolytic cascades, caspases can be positioned as either upstream 'initiators' (caspases 2, 8, 9, 10, 11 and 12) or downstream 'effectors (executioner caspases 3, 6 and 7)' of apoptosis. Initiator caspases activate executioner caspases that frequently coordinate their activities to demolish key structural proteins and activate other enzymes.
- The caspases cleave key cellular components that are required for normal cellular function including cytoskeleton proteins and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus.

INITIATOR/INDUCER CASPASES

Initiator/inducer caspases (2, 8, 9, 10, 11, 12) are initially produced as inactive procaspase monomers that are activated by dimerization and often cleave for activation. Dimerization also facilitates autocatalytic cleavage of caspase monomers into one large and one small subunit, which results in stabilization of the dimer.

EXECUTIONER/EFFECTOR CASPASES

Executioner/effectors caspases (3, 6, 7) activate cellular enzymes such as proteases and endonucleases. Activation of cellular enzymes degrade the cells own nuclear DNA and cytoplasmic proteins.

- Proteases degrade cytoskeleton and endonucleases break down DNA of targeted cell resulting in impairment of transcription, DNA replication and DNA repair.
- Caspase 3 converts a cytoplasmic DNAase into an active form, which induces internucleosomal cleavage of DNA. Caspase 3 also degrades cytoskeleton proteins.
- Caspase 3 antibodies serve as excellent biomarkers to monitor induction of apoptosis by analyzing the levels of procaspase 5 and its active form by Western blotting technique.
- Cleavage and activation of caspase 3 is catalyzed by caspase 8, caspase 9 and granzyme B to generate the active heterodimer of caspase 3 subunits.

MORPHOLOGIC AND BIOCHEMICAL CHANGES IN APOPTOTIC CELLS

Apoptosis exhibits characteristic morphologic features, such as highly condensed chromatin within nucleus, breakdown of nucleus, plasma membrane blebs, fragmentation of cells forming membrane-bound apoptotic bodies containing intact cytoplasmic organelles sometimes chromatin. Apoptotic bodies are phagocytosed by macrophages and surrounding cells within an hour, rendering their appearance very transient. Morphologic and biochemical alterations associated with apoptosis and their consequences are given in Table 1.39.

HISTOLOGIC CHANGES

On histologic examination, apoptotic cell exhibits various morphologic changes that occur during apoptosis. During early phase of apoptosis, cell shrinkage and pyknosis are visible by routinely hematoxylin and eosin-stained by light microscopy. The apoptotic cell appears as a round or oval mass with dark eosinophilic cytoplasm and dense chromatin fragments.

ULTRASTRUCTURAL CHANGES

Apoptotic cell demonstrates following ultrastructural changes in plasma membrane, cell shrinkage, nuclear changes, fragmentation of cell forming apoptotic bodies and clearance of apoptotic bodies by macrophages and surrounding epithelial cells.

Plasma Membrane Blebbing

Plasma membrane blebbing is the well-defined feature of apoptosis. During apoptosis, the cell's cytoskeleton breaks up and causes the membrane to bulge outward. These bulges may separate from the cell taking as portion of cytoplasm with them, to become known as apoptotic blebs.

Table 1.39 Morphologic and biochemical alterations associated with apoptosis and their consequences

Biochemical Alterations	Consequences
Activation of endonuclease	
Nonidentifies enzyme(s): internucleosomal DNA cleavage	'Ladder' aspect of DNA migrating in agar gel electrophoresis
Cytoskeleton changes	
Transglutamase and protease activation that results in the formation of an insoluble protein network	Prevent lysosomal and membrane rupture and the appearance of an inflammatory reaction 'cage effect'
Plasma membrane changes	
▪ Increased isoprenoid synthesis and loss plasma membrane dissymmetry ▪ Alterations in plasma membrane surface receptors especially their sugar composition ▪ Probable activation of ATP-dependent pumps, allowing influx of water against a concentration gradient	▪ Differentiate apoptotic cells from phagocytic cells ▪ Reduction in size of apoptotic cells being useful in isolation of these cells on Percoll gradient (low density gradient medium for preparation of cells)
Inconsistent changes	
Increased intracellular calcium	Transduction of a membrane signal or transcription of a specific gene (e.g. calmodulin)
▪ Increased synthesis of 3-galactoside, a link protein ▪ Increase in TRPM-2 gene transcription ▪ Activation of collagenases and metalloproteinases, TGF 31 synthesis	Inhibition of cell proliferation, increased intracellular calcium, separation of cells from neighboring cells and regulation of apoptosis/proliferation balance of cells and epithelia

Shrinking of Cytoplasm

Cell shrinkage or decrease in cell volume, is a ubiquitous characteristic of programmed cell death that is observed in all examples of apoptosis.

Nuclear Changes

Condensation of chromatin (**pyknosis**) occurs at the nuclear membrane followed by DNA fragmentation (**karyorrhexis**) by endonucleases.

Fragmentation of Cell into Apoptotic Bodies

During execution phase, cell's cytoskeleton breaks up and causes the plasma membrane to bulge outward and separate from the cell, taking a portion of cytoplasm with them, to become membrane-bound apoptotic bodies, which are tightly packed and contain intact mitochondria, lysosomes and ribosomes and other cell organelles, with or without nuclear fragments.

Clearance of Apoptotic Bodies

Apoptotic bodies are phagocytosed by macrophages and surrounding healthy cells without inducing inflammatory reaction. Macrophages and surrounding healthy cells prevent spillage of cellular lysosomal enzymes into surrounding tissue, thus protecting tissue from harmful exposure to the inflammatory and immunogenic contents of the dying cells. Glucocorticoids induce apoptosis; and sex steroid hormones inhibit apoptosis.

- Several receptors are implicated in the uptake of apoptotic bodies by macrophages and surrounding healthy cells. These receptors interact with their ligands on the apoptotic cells either directly or via bridging proteins. C1q serves as a bridging molecule for apoptotic cell clearance by binding to the apoptotic cell and the phagocyte and stimulating phagocytosis, independent of the classical complement pathway.
- Several studies revealed that alterations in cell surface receptors of phagocytes induced by apoptotic cells, externalization of phosphatidylserine and qualitative changes in the intercellular adhesion molecule 3 (ICAM-3) play an important role in the clearance of apoptotic bodies.
- Phosphatidylserine is normally present on the cytosolic surface of the plasma membrane. During apoptosis, scramblase causes redistribution of phosphatidylserine to the extracellular surface. These molecules mark the cell for phagocytosis by phagocytic cells possessing appropriate receptors. Upon recognition, the phagocyte recognizes its cytoskeleton for engulfment of the cell. The removal of dying cells by phagocytes occurs in an orderly manner without eliciting an inflammatory response.
- Apoptotic cells release various anti-inflammatory molecules such as transforming growth factor β (TGF-β), IL-10, annexin I, thrombospondin 1 (TSP-1), fractalkine, which inhibit proinflammatory cytokines synthesis of the phagocytes such as tumor necrosis factor α (TNF-α), IL-12, IL-1β, IL-18. Defining the ligands on apoptotic cells and corresponding receptors on phagocytes (macrophages and surrounding cells) with which they engage, is likely to lead to the development of novel anti-inflammatory protherapeutic agents.

BIOCHEMICAL CHANGES

Apoptosis is ATP-dependent programmed cell death mediated by caspases and characterized by nucleus fragmentation (karyorrhexis) resulting from the cleavage of double-stranded nuclear DNA via activation of calcium-magnesium-dependent endonuclease enzyme.

- Cleavage of DNA into high molecular weight structures may be detected with histochemical techniques or agarose gel electrophoresis (nonrandom mono- and oligonucleosomal length fragmentation of DNA giving 'ladder pattern').
- Another biochemical alteration is the translocation of phosphatidylserine to the outer surface of the plasma membrane. This translocation constitutes one of the principal targets of phagocytic recognition of apoptotic body.
- Mitochondria release '**cytochrome c**' and apoptosis-inducing factor (AIF) and other factors into cytoplasm.

APOPTOSIS IN HEALTH

Apoptosis is a programmed cell death by which a single cell or group of cells in a tissue is eliminated from the living tissue, which plays an important role for tissue modeling during embryogenesis and cellular/tissue hemostasis during adult life. Apoptosis during embryogenesis and adult life is given in Table 1.40.

APOPTOSIS DURING EMBRYOGENESIS

Apoptosis plays an important role in the processes of gamete maturation as well as in embryo development by removing cells, contributing to the appropriate formation of various organs and structures.

Involution of Embryonic Wolffian or Müllerian Structures

Apoptosis begins in the early blastocyst and plays an important role in the processes of embryonal and fetal development, contributing to the appropriate formation of various organs and structures.

Table 1.40 Apoptosis during embryogenesis and adult life

Apoptosis during Embryogenesis
▪ Involution of embryonic Wolffian or Müllerian structures
▪ Digits formation in fetus
▪ Lumen formation in hollow organs
▪ Development of nervous system
Apoptosis during Adult Life
▪ Apoptosis of self-reactive B and T cells in immune system
▪ Apoptosis of endometrial cells during menstrual cycle
▪ Atrophy of thymus
▪ Apoptosis of hepatocytes in viral hepatitis and formation of Councilman bodies
▪ Apoptosis of neuron and formation of red neurons
▪ Keratinocyte apoptosis in epidermis
▪ Apoptosis of neutrophils in acute inflammation

- Hormonal effect on apoptosis is also important in the maturation of the human reproductive system. The reproductive system has an early indifferent phase when it is neither male or female.
- Wolffian duct forms epididymis and vas deferens in males. Müllerian duct differentiates into uterus and fallopian tubes in female. It is well known that administration of estrogens will feminize the male, while administration of testosterone in females will musculinize the female. In order for that to happen, there has to be regression of the primitive Wolffian or Müllerian structures via apoptosis.

Digits Formation in Fetus

Interdigital cells are eliminated by apoptosis without affecting the living cells forming the digits, thus contributing to digit individualization by restricting interdigital tissue growth.

Lumen Formation in Hollow Organs

Lumen formation in hollow organs occur *de novo* by three processes: apoptosis of centrally located cells, autophagy of centrally located cells, and membrane separation by epithelial remodeling. The lumens present in the ductal structures are required for transport of fluids and air.

Development of Nervous System

The development of the nervous system is also dependent on apoptosis. Neural cell death has a pivotal role in the development and pathophysiology of the nervous system. About 50% of neurons are eliminated by programed cell death (apoptosis). Remaining 50% of neurons are produced with correct synaptic connections in target cells.

APOPTOSIS DURING ADULT LIFE

Apoptosis is programmed cell death that has been damaged beyond repair. Apoptosis also plays a role in preventing cancer. If apoptosis does not occur in DNA damaged cell, it can lead to uncontrolled cell division and subsequent development of cancer.

Apoptosis of Self-reactive B and T Cells in Immune System

Apoptosis is required for the development and maintenance of a healthy immune system.

- Self-reactive B and T cell antigen receptors are eliminated by apoptosis during their development in the thymus, a process known as negative selection.
- If self-reactive B and T cell antigens are not eliminated by apoptosis during their development, then these B and T cells may be released into the body, which can attack tissues resulting in autoimmune disorders [rheumatoid arthritis, systemic lupus erythematosus (SLE), autoimmune lymphoproliferative syndrome, and others].

Apoptosis of Endometrial Cells during Menstrual Cycle

The endometrium is a hormone-dependent tissue that undergoes cyclic changes during reproductive period as well as involutionary changes after menopause. The endometrial cycle in regularly menstruating women consists of three distinct phases: proliferative, secretory and menstrual.

- Apoptosis helps to maintain cellular homeostasis during the menstrual cycle by eliminating senescent endometrial cells from the functional layer of the uterine endometrium during the late secretory and menstrual phase of the cycle. Fas death receptor (CD95) and Fas ligand (FasL) are expressed in endometrium throughout the menstrual cycle.
- During late proliferative phase, Fas and FasL proteins are retained within cell's Golgi apparatus and cytoplasm, hence these are unable to interact and thus apoptotic signal is turned off. During secretory phase, Fas and Fas proteins are extruded as a part of cellular membranes, where Fas can bind FasL and 'turn on' apoptotic signal.
- Fas immunostaining on endometrial glandular cells is stronger during secretory phase than during proliferative phase of endometrium.

Thymus Gland Atrophy

The thymus is large in the fetus and infant, that undergoes atrophy before adulthood. This involution of thymus occurs via apoptosis, which is thought to be steroid sensitive.

- The steroid hormones are synthesized in the adrenal gland so that, in this case, one organ is responsible for the involution of another via its secreted product. This observation is useful in perinatal autopsies to study whether the baby *in utero* has been under stress or not.
- Adrenal steroid hormones in stressed baby *in utero* results in premature involution of thymus. If the baby has been normal *in utero*, but has suffered birth asphyxia, the thymus gland will be of normal size.

APOPTOSIS IN DISEASES

Apoptosis eliminates potentially harmful DNA-damaged cells that are injured beyond repair by activating DNA damage checkpoint pathways without eliciting a host reaction, thus limiting collateral tissue damage. Hence, apoptotic signaling pathways, help to safeguard the 'genomic stability'. Evidence indicates that dysregulation of apoptotic signaling pathways can cause cancerous growth or autoimmune disorders, while accelerated apoptosis is evident in degenerative disorders, immunodeficiency disorders and infertility. Dysregulation of apoptosis-associated disorders is given in Table 1.41.

APOPTOSIS INDUCED BY VIRUSES

Apoptosis can be induced by viruses either by stimulating the immune response or by introducing viral suicidal genes that kill the host cells.

- Immune response is mediated by CD8+ cytotoxic T cells/natural killer cells (perforin/granzyme) associated pathway of apoptosis. Many cells undergo apoptosis in response to viral infection, with a consequent reduction in the release of progeny virus.

Table 1.41 Dysregulation of apoptosis-associated disorders

Categories	Disorders
Human cancers	Breast carcinoma, lung carcinoma, renal cell carcinoma, ovarian carcinoma, endometrial carcinoma, head and neck carcinoma, melanoma, follicular lymphoma and leukemia
Viruses	Herpesvirus, adenovirus and poxvirus
Neurological disorders	Alzheimer disease, Parkinson disease, Huntington disease, amyotrophic lateral sclerosis, cerebral stroke, cerebellar degeneration
Cardiovascular disorders	Ischemic heart disease, heart failure, infectious diseases, bacteria, viruses
Autoimmune disorders	Systemic lupus erythematosus, rheumatoid arthritis, thyroiditis, autoimmune lymphoproliferative syndrome, immune-mediated glomerulonephritis

- The viral genome hijacks the host cell's machinery, forcing host cell to replicate the viral genome and produce viral proteins to make new capsids. After maturation, the virus induces host cell apoptosis and propagation of viral particles to neighboring uninfected cells.

Councilman Bodies in Hepatocytes in Viral Hepatitis

In pathology, Councilman body also known as Councilman hyaline body has been named after American pathologist William Thomas Councilman.

- Councilman body is an eosinophilic/pink globule in hepatocytes on hematoxylin and eosin-stained sections examined under light microscope, that represents a hepatocellular apoptosis often surrounded by normal liver parenchyma.
- Apoptotic cells represent membrane-bound cellular chromatin remnants that are extruded into the hepatic sinusoids demonstrated in cases of viral hepatitis, yellow fever and viral hemorrhagic fever. Liver biopsy in viral hepatitis shows apoptosis of virus infected hepatocytes, mild zonal necrosis and lymphocytic infiltration.

Apoptosis of HIV Infected Cells

Significant apoptosis during HIV-1 infection can be attributed to the direct viral killing of peripheral infected mononuclear cells in AIDS patients. In HIV infected persons, viral load is a good predictor of disease progression; the higher the viral load the faster the disease progression.

NEURONAL APOPTOSIS AND FORMATION OF RED NEURONS IN BRAIN

A 'red neuron' is a pathologic finding in the central nervous system, indicative of acute neuronal injury and subsequent apoptosis demonstrated as eosinophilic neuron on observed on hematoxylin and eosin-stained sections under microscope.

- Since neurons are permanent cells, these cells are most susceptible to hypoxic injury often found after 12–24 hours in cerebral stroke.
- The red coloration of red neuron occurs due to pyknosis or degradation of the nucleus and loss of Nissl bodies.

KERATINOCYTE APOPTOSIS IN EPIDERMIS

Keratinocyte apoptosis plays a critical role in regulating epidermal development and restraining carcinogenesis. Keratinocyte apoptosis can also be triggered by ultraviolet radiation and other stimuli.

- The stratum granulosum, the thin middle layer of epidermis, initiates keratinization (constant production of keratin). This process starts with apoptosis of epithelial cells in stratum granulosum of skin.
- The keratinocytes become metabolically inactive with degradation of organelles resulting in constant turnover of differentiated flattened dead squamous cells, that resemble 'protein sacs' containing more than 80% of keratins cross-linked to other cornified envelope proteins.
- Dysfunctional apoptosis occurs in some skin diseases (e.g. psoriasis and skin cancer). Acanthosis, the hallmark of psoriatic skin is an example of diminished epidermal cells apoptosis.

APOPTOSIS OF NEUTROPHILS IN ACUTE INFLAMMATION

Neutrophils respond to acute inflammation and kill bacteria by phagocytosis. Activated neutrophils generate vast amounts of reactive oxygen species, which kill microorganisms and modulate life span as well as clearance of the neutrophils themselves.

- After elimination of bacteria, neutrophil apoptosis occurs to limit destructive capacity of neutrophilic products to the surrounding tissue.
- Apoptosis of neutrophils is important for the resolution of inflammation, as this allows for phagocytosis and removal of senescent cells prior to their necrotic disintegration.
- Apoptotic neutrophils stimulate macrophages, which clear apoptotic cells as well as cell debris, and reduce the inappropriate inflammatory response further.

APOPTOSIS OF PARENCHYMAL ORGAN'S CELLS DUE TO OBSTRUCTION IN DUCTS

Obstruction in ducts of pancreas and parotid glands result in pathologic organ shrinkage by the process of apoptosis of parenchymal cells. After pancreatic duct obstruction, pancreatic acinar cells progressively disappear and pancreatic islets of Langerhans are preserved.

APOPTOSIS OF CELLS WITH DAMAGED DNA

Hypoxia, ionizing radiation, cytotoxic chemotherapeutic drugs and viruses can cause cellular DNA damage, either directly or via production of free radicals. The TP53 tumor suppressor gene recognize cells with DNA damage and analyze whether these can be repaired.

- If DNA repair mechanisms cannot cope with the injured cells, the cell triggers intrinsic mechanisms that induce apoptosis. In these situations, elimination of these cells with DNA damage may be better alternative than risk of malignant transformation in these cells.
- The injurious stimuli can cause apoptosis if the insult is mild, but large doses of the same injurious stimuli may result in necrotic death. In certain cancers, where TP53 tumor suppressor gene is mutated or deleted, the apoptosis is not induced in cells with DNA damage.
- People with **Li-Fraumeni syndrome** have only one functional copy of TP53 tumor suppressor gene, so they are more likely to develop a malignant tumor in early adulthood.
- Follicular lymphoma is a low-grade B cell neoplasm, which usually occurs in elderly patients. About 90% of cases show t(14;18) (q32;q21) chromosomal translocation which juxtaposes the IgH locus on chromosome 14 with BCL-2 gene on chromosome 18 resulting in overexpression of BCL-2. BCL-2, which is located in the mitochondria, promotes cell survival by opposing apoptosis in the follicles. Overexpressed BCL-2 inhibits APAF1 and inactivates caspases leading to survival of neoplastic cells in follicles.

APOPTOSIS OF CELLS WITH ACCUMULATED MISFOLDED PROTEINS

Misfolded proteins may arise due to mutations in the genes encoding these proteins or damage caused by oxygen-derived free radicals. Excessive accumulation of misfolded proteins in the endoplasmic reticulum results in endoplasmic reticulum stress, which culminates in apoptotic cell death. Apoptosis caused by the accumulation of misfolded proteins in the cells has been invoked as the basis of several degenerative diseases of nervous system (Creutzfeldt-Jakob disease, Alzheimer disease, Parkinson disease, Huntington disease and muscle system atrophy) and other organs damage (amyloid deposits in liver, spleen).

APOPTOSIS OF CANCER STEM CELLS

Apoptosis is often a self-defense mechanism for destruction of cells infected with viruses or irreparable DNA damaged cells by CD8+ cytotoxic T cells, thus protecting against neoplastic transformation. Radiation and cytotoxic anticancer drugs induce apoptosis of cancer cells.

Recent Concepts: Nucleolin and Apoptosis

- NCL gene encodes a multifunctional RNA-binding nucleolin protein, that is primarily distributed in the nucleolus (90%), cytoplasm and cell membrane.
- Cell surface nucleolin can bind to various ligands to affect many physiologic functions including ribosomal biogenesis, processing of ribosomal RNA (rRNA), messenger RNA stability, downstream target of several regulation of signal transcription pathway and cell proliferation.

- Nucleolin interacts with DNA repair proteins to maintain genomic stability via stability of apoptosis-related mRNAs by binding to nucleolin RNA-binding domain (RBD) to the 5'- and 3'-UTR of mRNA and inhibit apoptosis. Increased nucleolin expression can also elevate BCL-2 protein levels in cancer cells.
- Nucleolin also interacts with 15 and 6 microRNAs, which are negative regulators of BCL-2 expression.
- Cell surface nucleolin can bind to Fas and block the interaction of Fas/FasL, which prevents cells from entering Fas-induced apoptosis. The interaction of cell-surface nucleolin with ErB1 and RAS also favors cell proliferation.
- Therefore, cell-surface nucleolin can facilitate antiapoptotic phenotype and induce initiation and survival or cancer cells.

LABORATORY DIAGNOSIS

Techniques for detection of apoptotic cells include: cytomorphologic changes (light and electron microscopy), DNA discontinuous fragmentation ladder assay (conventional agarose gel electrophoresis), terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay, executioner caspase 3 (immunohistochemistry), annexin V (immunohistochemistry), p53 (immunohistochemistry and RT-PCR, Western blot, enzyme-linked immunosorbent assay, flow cytometry), executioner 3 activation (immunohistochemistry, Western blot, enzyme-linked immunosorbent assay), Fas, TNF-α (tumor necrosis factor α), TRAIL: tumor necrosis-related apoptosis-inducing ligand (RT-PCR, Western blot, immunohistochemistry) and '**cytochrome c**' release (ELISA: enzyme-linked immunosorbent assay).

LIGHT MICROSCOPY

Light microscopy has aided in analyzing the various cytomorphological changes that occur during apoptosis on routinely hematoxylin and eosin-stained sections.

- In the initial stage of apoptosis, the apoptotic cells exhibit cell shrinkage with dark dense eosinophilic cytoplasm, highly condensed chromatin with nuclear pyknosis. In the later stage, cell undergoes fragmentation with formation of apoptotic bodies containing cytoplasmic organelles.
- The apoptotic bodies are phagocytosed by macrophages and surrounding cells within an hour, rendering their appearance very transient. There is essentially no inflammatory response because of these reasons: (a) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue, (b) apoptotic cells are quickly phagocytosed by macrophages and surrounding healthy cells thus likely preventing secondary necrosis, and (c) phagocytosed apoptotic bodies do not induce production of cytokines.

ELECTRON MICROSCOPY

Electron microscopy is still considered the 'gold standard' for the identification of apoptotic cells. Different cytomorphological features of cells undergoing apoptosis are analysis by transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

- Transmission electron microscopy analysis is essentially qualitative ultrastructural changes in apoptotic cells such as plasma membrane blebbing, chromatin condensation in nucleus and cytoplasmic reorganization, whereas SEM can provide information of the cell surface (extensive plasma membrane blebbing, loss of microvilli), cell–cell and cell–substrate interactions, but it is very difficult to analyze apoptotic features by SEM.
- Extensive plasma membrane blebbing is followed by karyorrhexis and separation of cell fragments into apoptotic bodies by budding. Apoptotic bodies consist of tightly packed cytoplasmic organelles with or without nuclear fragment. Electron microscopy is useful for subsequent biochemical or molecular analysis.

ELECTROPHORESIS

Classical apoptotic cell death can be defined by certain cytomorphological and biochemical characteristics that distinguish it from other forms of regulated cell death. One such feature, which is hallmark of apoptosis, is DNA fragmentation.

- In dying apoptotic cells, DNA is cleaved by an endonuclease that induces fragmentation of chromatin into nucleosomal units, which are multiples of about 180 to 200 bp oligonucleosomal multimers and appear as a 'DNA ladder pattern' of discontinuous DNA fragments, when run on conventional agarose gel electrophoresis.
- DNA ladder remains the hallmark of apoptosis. DNA from apoptotic cell is extracted from the culture and is precipitated with polyethylene glycol (PEG) or agarose or polyacrylamide. The fragmented DNA remains in the supernatant and can be easily subjected for gel electrophoresis or quantification using fluorescent dyes (fluorescein and rhodamine).
- Pulse field gel electrophoresis is a specialized technique for resolving DNA molecules in the range of 50 kb to 10 Mbp. Single cell gel electrophoresis (SCGE) visualizes DNA damage analyzed at the levels of individual apoptotic cell, that exhibits comet-like

structures with small head and large tail. Comet assay has higher sensitivity than DNA ladder assay and TUNEL staining.

FLOW CYTOMETRY

Flow cytometry is one of the most popular and versatile applications for accurate quantification of apoptosis from induction via surface receptors to late stage where DNA fragmentation occurs.

- Apoptotic cells are stained with fluorescent dyes and passed through beam of light of single wavelength.
- Cells flowing through beam of single wavelength scatter light to some extent in the optical and electronic detection apparatus of the flow cytometer.
- Such forward scatter (FSC) versus side scatter (SSC) distinguishes apoptotic cells from nonapoptotic cells and determination of the immunophenotype of cells undergoing apoptosis.

TUNEL ASSAY

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) is the most common method used to assay DNA fragmentation in individual cells caused by apoptosis.

- The assay relies on the use of the terminal deoxynucleotidyl transferase, an enzyme that catalyzes attachment of deoxynucleotides, tagged with a fluorochrome or another marker to 3'-hydroxyl termini of DNA double strand breaks. It may also label cell having DNA damaged by other mechanisms than in the course of apoptosis.
- The amount of fluorescence can be detected by fluorescence microscopy, flow cytometry and immunohistochemical techniques.

IMMUNOHISTOCHEMISTRY

Apoptosis competence is central to the prevention of cancer. Gold standard for evaluation of apoptosis is morphologic evaluation. Immunohistochemical detection of apoptotic cells is performed on paraffin-embedded sections by using commercially developed antibodies against a wide range of substrates most importantly cleaved caspase 3, cleaved cytokeratin 18 (c-CK18), cleaved laminin A (c-lam-A), phosphorylated histone 2AX (γ-H2AX), cleaved poly(ADP ribose) polymerase, apoptosis-inducing factor (AIF), p53, annexin V, and M30 neoantigen.

Executioner Caspase 3 Analysis

Caspase 3 belongs to the family of cysteine proteases, which is known as executioner caspase in apoptosis by coordinating the destruction of cellular structures such as DNA fragmentation or degradation of cytoskeletal proteins. Caspase 3 is responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP).

- Caspase 3 is generated as zymogen in inactive form. Cleavage and activation of inactive form of procaspase 3 is catalyzed by caspase 8, caspase 9 and granzyme B to generate active caspase 3.
- Caspase 3 antibodies serve as excellent biomarker to monitor induction of apoptosis by detecting the levels of procaspase 3 and its active form.

Monoclonal Antigen M30 Analysis

The monoclonal antigen M30 recognizes a neoepitope of cytokeratin 18, that is produced during the process of apoptosis. It is early indicator of apoptosis in epithelial cells. Monoclonal antigen M30 is immunoreactive in formalin-fixed paraffin-embedded tissue in colon, trophoblastic tissue in human placenta and salivary glands. Monoclonal antigen M30 is absent in nonapoptotic cells.

Annexin V Analysis

Annexin V is a calcium-dependent, phospholipid-binding protein with a high affinity for phospholipid phosphatidylserine, thus can be used a probe for detecting apoptosis.

- The phosphatidylserine residues are normally hidden within the plasma membrane. The appearance of phosphatidylserine residues on the cell is an early event in apoptosis and can be used to detect apoptosis.
- During apoptosis, phosphatidylserine is translocated from the cytoplasmic face of the plasma membrane to the cell surface.

TP53 Gene Mutation Analysis

TP53 gene is a well-defined tumor suppressor gene mapped on chromosome 17p13.1. TP53 gene encodes phosphoprotein that plays a key role in regulating cell proliferation and apoptosis in response to DNA injury. It also suppresses angiogenesis.

- TP53 gene is the most frequently mutated in human cancers. The p53 protein exists as wild type and various types of mutant forms.
- Wild type p53 protein without TP53 mutation is found in normal cells and is critically important in repair of DNA damage. DNA damage activates TP53 gene resulting in either cell cycle arrest allowing time for the cell to repair the damage or apoptosis.
- Loss of TP53 gene function indirectly decreases genomic stability. Manipulation of the apoptotic functions of TP53 gene constitutes an attractive target for cancer therapy.

INTRACELLULAR ACCUMULATION OF SUBSTANCES

Residual effects of reversible cell injury persist in the cell as an evidence of cell injury at subcellular level. One of the manifestations of metabolic derangements in cells is the intracellular accumulation of abnormal amounts of various substances. A normal endogenous substance is produced at a normal rate, but the rate of metabolism is inadequate to remove it. Defective protein folding, packaging and transport, both genetic or acquired, also produce substance accumulation in excess in the cells.

- The intracellular accumulation of substances falls into three categories: (a) one of the normal cellular constituents is accumulated in excess, such as carbohydrates, lipids, proteins and water, (b) one of abnormal substances is accumulated in excess in the cells, either exogenous substance (mineral) or endogenous excess product of metabolism, (c) one of the endogenous or exogenous pigments is accumulated in excess in the cells.
- The intracellular substances may accumulate in the cytoplasm frequently in the phagolysosomes either in transient or permanent manner. These substances may be harmless to the cells, but can be severely toxic to cells on occasion. If the intracellular overload of substance occurs due to systemic derangement, it can be brought under control, the intracellular accumulation is reversible. In genetic disorders, intracellular overload of substance is progressive resulting in secondary tissue/organ damage, that may have a fatal outcome. In some instances, cells may produce the abnormal substance, and in other instances, cells store products of pathologic process elsewhere in the body.
- Most of intracellular accumulation of substances are attributable to three types abnormalities: (a) a normal endogenous substance is produced at a normal or increased rate, but the rate of metabolism is inadequate to eliminate it. For example, fatty change in liver is due to intracellular accumulation of triglycerides, (b) appearance of reabsorption protein droplets in renal tubules occurs due to increased leakage of proteins from the glomerulus in nephrotic syndrome, (c) intracellular accumulation of normal or abnormal endogenous substance due to genetic or acquired defects in the metabolism, packaging, or secretion of these substances.
- For example, genetic defect in the specific enzyme is involved in the metabolism of carbohydrates and lipids leading to intracellular accumulation of these substances largely in lysosomes.
- Misfolding proteins are accumulated in the form of globular eosinophilic inclusions in the endoplasmic reticulum of hepatocytes in cases of α_1-antitrypsin deficiency caused by single amino acid substitution in the enzyme.
- Another example is intracellular accumulations of substances such as carbon or silica particles, which are neither degraded by enzyme or metabolized in the liver. Whatever is the nature and origin of the intracellular accumulations, it implies the storage of some product by individual cells.

Pathology Pearls: Intracellular Accumulations of Substances

- Lipid accumulate in the liver of obese persons and persons with chronic alcoholism.
- Glycogen accumulates in the liver, skeletal muscles, or kidneys in patients with inborn errors of glycogen metabolism and diabetes mellitus.
- Protein accumulates in the proximal renal tubules in patients with proteinuria. Protein accumulation generally appears as discrete eosinophilic cytoplasmic droplets, vacuoles, or aggregates.
- Exogenous and endogenous pigments accumulate in various cells.
  - Exogenous carbon pigment is deposited in alveolar macrophages. When a person gets a tattoo, the pigment is ingested by dermal macrophages, usually without an inflammatory response.
  - Endogenous pigments that accumulate in various cells include lipofuscin (brown pigment formed in the lysosomes of the elderly persons), melanin (brown-black pigment formed during the oxidation of tyrosine to dihydroxy-phenylalanine [DOPA] by tyrosine in melanocytes), hemosiderin (iron-rich yellow brown pigment derived from hemolyzed red blood cells and demonstrated by Perls Prussian histochemical stain) and bilirubin (also derived from hemoglobin but contains no iron). Excess of bilirubin in cells and tissues is called jaundice.
- Hereditary hemochromatosis is a genetic disorder of iron absorption characterized by the deposition of hemosiderin in the liver, spleen, and bone marrow. Patient presents with cirrhosis, diabetes mellitus and skin discoloration (bronze diabetes).

LIPID ACCUMULATION

Lipid accumulation is chemically defined as a substance that is insoluble in water and soluble in alcohol, chloroform and ether. Lipids together with carbohydrates and proteins are main constituents of cells. Cholesterol and triglycerides are lipids, which are easily stored in the body, which are important constituents of cells. These serve as a source of fuel. Lipids include fatty acids, waxes and steroids.

- Compound lipids are complexed with another type of chemical compound and these include lipoproteins, phospholipids and glycolipids. Lipid accumulation results from an imbalance between lipid acquisition and lipid disposal in parenchymal cells of liver, heart and skeletal muscle due to defects in uptake, catabolism or secretion.
- Fatty change in liver is common example. Skeletal muscle may show mild fatty change as a result of ischemia and severe fatty change due to diphtheria.

TRIGLYCERIDE ACCUMULATION INDUCING FATTY CHANGE IN LIVER

Liver is the major organ involved in fat metabolism. Hepatic fat accumulation results from an imbalance between lipid acquisition and lipid disposal, which are regulated through four major pathways: uptake of circulating lipids, *de novo* lipogenesis, fatty acid oxidation (FAO) and export of lipids in the form of very low-density lipoprotein (VLDL).

- Fatty change in liver most often occurs due to accumulation of triglycerides. The triglycerides accumulate when lipoprotein transport is disrupted and/or when fatty acids accumulate in the liver.
- Alcohol abuse, diabetes mellitus and obesity are the most common causes of fatty change in liver. Alcohol interferes with mitochondrial and microsomal function in hepatocytes, leading to an accumulation of lipid.
- Other causes of fatty change in liver include toxins, protein malnutrition (kwashiorkor), anoxia and severe gastrointestinal malabsorption. Tetracycline can cause microvesicular fatty change in liver.

Pathogenesis

Normally, free fatty acids (FFAs) derived from adipose tissue or dietary source are normally transported into hepatocytes, where free fatty acids are esterified to triglycerides, converted into cholesterol or phospholipids, or oxidized to ketone bodies. Some of the free fatty acids are synthesized from acetate within hepatocytes as well. Egress of the triglyceride requires the formation of complexes with apoproteins to form lipoproteins, which are able to enter the circulation.

- Defects in any of the steps of uptake, catabolism, or secretion can lead to accumulation of triglycerides in fatty liver. Alcohol is a hepatotoxin that alters mitochondrial and smooth endoplasmic reticulum function and thus inhibits oxidation of free fatty acids.
- Carbon tetrachloride and protein malnutrition decrease the synthesis of apoproteins. Anoxia inhibits fatty acid oxidation, and starvation increases free fatty acid mobilization from peripheral stores.
- Mild fatty change in liver has no effect on cellular functions. Severe fatty change may transiently impair cellular functions. But in carbon tetrachloride poisoning, fatty change in liver is irreversible.

Surgical Pathology: Fatty Change in Liver

Gross Morphology

Mild fatty change in liver may not affect the gross appearance. Severe fatty change results in enlargement of liver, which becomes progressively yellow on cut surface until it may weigh 3–6 kg. This uniform change is consistent with fatty metamorphosis (fatty change).

Frozen/Cryostat Section and Fat Stains

- Fat cannot be demonstrated in the paraffin-embedded sections because fat is dissolved during processing of tissue in tissue processor.
- Fat can be demonstrated by frozen/cryostat section technique with the fat stains such as Sudan III, Sudan IV, Sudan black, Oil red O and osmic acid.

Light Microscopy

- Initially, hepatocytes show small fat vacuoles around nucleus (mild fatty change or microvesicular) in hematoxylin and eosin-stained sections.
- Progressive accumulation of fat forming vacuoles in the cytoplasm coalesce to create spaces that displace the nucleus toward periphery (severe fatty change).
- Occasionally contiguous cells rupture, and the enclosed fat globules unite to produce so-called fatty cysts.
- Distribution of lipid vacuoles may be diffuse, focal or zonal, midzonal or peripheral depending on severity and etiology. Histology of fatty change in liver is shown in Fig. 1.36.

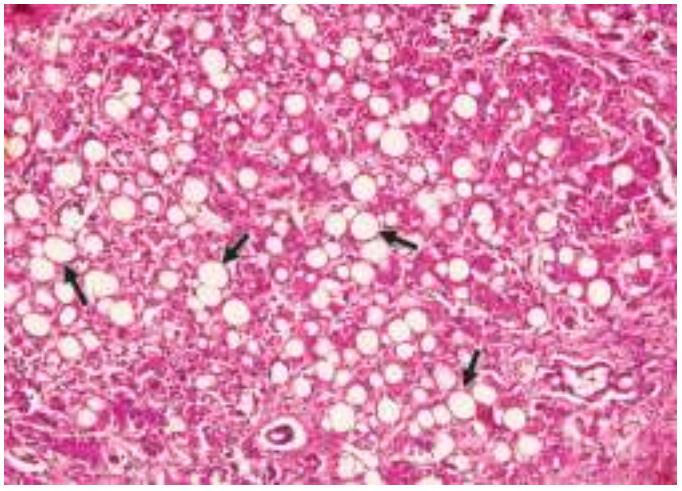


Fig. 1.36: Histology of fatty change in liver. Histologically, fatty liver is characterized by fat accumulation, which is most prominent in the centrilobular zone. Macrovesicular steatosis is the rule. Hepatocytes containing one or numerous fat droplets that displace the nucleus to an eccentric position with abundant clear looking cytoplasm (arrows) (400X).

CHOLESTEROL AND ITS ESTERS ACCUMULATION

Most cells utilize cholesterol for the synthesis of cell membranes without intracellular accumulation. Accumulation of cholesterol and its esters is demonstrated in various disorders such as atherosclerosis, xanthomas, cholesterolosis in gallbladder and Niemann-Pick disease.

Atherosclerosis

Atherosclerosis means hardening (sclerosis) or loss of elasticity of large elastic arteries and medium-sized arteries as a result of fat deposition on their intima.

- Most common sites of atheromatous plaques are abdominal aorta, proximal region of coronary arteries, carotid arteries, vertebral arteries (circle of Willis), iliac arteries, popliteal artery, anterior tibial artery, posterior tibial artery and renal artery; but sparing internal mammary arteries and arteries of upper extremities.
- Major modifiable risk factors of atheromatous plaques include hyperlipidemia, hypertension, tobacco smoking and diabetes mellitus; and nonmodifiable factors are increasing age, male gender, family history of atherosclerosis and genetic abnormalities (ApoA1, ApoE, hepatic lipase and LDL receptor).
- Minor risk factors of atheromatous plaques include obesity, stress type A personality, thrombophilia, high intake of carbohydrates, homocysteinemia, racial factors and infectious agents.
- Atheromatous plaque is composed fibrous cap covering central core comprising smooth muscle cells, foam cells, fibrin, and extracellular matrix material, such as collagen, elastin, glycosaminoglycans, and proteoglycans. Endothelium over the surface of the fibrous cap frequently appears intact.

Xanthomas

Xanthomas are formed due to accumulation of cholesterol or cholesterol esters within macrophages under the skin and tendons on joints (knees and elbows), feet, hands and buttocks.

- Xanthomas can vary in size from pinhead to large grape size, which often appear like a flat mump or yellow to orange. These are usually painless about may be tender or itchy.
- Xanthomas are caused by elevated blood lipids (familial or acquired hyperlipidemia) or fat in the body in the settings of diabetes mellitus, hypothyroidism, primary biliary cirrhosis, nephrotic syndrome, cholestasis and corticosteroid administration.

Gallbladder Cholesterolosis

Gallbladder cholesterolosis is characterized by mucosal villous hyperplasia, excessive accumulation of cholesterol and cholesterol esters in macrophages within the lamina propria and in mucosal epithelium in gallbladder. It is present in 20% of cholecystectomy specimens, usually affecting adult women. It is associated with bile supersaturation with cholesterol but not with increased cholesterol. The gallbladder may be affected in a patchy or diffuse form. Usually, cholesterolosis remains clinically silent.

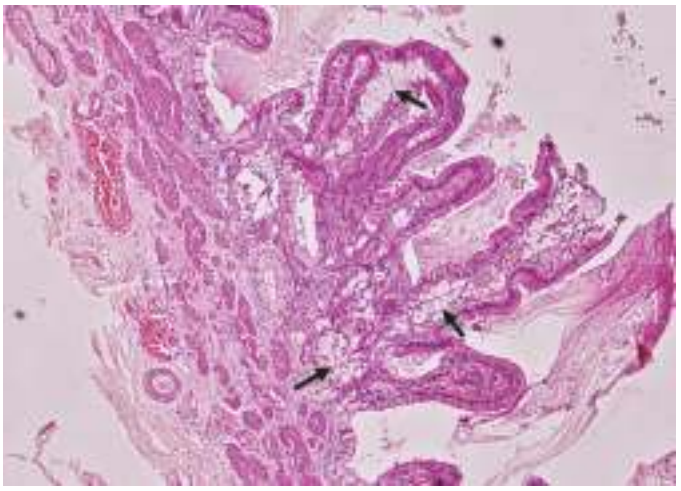


Fig. 1.37: Histology of cholesterolosis in gallbladder. Histologic examination of cholesterolosis shows presence of cholesterol laden macrophages in the lamina propria of the gallbladder mucosa. There is no association with inflammatory changes or presence of gallstones. This finding is incidental without clinical significance (arrows) (100X).

Surgical Pathology: Gallbladder Cholesterolosis

Gross Morphology

Mucosal surface of gallbladder shows yellow, flat deposits, flat or diffuse or speckled appearance (strawberry gallbladder). About 20% of cholecystectomy specimens are associated with cholesterol polyps.

Light Microscopy

- In cholesterolosis, mucosa shows villous hyperplasia with macrophages at the tips of villi and foam cells in the lamina propria and epithelium.
- There is no association with inflammatory changes and cholelithiasis. There may be presence of polypoid lesion with heterotopic bone (osseous metaplasia).
- The changes are usually restricted to gallbladder and not involving extrahepatic bile ducts. Histology of cholesterolosis in gallbladder is shown in Fig. 1.37.

GLYCOGEN ACCUMULATION

Glycogen accumulation is a multibranched polysaccharide of glucose that serves as a buffer to maintain blood glucose levels. It is synthesized from glucose when blood glucose levels are high. It is as an important energy

reservoir in the liver (6–10% of the liver mass) and skeletal muscles (1–2% of skeletal muscle mass).

- In addition to liver and skeletal muscle, glycogen is found in smaller amounts in other tissues such as red blood cells, white blood cells, renal tubules, and some glial cells in brain; except during prolonged starvation. Additionally, glycogen is used to store glucose in the uterus to provide energetic needs of the embryo.
- When energy is required by the body, glycogen is broken down via glycogenolysis into glucose, which then enters the glycolytic or pentose phosphate pathway and then released into the bloodstream.
- In addition to glucagon, cortisol, epinephrine, and norepinephrine also stimulate glycogen breakdown. Periodic acid–Schiff (PAS) is a staining method used to detect polysaccharides such as glycogen, glycoproteins, glycolipids and mucins in tissues. PAS stain with diastase digestion has histochemical specificity for glycogen. Skeletal muscle normally contains glycogen, and is often recommended as a positive control tissue.

GLYCOGEN STORAGE DISEASES

Glycogen storage diseases (GSDS) are the result of deficiency of lysosomal enzymes that cause the alteration of glycogen metabolism (glycogen synthesis or glycolysis) in the liver and skeletal muscle.

- The liver forms of glycogen storage diseases (types 1, 3, 4 and 6) are marked by inability to glycogen to glucose leading to hepatomegaly and hypoglycemia.
- The skeletal muscle forms of glycogen storage diseases (types 2, 3A, 5 and 7) have mild symptoms appearing during strenuous exercise owing to inability to provide energy for skeletal muscle contraction.
- Examples of glycogen storage diseases include von Gierke disease, Pompe disease, Forbes-Cori disease, Cori disease, Anderson disease, McArdle disease, and Hers disease.
- Glycogen storage diseases are given in Table 1.42. Histology of hepatic glycogen storage disease is shown in Fig. 1.38.

Table 1.42 Glycogen storage diseases

Defective Enzyme	Organ Affected	Glycogen Storage in the Affected Organ	Clinical Features
von Gierke disease (GSD type 1)			
Glucose-6-phosphatase or transport system	▪ Liver ▪ Kidney	Increased amount of normal structure	Massive hepatomegaly, failure to thrive, severe hypoglycemia, ketosis, hyperuricemia, hyperlipidemia
Pompe disease (GSD type 2)			
α-1,4-Glucadase (lysosome)	All organs (liver, kidney, skeletal muscle)	Massive increase in amount, normal structure	Hypotonia (floppy baby), hypertrophic cardiomyopathy, cardiorespiratory failure causes death usually before age of two years
Cori disease (GSD type 3)			
Amylo-1,6-glucosidase (debranching enzyme)	▪ Skeletal muscles ▪ Liver	Increased amount, short outer branch	Like type 1, but milder form of disease
Anderson disease (GSD type 4)			
Branching enzyme (α-1,4 → α -1,6)	Liver and spleen	Normal amount, very long outer branches	Progressive liver cirrhosis, hepatocellular failure with fatal outcome before age of two years
McArdle disease (GSD type 5)			
Phosphatase	Skeletal muscles	Moderately increased amount, normal structure	Limited ability to perform strenuous exercise due to painful muscle cramps, otherwise, patient's development normal
Hers disease (GSD type 6)			
Phosphorylase	Liver	Increased amount	Like type 1, but milder form of disease
Tarui disease (GSD type 7)			
Phosphofructokinase	Skeletal muscles	Increased amount, normal structure	Like type 5, but moderately severe disease
GSD type 8 due to hepatic phosphorylase kinase deficiency			
Phosphorylase kinase	Liver	Increased amount, normal structure	Mild hepatomegaly, mild hypoglycemia

Types 1 through 7 are inherited disorder; and type 8 is sex-linked disorder.

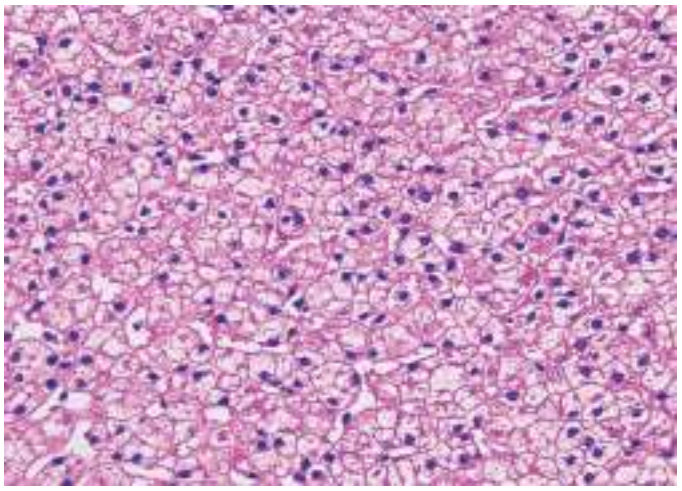


Fig. 1.38: Histology of hepatic glycogen storage disease. Glycogen storage disorders are inborn errors of metabolism with abnormal storage or utilization of glycogen characterized by hypoglycemia, hepatomegaly, stunted growth and chronic lactic acidosis. Histologic examination shows large hepatocytes with prominent cell membrane and glycogenated nuclei with periodic acid–Schiff positive glycogen (400X).

Glycogen Storage Disease Type 0

Glycogen storage disease type 0 (GSD 0) is inherited as an autosomal recessive lysosomal storage disorder, which occurs due to deficiency of glycogen synthase encoded by glycogen synthase 2 (GYS 2) gene. GSD 0 is inherited within families in an autosomal recessive fashion. Normally, glycogen synthase participates in the generation glycogen, that is stored in the liver. Glycogen synthase deficiency leads to low amount of glycogen in the liver resulting in low blood sugar (hypoglycemia) and hyperketonemia.

- **Clinical features:** Early in infancy, patients usually have no symptoms. Any child with a history of needing frequent meals with hypoglycemia and presence of ketone bodies in the urine is suggestive of glycogen storage disease type 0.
- **Laboratory diagnosis:** Genetic testing on blood sample is now available. Aim of treatment for glycogen storage disease type 0 (GSD 0) is to prevent low blood sugar (hypoglycemia) by avoiding fasting. Frequent meals and snacks should be given every 3–4 hours during the day.

von Gierke Disease

Glycogen storage disease type 1 (GSD 1), also called von Gierke disease, is most common autosomal recessive lysosomal glycogen storage disorder caused due to deficiency of glucose-6-phosphatase, in which body cannot break glycogen stored in the liver and skeletal muscle. Normally, glycogen stored in the liver and skeletal muscle is broken down to glucose by glucose-6-phosphatase to generate energy. von Gierke disease is also called type 1 glycogen storage disease.

- **Clinical features:** Patient presents with severe hypoglycemia that coincides with ketonuria, metabolic acidosis and elevated lactate (due to excessive glycolysis) and alanine. Building up of glycogen in liver results in hepatomegaly. Decreased glucose level causes lipolysis resulting in hyperlipidemia. Uricemia is caused by competitive inhibition by lactate of renal tubular urate secretion and increased uric acid production. Patient has growth retardation. Epinephrine and glucagon cannot produce hyperglycemia but result in lipolysis and increased lactate concentration.
- **Laboratory diagnosis:** von Gierke disease is diagnosed by liver biopsy and DNA testing. Patients are treated by cornstarch, allopurinol and granulocytes colony stimulating factor (GCSF).

Pompe Disease

Glycogen storage disease type 2 (GSD 2), also called Pompe disease, is an autosomal recessive lysosomal glycogen storage disorder, which damages skeletal muscle, cardiac muscle, liver and nerves throughout the body. Pompe disease is caused by mutations in the GAA gene, which encodes lysosomal acid α_1-glucosidase and α_4-glucosidase resulting in accumulation of glycogen in the lysosomes of skeletal muscle, cardiac muscle and nerves throughout the body. Normally, lysosomal acid α-glucosidase is involved in glycogen breakdown.

- **Clinical features:** Patient develops intractable hypoglycemia, muscle weakness and cardiomegaly with normal liver functions. Pompe disease is of two types: (a) infantile type and (b) delayed onset type (childhood and juvenile/adult types).
  - **Infantile-onset Pompe disease:** It is most severe form of Pompe disease. Infants are normal at birth, and manifest within the first two to three months with rapidly progressive skeletal muscle weakness, diminished skeletal muscle tone, respiratory problems, feeding problems, hypertrophic cardiomyopathy progressing to cardiorespiratory failure before attaining 3 years of age.
  - **Delayed-onset Pompe disease:** Pompe disease manifests during infancy or early childhood with delayed motor milestones. Juvenile/adult form of Pompe disease presents during in second and seventh decades of life with muscle weakness.
- **Laboratory diagnosis:** Patients with Pompe disease are diagnosed by skeletal muscle biopsy, liver biopsy, lysosomal acid maltase assay and DNA testing. Patients are treated by lysosomal acid maltase replacement.

Cori Disease

Glycogen storage disease type 3 (GSD 3), also known as Cori disease, is an autosomal recessive lysosomal glycogen storage disorder caused due to deficiency of glycogen debranching enzymes (amylo-1,6-gluco--sidase) resulting in storage of abnormal form of glycogen in the liver, heart, or skeletal muscle. Patient presents with stunted growth, hepatomegaly, and hypoglycemia.

- **Clinical features:** Beginning in infancy, any type of GSD III is characterized by low blood sugar (hypoglycemia), hyperlipidemia and elevated blood concentration of liver transaminases and creatinine kinase. As the infant gets older, the child develops stunted growth, hepatomegaly. Liver size returns to normal during adolescence. Some affected persons develop cirrhosis, hepatocellular failure and liver adenoma in life.
- **Laboratory diagnosis:** Beginning in infancy, any type of GSD 3 is characterized by low blood sugar (hypoglycemia), hyperlipidemia and elevated blood concentration of liver transaminases and creatinine kinase.

Anderson Disease

Glycogen storage disease type 4 (GSD 4), also called Anderson disease, is an autosomal recessive trait lysosomal glycogen storage disorder. It is caused by a mutation in GBE1 gene, which encodes glycogen branching enzyme resulting in accumulation of abnormal glycogen in the liver, muscle, and/or other tissues. Normal glycogen branching enzyme is involved in glycogen breakdown in skeletal muscle and liver. Synonyms of GSD 4 are Anderson disease or glycogenosis, amylopectinosis, branching enzyme deficiency and glycogenosis type 4.

- **Clinical features:** Infant presents with failure to thrive, stunted growth and hepatomegaly and cirrhosis progressing to portal hypertension and hepatocellular failure. Death occurs from heart or hepatocellular failure before attaining 5 years of age.
  - In addition, several neuromuscular variants of Anderson disease have been described that may be evident at birth, in late childhood or adulthood.
  - Patient develops isolated muscle involvement with skeletal myopathy and/or dilated cardiomyopathy leading to muscle weakness, fatigue, exercise intolerance, shortness of breath with exertion, edema and cardiac arrhythmias.
  - Neuromuscular variant of GSD 4 in adults, known as polyglucosan body disease, is characterized by dysfunction of the central nervous system (brain and spinal cord) and peripheral nervous system (motor, sensory and autonomic nervous system).
- **Laboratory diagnosis:** Diagnosis of GSD 4 is established by clinical evaluation, history and physical examination. Biopsy obtained from liver, skeletal muscle, heart, skin or peripheral nerve is examined under light microscope, which demonstrates abnormal deposition of amylopectin-like material. Patients are diagnosed by liver biopsy and DNA testing. Patients are treated by liver transplantation.

McArdle Disease

Glycogen storage disease type 5 (GSD 5), also called McArdle disease, is the most common metabolic autosomal recessive lysosomal glycogen storage disorder of skeletal muscle carbohydrate metabolism and one of the most frequent genetic myopathies. It is caused by mutations in PYGM gene located on chromosome 11q13 that codes for the **phosphorylase enzyme**. Normal phosphorylase enzyme causes degradation of glycogen in skeletal muscle for energy. Deficiency of phosphorylase results in accumulation of glycogen in skeletal muscle. Synonyms of GSD 5 are McArdle disease, glycogenosis type 5, myophosphorylase deficiency or muscle glycogen phosphorylase deficiency.

- **Clinical features:** McArdle disease manifests during second or third decade of life. Patient presents with skeletal muscle cramps, muscle stiffness and muscle weakness after strenuous exercise, hypoglycemic seizures or cardiomegaly; increased blood concentration of creatine kinase, myoglobinuria (dark burgundy-colored urine due to presence of myoglobin, a protein found in heart and skeletal muscles), exaggerated increase in ammonia and diminished activity of muscle phosphorylase.
- **Laboratory diagnosis:** Patient is diagnosed by skeletal muscle enzyme assay and DNA testing. Patient is advised to take sucrose prior to strenuous activity.

Hers Disease

Glycogen storage disease type 6 (GSD 6), also known as Hers disease, is a genetic disorder caused by PYG 1 gene mutation and deficiency of the hepatic phosphorylase enzyme resulting in excessive accumulation of glycogen in the liver. Normally, hepatic phosphorylase enzyme is essential to break down glycogen stored in the liver and skeletal muscle and used for energy.

- **Clinical features:** Infant or child presents with failure to thrive, hypotonia, hepatomegaly, ketotic hypoglycemia, elevated hepatic transaminases, hyperlipidemia and low prealbumin level. Most common

complications include short stature, delayed puberty, osteopenia and osteoporosis. Liver fibrosis commonly develops in GSD 6, but cirrhosis and hypertrophic cardiomyopathy rarely develop. Clinical and biochemical alterations may decline with age, but ketosis and hypoglycemia can continue to occur.

- **Laboratory diagnosis:** Diagnosis of Hers disease is established by clinical evaluation, history and physical examination; and molecular testing of **PYG1** gene mutation.

Tarui Disease

Glycogen storage disease type 7 (GSD 7), also known as Tarui disease, is an autosomal recessive lysosomal glycogen storage disorder. It is more often in individuals of Japanese and Ashkenazi Jewish descent equally affecting males and females. It is caused by mutations in the muscle phosphofructokinase gene coding for phosphofructokinase enzyme resulting in deficiency of phosphofructokinase enzyme in skeletal muscle and erythrocytes phosphofructokinase, which leads to reduced amount of energy available to skeletal muscles during exercise.

- **Clinical features:** Tarui disease usually begins in childhood. Patient presents with weakness, pain, stiffness of skeletal muscles during exercise; sometimes associated with nausea, vomiting and dark-burgundy colored urine due to the presence of myoglobin (myoglobinuria). Tarui disease can rarely affect infants and adults.
- **Laboratory diagnosis:** Tarui disease is diagnosed by a skeletal muscle biopsy for the measurement of phosphofructokinase enzyme level or analysis of phosphofructokinase enzyme in red blood cells. Molecular genetic testing is done to analyse mutation in phosphofructokinase gene encoding phosphofructokinase enzyme in Japanese and Ashkenazi Jewish descent. The patients should be advised to avoid strenuous exercise for prevention of skeletal muscle pain and cramps. Consumption of carbohydrates should be avoided. Genetic counseling is recommended for affected individuals and their families.

Glycogen Storage Disease due to Hepatic Phosphorylase Kinase Deficiency

Glycogen storage disease type 8 (GSD 8) is an X-linked recessive hepatic glycogen storage disorder resulting from lack of expression of phosphorylase-β-kinase activity. This is a regulatory enzyme in the activation cascade of glycogenolysis. GSD 8 is mildest form of glycogenoses and characterized by hepatomegaly, increased glycogen in liver, growth retardation and decreased leukocyte phosphorylase, elevation of glutamate-pyruvate transaminase and glutamate-oxaloacetate transaminase, hypercholesterolemia and hyperglyceridemia. Liver shrinkage occurs in response to glucagon.

Glycogen Storage Disease Type 9

Glycogen storage disease type 9 (GSD 9) is an autosomal recessive lysosomal glycogen storage disorder caused by the inability to breakdown glycogen by phosphorylase enzyme in the liver. The signs and symptoms such as hepatomegaly and slow growth begin in the early childhood.

- **Clinical features:** Affected children may have delayed development of milestones (sitting, standing, walking) and mild skeletal muscle weakness. Adolescents may have delayed puberty and liver fibrosis, that can rarely progress to cirrhosis. During prolonged periods of fasting, patient develops hypoglycemia or elevated levels of ketone bodies in the blood (ketosis). Ketones are molecules produced during lipolysis, which occurs when stored sugars are not available. GSD 9 can affect skeletal muscle tissue in children and adults. Patient experiences fatigue, skeletal muscle weakness, skeletal muscle pain, and cramps during strenuous exercise.
- **Laboratory diagnosis:** GSD 9 can cause breakdown of skeletal muscle resulting in release of myoglobin and its excretion in the urine. Myoglobinuria can cause the urine to be red or brown. In a small number of patients with GSD 9 both liver and skeletal muscles are affected, however skeletal muscle problems are usually mild.

Glycogen Storage Disease Type 10

Glycogen storage disease type 10 (GSD 10) is an autosomal recessive lysosomal glycogen storage disorder of glycogen metabolism caused by mutation in PGAM2 gene encoding skeletal muscle phosphoglycerate mutase enzyme. GSD 10 primarily affects skeletal muscles. Patient presents with muscle cramps following strenuous physical activity, recurrent episodes of myoglobinuria as a result of breakdown of skeletal muscle. Untreated cases of myoglobinuria can result in renal failure. In some cases of GSD 10, microscopic tube-shaped structures called tubular aggregates are demonstrated in skeletal muscle fibers. It is not clear how tubular aggregates are associated with the signs and symptoms of the disorder.

PROTEIN ACCUMULATION

Excessive intracellular accumulation of proteins occurs in the settings of protein droplets in proximal tubules in renal disease, Russell bodies in plasma cells, α_1-antitrypsin deficiency, accumulation of cytoskeletal proteins and misfolded proteins in amyloidosis.

INCREASED PROTEIN DROPLETS REABSORPTION IN THE PROXIMAL RENAL TUBULE IN RENAL DISEASE WITH PROTEINURIA

Excessive intracellular accumulation of protein droplets in proximal tubules occurs in renal disease with heavy protein leakage across glomerular filter in renal disease.

- Protein droplets in proximal tubules appear as rounded eosinophilic droplets, vacuoles or aggregates. This change is reversible, if proteinuria diminishes.
- Proteins filtered through glomerular filter are reabsorbed in the proximal tubules via processes that involve at the apical pole of the cells, internalization by receptor-mediated endocytosis involving high molecular proteins (megalin and cubulin) and subsequent lysosomal degradation into constituent amino acids and smaller peptides.

INCREASED PROTEIN PRODUCTION BY PLASMA CELLS FORMING RUSSELL BODIES

Russell bodies are eosinophilic, large, homogenous immunoglobulin-containing hyaline inclusions in the plasma cell undergoing excessive synthesis of a mutant immunoglobulin, which can neither exit nor be degraded in the endoplasmic reticulum.

- Russell bodies lie in distended of rough endoplasmic reticulum, which may be observed in reactive plasmacytosis (e.g. chronic infections) and plasma cell neoplasms on bone marrow aspirates.
- Dilated cisternae of the endoplasmic reticulum resembling Russell bodies are induced in light chain producing myeloma cell lines by transfection of μ heavy chain gene lacking the first constant domain. Plasma cells containing Russell bodies are sometimes referred to a Mott cells.

ACCUMULATION OF CYTOSKELETON PROTEINS

Cytoskeleton of a cell represents network of protein filaments in the cytosol that maintains cell shape, cell movement, cell division, intracellular organization and intracellular transport of organelles and molecules. There are three main components of the cytoskeleton: microtubules, microfilaments and intermediate filaments, along with other proteins that support these components. All three components of cytoskeleton interact with each other noncovalently.

Mallory Hyaline (Mallory-Denk Body)

In histopathology, Mallory hyaline also known as Mallory-Denk body or Mallory body, is an irregular eosinophilic intracytoplasmic inclusion, that represents damaged intermediate filaments (cytokeratins 8 and 18) within hepatocytes in various liver disorders such as alcoholic liver disease, primary biliary cirrhosis, extrahepatic bile duct obstruction, α_1-antitrypsin deficiency, Wilson disease, amiodarone drug-induced liver injury, Indian childhood cirrhosis and nonalcoholic steatohepatitis. Liver disorders associated with Mallory-Denk bodies are given in Table 1.43.

α_1-ANTITRYPSIN PROTEIN ACCUMULATION

The α_1-antitrypsin is an acute phase plasma protein encoded on the proteinase inhibitor locus (Pi) on chromosome 14.

- Normal amount of α_1-antitrypsin protein is synthesized in the liver in normal genotype persons (PiMM phenotype), which inhibits neutrophil elastase. It is most abundant circulating serine protease inhibitor (elastase) secreted by neutrophils during inflammation, which digests the lung parenchyma in tobacco smokers and accelerates the appearance of emphysema symptoms.
- α_1-Antitrypsin deficiency is the most common genetic disorder of liver affecting infants and children. In liver, mutant Z gene in homozygous PiZZ genotype synthesizes mutant Z protein, which folds abnormally and accumulates in endoplasmic reticulum as hyaline globules demonstrated with periodic acid–Schiff (PAS) stain resulting in defective intracellular transport and secretion of proteins in the hepatocytes. Mutated Z protein causes lung disease (emphysema), hepatocellular injury, cirrhosis and hepatocellular carcinoma.

Table 1.43 Liver disorders associated with Mallory-Denk bodies

Category	Disorder
Excessive alcohol consumption	Alcoholic liver disease
Biliary tract disease	▪ Biliary atresia ▪ Extrahepatic bile duct obstruction ▪ Primary biliary cirrhosis ▪ Primary sclerosing cholangitis
Inherited disorders	▪ α_1-antitrypsin deficiency ▪ Abetalipoproteinemia ▪ Glycogen storage disease 1α ▪ Wilson disease
Drug-induced disorders	▪ Ethanol ▪ Amiodarone
Cirrhosis of unknown etiology	▪ Indian childhood cirrhosis ▪ Nonalcoholic steatohepatitis
Hepatocellular tumors	▪ Hepatic adenoma ▪ Hepatocellular carcinoma
Other cause	Focal nodular hyperplasia

- Patients with α_1-antitrypsin deficiency usually develops the first signs and symptoms of lung disease (emphysema) between 20 and 50 years of age.
- Treatment of emphysema includes intravenous administration of prolastin, which replaces α_1-proteinase inhibitor to prevent further lung damage.
- Evaluation for genetic defect involves quantitation of serum α_1-antitrypsin protein and characterization of genetic polymorphism.

PIGMENT ACCUMULATION

Pigments are colored substances which absorb visible light. Pigments are either present in normal cells or introduced from exogenous source. Examples of endogenous pigments synthesized within the body are melanin, bilirubin, hemosiderin, lipofuscin, homogentisic acid. Exogenous pigments introduced from exogenous source include carbon (anthracotic) pigment, tattooing, β-carotene and arsenic. Type of endogenous and exogenous pigments are shown in Table 1.44.

ENDOGENOUS PIGMENTS

Endogenous pigments include melanin, bilirubin, hemosiderin, hematin, lipofuscin, homogentisic acid and Hamazaki-Weissenberg bodies.

Lipofuscin Pigment

The term 'lipofuscin' is derived from the Latin (*fuscus* means brown), thus brown lipid. It is an insoluble pigment, also known as lipochrome or wear-and-tear or aging pigment.

- Lipofuscin is composed of polymers of lipids and phospholipids complexed with proteins, which pigment accumulates in liver, heart muscle, adrenal gland and ganglion cells, producing brown atrophy.
- Lipofuscin is derived from continuing lipid peroxidation of lipids present in cellular membranes as a result of inadequate defenses against activated oxygen-derived free radicals. Lipid peroxides are unstable and breaking down into smaller molecules. Lipofuscin does not injure cells or impair their functions.

Table 1.44 Type of endogenous and exogenous pigments

Endogenous Pigments
Lipofuscin (aging pigment)
Melanin (melanocytes)
Bilirubin (in the settings of hemolytic anemia, cirrhosis, cholestasis)
Hemosiderin (ferritin aggregates)
Hematin
Homogentisic acid
Hamazaki-Wesenberg bodies
Exogenous Pigments
Carbon (anthracotic) pigment
Tattooing
β-Carotene
Arsenic

Pathology Pearls: Lipofuscin Pigment

Light Microscopy

Lipofuscin appears as a yellow brown finely granular pigment in cytoplasm often in perinuclear region in liver, heart muscle, adrenal glands and ganglion cells especially of aging persons or patients with cancer cachexia or severe malnutrition. Histology of myocardium laden with lipofuscin is shown in Fig.1.39.

Histochemistry

Lipofuscin is acid-fast and demonstrated by Sudan black and basic fuscin stains.

Electron Microscopy

Lipofuscin pigment deposition appears as electron dense granules located in a perinuclear region of organs.

Melanin Pigment

Melanin is formed in melanocyte or its precursor derived from neural crest. Melanin biosynthesis begins with the amino acid tyrosine enzyme present in the epidermis, that oxidizes tyrosine to form DOPA (3,4-dihydroxyphenylalanine) in the rate-limiting step of melanin biosynthesis.

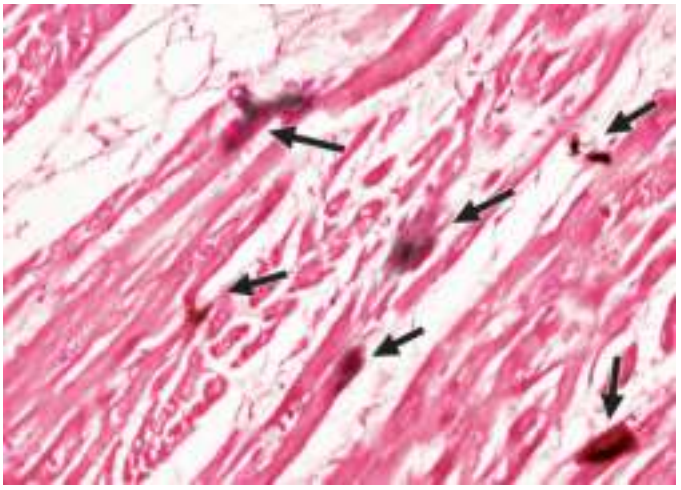


Fig. 1.39: Histology of myocardium laden with lipofuscin. Lipofuscin is fine yellow-brown-golden pigment granules composed of lipid containing residues of lysosomal digestion. It is considered to be one of the aging or 'wear-and-tear' pigments, found in the liver, heart, kidney, retina, adrenal glands, nerve cells and ganglion cells (arrows) (400X).

- Melanin is transferred to adjacent clusters of keratinocytes in the epidermis and macrophages (melanophores) in the dermis.
- Melanin combines with proteins to form melanoprotein. Melanin pigment is responsible for producing color in the body in areas such as skin, hair and eyes.

Hyperpigmentation Disorders

Hyperpigmentation refers to darkening of skin caused by increased melanin, which may be diffuse or focal, affecting face and back of the hands. Ultraviolet rays increase melanin production resulting in hyperpigmentation. Hyperpigmentation is associated with various diseases such as Addison disease, Cushing syndrome. Acanthosis nigricans (hyperpigmentation of intertriginous region associated with insulin resistance), melasma on face during pregnancy, linea nigra on the abdominal region during pregnancy, acne scarring, Peutz-Jeghers syndrome (GIT polyps and hyperpigmented macules on lips), Cronkhite-Canada syndrome and Nelson syndrome (hyperpigmentation, headache, vision impairment and cessation of menstrual cycle in patient with bilateral removal of adrenal glands). Hyperpigmentation associated disorders are given in Table 1.45.

Hypopigmentation Disorders

Hypopigmentation refers to patches of skin that are lighter than the baseline skin color, which occurs due to depletion of melanocyte or melanin, or decrease in the amino acid tyrosine, which is used by melanocytes to manufacture melanin. Genetic disorders caused by mutation in tyrosine gene or OCA2 gene are associated with hypopigmentation. Hypopigmentation is observed in albinism and vitiligo.

Table 1.45 Hyperpigmentation associated disorders

Addison disease (usually associated with elevated melanocyte stimulating hormone)
Cushing syndrome
Acanthosis nigricans (hyperpigmentation of intertriginous region associated with insulin resistance)
Melasma on face during pregnancy
Linea nigra on the abdominal region during pregnancy
Acne scarring
Peutz-Jeghers syndrome (autosomal dominant disorder with GIT polyps and hyperpigmented macules on lips)
Cronkhite-Canada syndrome
Nelson syndrome (hyperpigmentation, headache, vision impairment and cessation of menstrual cycle in patient with bilateral removal of adrenal glands)

- **Albinism:** Albinism is a genetic disorder characterized by the complete or partial absence of melanin production by melanocytes in the epidermis; and associated with vision defects such as photophobia, nystagmus and amblyopia. Oculocutaneous albinism (OC1) results from genetic defect in tyrosinase enzyme.
- **Vitiligo:** Vitiligo is an autoimmune disorder in which cause is an attack by CD8+ cytotoxic T cells on melanocytes in the dermis. Oxidative stress causes cellular disruption including interruption of protein maturation in the endoplasmic reticulum (ER) resulting in the activation of unfolded protein response (UPR) and expression of UPR-regulated chemokines such as interleukin 6 (IL-6) and interleukin 8 (IL-8), which recruit CD8+ cytotoxic T cells, which attack melanocytes in the epidermis. Patient presents with loss of skin color in the form of depigmented, or white patches of skin in any location of the body, that can be focal or multiple in several different areas.

Bilirubin Pigment

Bilirubin is the normal pigment present in bile derived from hemoglobin but contains no iron. RBCs are normally destroyed by reticuloendothelial system (spleen and bone marrow) and liberate hemoglobin, which dissociates to form heme moiety porphyrin and iron and globin.

- Bilirubin derived from porphyrin is an unconjugated (lipid soluble) form, which is conjugated into water-soluble form in the liver by glucuronyl transferase enzyme and excreted into bile. Its normal synthesis and excretion are vital to health.
- Jaundice (yellow discoloration) is a clinical disorder caused by excess of this pigment in the body, which gets deposited in sclerae, mucosae, and internal organs.
- Bilirubin level is increased due to hemolytic anemia (spherocytosis), liver etiology (uptake and conjugation defect of unconjugated bilirubin) and posthepatic obstruction.
  - Hemolytic jaundice is associated with the destruction of red blood cells in hemolytic anemia. In hemolytic disease of newborns, an increase in unbound conjugated bilirubin may result in 'kernicterus' when it enters the central nervous system and dissolves in brain tissue.
  - Conjugated bilirubin is primarily increased in viral hepatitis (hepatocellular injury) and obstructive jaundice (intrahepatic or extrahepatic obstruction of the biliary tract). Bilirubin may accumulate in hepatocytes.

Hemosiderin Pigment

Hemosiderin pigment is a hemoglobin-derived golden brown granular or crystalline pigment, also known as iron pigment. Hemosiderin consists of partially degraded apoprotein, lipid and iron. Iron is stored mostly in the liver, as ferritin and hemosiderin.

- Hemosiderin is a partially denatured form of ferritin present in the form of aggregates in tissues. Apoferritin takes up excess of iron and oxidizes ferrous to ferric form and surrounded by apoprotein known as ferritin. Then ferritin is broken down to hemosiderin.
- Ferritin level in the blood is an index of body iron stores, which is an acute phase reactant protein elevated in inflammatory diseases.
- Hemosiderin is another iron storage protein, which can hold about 35% of iron by weight. Hemosiderin accumulates when iron levels are increased.
- Normally, hemosiderin and ferritin exist in small amounts within tissue macrophages of the iron storage sites such as bone marrow, liver, and spleen, all actively engaged in red cell breakdown.
- Hemosiderin differs from ferritin: (a) unlike ferritin, hemosiderin is visible, water insoluble crystalline protein–iron complex, (b) hemosiderin has higher iron/protein ratio than ferritin, (c) hemosiderin is more stable and less available form of storage iron than ferritin, (d) unlike ferritin, iron in the hemosiderin goes back to metabolic pool of the body in a slow fashion, and (e) iron in hemosiderin is chemically reactive and turns blue-black when exposed to potassium ferrocyanide, which forms the basis for the Perls Prussian blue stain.

Hemosiderosis

Hemosiderosis refers to intracellular storage of hemosiderin located in the fixed macrophages of the bone marrow without associated tissue or organ damage. In congestive heart failure, small hemorrhages occur in the lungs.

- Red blood cells are phagocytosed by alveolar macrophages laden with hemosiderin are known as 'heart failure cells.
- Pulmonary hemosiderosis along with fibrosis is known as **brown induration of lung**. Examples of localized hemosiderosis include organized hematoma, hemorrhagic infarcts and bone fractures.

Hemochromatosis

Hemochromatosis refers to excessive accumulation of hemosiderin primarily within parenchymal cells with accompanying tissue damage, scarring, and organ dysfunction. Excessive iron deposition occurs in primary or secondary hemochromatosis.

- **Primary hemochromatosis:** It is autosomal dominant inborn disorder of iron metabolism resulting in dysregulation of iron metabolism. It most often occurs due to mutation of HFE gene on chromosome 6. Other gene mutations include C282Y, followed by the H63D. Mutations in HFE, C282Y and H63D genes increase iron absorption in the small intestine, excess iron is stored mostly in the form of hemosiderin, primarily in the liver, pancreas, myocardium, and multiple endocrine glands. Perls Prussian blue staining marks the intraparenchymal deposition of hemosiderin. Primary hemochromatosis results in 'bronze diabetes' characterized by micronodular cirrhosis, diabetes mellitus, and skin pigmentation (melanin).
- **Secondary hemochromatosis:** It is most often caused by multiple blood transfusions especially in β-thalassemia major. Iron is deposited in parenchyma of various organs.

Pathology Pearls: Hemosiderin Pigment

Hemosiderin differs from ferritin: (a) unlike ferritin, hemosiderin is visible, water insoluble crystalline protein–iron complex, (b) hemosiderin has higher iron/protein ratio than ferritin, (c) hemosiderin is more stable and less available form of storage iron than ferritin, (d) unlike ferritin, iron in the hemosiderin goes back to metabolic pool of the body in a slow fashion, and (e) iron in hemosiderin is chemically reactive and turns blue-black when exposed to potassium ferrocyanide, which forms the basis for Perls Prussian blue stain.

Light Microscopy

Hemosiderin appears as globular and golden-brown granules. This pigment becomes refractile, when condenser of the microscope is lowered down. Histology of hepatic hemosiderin deposition is shown in Fig. 1.40.

Histochemical Stain

Perls Prussian blue stain is also known as iron stain. Perls Prussian blue stain turns hemosiderin to blue by Prussian blue reaction. Histology of hepatic hemosiderin pigment demonstrated by Perls Prussian blue stain is shown in Fig. 1.41. Distribution of iron in adults is given in Table 1.46. Difference between hemosiderin and ferritin is given in Table 1.47. Hemosiderin is absent in bone marrow macrophages in iron deficiency. It accumulates pathologically in tissues in excess amounts.

Hematin Pigment

Hematin, also known as ferriheme, is a dark bluish or brownish, Perls Prussian blue negative pigment, containing iron in the ferric state, obtained by oxidation of heme.

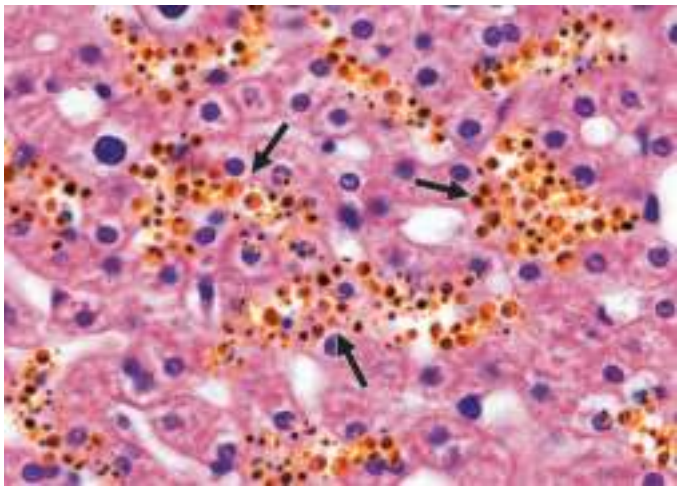


Fig. 1.40: Histology of hepatic hemosiderin deposition. Hemosiderin is a golden-brown iron-containing pigment derived from ferritin, the initial iron storage protein, that tends to be more clumped than melanin. Distinction can be difficult and at times, requiring special iron and melanin pigments. Hemosiderin granules in liver are seen as golden brown finely granular pigment (arrows). In Perls Prussian blue reaction, the tissue section is treated with dilute hydrochloric acid to release ferric ions from binding proteins. These ions will react with potassium ferrocyanide to produce an insoluble blue compound.

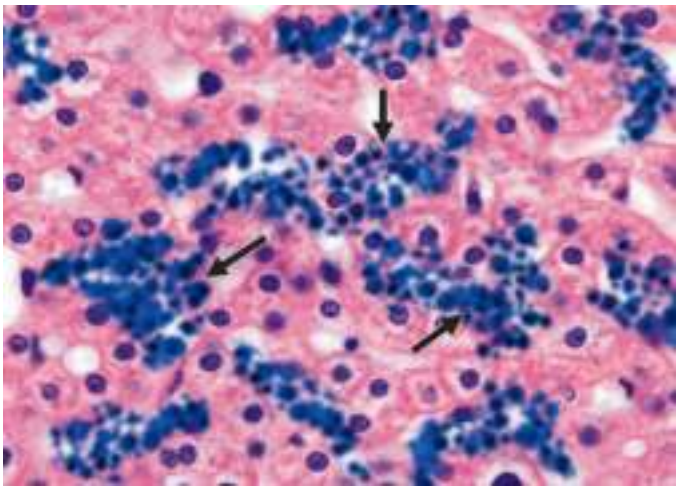


Fig. 1.41: Histology of hepatic hemosiderin pigment demonstrated by Perls Prussian blue stain. The hepatocytes and Kupffer cells are full of granular brown deposits of hemosiderin from accumulation of excess ion in the liver. Hemosiderin granules in liver are demonstrated by Perls Prussian blue stain specific for iron-containing, golden-brown, granular pigment derived from ferritin (arrows). In Perls Prussian blue reaction, the tissue section is treated with dilute hydrochloric acid to release ferric ions from binding proteins. These ions will react with potassium ferrocyanide to produce an insoluble blue compound.

- Hematin is derived from the breakdown of hemoglobin by macrophages in the spleen and bone marrow that have phagocytosed red blood cells (RBCs) in the setting of malaria, methemoglobinemia and schistosomiasis.
- Hematin crystallization is the main mechanism of detoxification of heme that is released in malaria-infected red blood cells as a byproduct of the hemoglobin catabolism by the malarial parasite.
- Functions of hematin include: (a) it inhibits the synthesis of porphyrin, (b) it stimulates the synthesis of globin, (c) it is a component of cytochromes and peroxidases, and (d) it is also used as a reagent.

Homogentisic Acid Pigment

Homogentisic acid is a black pigment that occurs in patients with alkaptonuria, a rare inborn error of metabolism disorder due to homogentisic oxidase deficiency.

- The term alkaptonuria refers to urinary excretion of metabolized homogentisic acid imparting a dark color to urine on standing.

Table 1.46 Distribution of iron in adult male and female

Distribution of Iron Form	Distribution of Iron in Adult Male	Distribution of Iron in Adult Female	Percentage of Total Body Iron
Functional iron			
Hemoglobin (functional iron)	2.4 gm	1.7 gm	65%
Heme enzymes for cellular respiration: functional iron (e.g. cytochromes, catalase, peroxidase, flavoproteins)	0.02 gm	0.015 gm	0.5%
Myoglobin (functional iron)	0.15 gm	0.12 gm	3.5%
Storage/available tissue iron			
Ferritin and hemosiderin (storage iron in bone marrow, liver and spleen)	1.0 gm (0.3–1.5 gm)	0.3 gm (0–1.0 gm)	30%
Plasma iron			
Transferrin-bound iron in plasma	0.004 gm	0.003	0.1%

Total body iron (3.0–5.0 gm)

Table 1.47 Differences between hemosiderin and ferritin

Parameters	Hemosiderin	Ferritin
Solubility in water	Water insoluble crystalline protein–iron complex	Water soluble
Visibility	Visible in tissues	Invisible in tissues
Iron/protein ratio	High	Low
Stability	More stable	Less stable
Storage sites	Bone marrow, liver and spleen	Bone marrow, liver and spleen
Availability form of iron	Less available form of storage iron	More available form of storage iron
Iron going back to metabolic pool	Slow fashion	Rapid fashion
Prussian blue stain	Positive	Negative

- The term **ochronosis** refers to deposition of this pigment prominently in cartilage and connective tissue and other structures (eyes, larynx and bronchi, heart and vessels, prostate, and sweat glands).
- Most symptoms result from joint involvement, which can lead to disabling arthritis as patient ages.

Hamazaki-Wesenberg Bodies

Hamazaki-Wesenberg bodies appear as small, yellow-brown spindle-shaped periodic acid–Schiff (PAS) positive structures in the sinuses of lymph nodes either lying free or as cytoplasmic inclusions deep abdominal mesenteric lymph nodes (most common), superficial lymph nodes and mediastinal lymph nodes. Their significance is not known.

EXOGENOUS PIGMENTS

Exogenous pigments are formed outside the body but found within tissues. Exogenous pigments enter the body in a variety of ways. Examples of exogenous pigments include carbon, asbestos fibers, tattoo ink pigment and metals.

- Copper is the most commonly observed exogenous pigment found in the liver tissue in Wilson's disease. Hematoxylin and eosin-stained tissue section usually does not reveal the presence of copper in liver tissues.
- Two most widely used methods for staining copper are the **rhodanine** and **rubeanic acid stains**, both of which require overnight incubation.
- Shikata stains (orcein and Victoria blue) which stain copper binding protein also require overnight incubation.

Coal Pigment

Coal dust is a fine powdered form of coal, which is created by crushing, grinding or pulverizing of coal. Burning of pulverized coal (powdered coal) in pulverized coal-fired boiler is used to generate thermal energy.

- Because of the brittle nature of coal, coal dust is created during mining, transportation or mechanically handling coal. Coal dust is hazardous to coal workers if it is suspended in air outside controlled environment of crushing grinding and combustion equipment.
- Coal dust (anthracotic pigment) is deposited in the alveolar macrophages, peribronchial lymphatics and hilar lymph nodes. The black streaks seen between lobules of lung beneath the pleural surface are due to accumulation of anthracotic pigment.
- Coal workers' pneumoconiosis, also known as 'black lung disease', is caused by prolonged exposure to coal dust. It develops after the initial mild form of disease known as anthracosis.
  - Prolonged exposure to coal mine dust can cause simple coal workers' pneumoconiosis and complicated coal workers' pneumoconiosis (pulmonary massive fibrosis), chronic bronchitis and emphysema.
  - Coal dust stimulates macrophages to release various products including cytokines, oxygen-derived free radicals and fibroblast growth factors, which are important in the inflammation and complicated coal workers' pneumoconiosis (pulmonary massive fibrosis).

Tattoo ink Pigment

Tattooing is a form of localized exogenous pigment inoculated into the skin. Tattoo ink pigment is phagocytosed by dermal macrophages. It resides in dermal macrophages forever. Inoculated exogenous pigment, as such does not evoke inflammatory response. Most common skin reactions to tattoo ink pigment include a transient acute inflammatory reaction of skin as a result of trauma induced by needles, superficial and deep infections, allergic contact dermatitis, photodermatitis, granulomatous and lichenoid reactions.

EXTRACELLULAR ACCUMULATION OF SUBSTANCES

INTRACELLULAR AND EXTRACELLULAR HYALINE CHANGE

The term 'hyaline' usually refers to an alteration within cells or in the extracellular space, which exhibits a homogenous, glassy, eosinophilic appearance in routine paraffin-embedded histologic sections stained with hematoxylin and eosin. It is widely used as descriptive histologic term rather than a specific marker for cell injury. It is almost always associated with accumulation of a protein in the tissue. Extracellular hyaline can be demonstrated in hyaline arteriosclerosis, uterine leiomyoma, hyaline membrane in the newborn, hyalinization of glomeruli in chronic glomerulonephritis and corpora amylacea in prostate gland. Intracellular and extracellular hyaline change in various disorders is given in Table 1.48.

- Intracellular hyaline change is demonstrated as Russell bodies in plasma cells, reabsorption plasma protein droplets in proximal tubules, tumoral hyaline globules and Mallory hyaline (Mallory-Denk body) in liver disorders.
- Extracellular hyaline change is somewhat more difficult to analyze. Collagenous fibrous tissue in old healed scars may appear hyalinized, but the physiochemical mechanism underlying hyaline change is not clear. In long-standing hypertension and diabetes mellitus, the walls of the arterioles, especially in the kidney, become hyalinized, owing to extravasated plasma protein and deposition of basement membrane material. Hyaline change in the renal arterioles is termed **hyaline arteriolosclerosis**.

Table 1.48 Intracellular and extracellular hyaline change in various disorders

Intracellular Hyaline Change
Mallory hyaline
Russell bodies (e.g. multiple myeloma)
Zenker's hyaline change in rectus abdominis and diaphragm
Crooke's hyaline change in ACTH producing cells
Extracellular Hyaline Change
Hyaline arteriosclerosis
Hyaline change in uterine leiomyoma
Hyaline membrane in the newborn
Hyalinization of glomeruli in chronic glomerulonephritis
Corpora amylacea in prostate

AGGREGATION OF MISFOLDED PROTEINS (AMYLOIDOSIS)

Amyloidosis is fundamentally a disorder of protein misfolding. More than 20 different proteins result in aggregation to form extracellular insoluble fibrils having an antiparallel, β-pleated sheet tertiary structure in organs such as kidney, spleen and liver, resulting in Congo red staining and 'apple green birefringence' under polarized light microscope. Amyloid material appears as beaded fibrillar appearance on electron microscopy.

- Amyloid is composed of fibrillar proteins, and non-fibrillary glycoproteins [amyloid P component also called serum amyloid P (SAP) glycosaminoglycans and apolipoprotein E]. Serum amyloid P component may contribute to stability of amyloid deposits.
- Radioactive iodine labeled SAP is used to assess amyloid deposition in nuclear medicine studies. Irrespective of molecular composition, amyloid always has the same characteristic histologic and ultrastructural appearance. Amyloid is resistant to digestion, which accumulates within tissue, interferes with function and destroys vital organs.
- Important clinical forms of amyloidosis include: (a) primary amyloidosis is a feature of multiple myeloma and characterized by deposits of AL amyloid in the kidneys, blood vessels and heart, which is derived from the immunoglobulin light chains AL, (b) secondary amyloidosis is characterized by deposits of AA amyloid in kidneys, liver and spleen which is derived from serum amyloid associated protein produced by liver in the settings of chronic inflammation, autoimmune disorders and renal cell carcinoma, (c) familial amyloidosis results from deposits of abnormal transthyretin in the nerves, and (d) localized amyloidosis is typical of Alzheimer disease (amyloid deposit in the cerebral cortex) and medullary carcinoma of the thyroid.

RENAL AMYLOIDOSIS

Renal amyloidosis is the most common serious complication in the disease. Kidneys are commonly involved in 80% cases of systemic amyloidosis. Patient develops renal failure associated with fatal outcome.

- Renal amyloidosis occurs due to deposition of amyloid material in primary (AL in multiple myeloma) and secondary amyloidosis (AA in chronic disorders).
  - In primary amyloidosis, κ or λ chains of immunoglobulins synthesized by neoplastic plasma cells are deposited in the glomerular basement membrane and mesangial matrix. These immunoglobulins can be detected in serum or urine by electrophoresis.

- In secondary amyloidosis, amyloidosis is a well-known complication of chronic inflammatory disorder such as tuberculosis, bronchiectasis, rheumatoid arthritis, and osteomyelitis, which stimulate the liver to synthesize AA protein, an acute-phase reactant secreted by the liver. The kidneys (80%), liver, spleen, and adrenals are the most common organs involved in secondary systemic amyloidosis.

- Renal amyloidosis is important cause of nephrotic syndrome in 5% of middle-age adults. About 50% of patients may have proteinuria in the nephritic range and hypertension followed by massive proteinuria in nephrotic range, weight loss, organomegaly, monoclonal light chains in serum or urine.
  - As the renal disease progresses, there will be renal insufficiency and chronic renal failure (small contracted kidneys) over 2–5 years.
  - Renal failure is a common cause of death in amyloidosis. Duodenal biopsy is sensitive technique for diagnosing amyloidosis in chronic kidney disease patients, and highly correlates with renal amyloidosis.

Pathology Pearls: Renal Amyloidosis

Gross Morphology

- Kidney is enlarged, pale, and waxy with smooth surface with firm consistency.
- In advanced cases, the kidneys are contracted and shrunken due to vascular narrowing induced by deposition of amyloid.
- Cut section of kidneys reveals pale firm and waxy amyloid material in the renal cortex.

Light Microscopy

- Glomeruli show thickening of glomerular basement membrane.
- Amyloid material appears as amorphous, homogenous and eosinophilic in mesangial region, tubular basement membranes, renal vessels and interstitial tissue.
- Expansion of mesangial region obliterates the glomerular capillary loops rendering the glomerular filter leaky to plasma proteins and impaired renal functions.
- There is no cellular response to the amyloid deposits. The amount of amyloid in the glomeruli correlate roughly with the clinical manifestations of the disease. Histology of renal amyloidosis is shown in Fig. 1.42.

Amyloid Staining

- Amyloid material in glomerular mesangial matrix, glomerular and tubular basement membrane is demonstrated by Congo red staining under polarized microscopy, which reveals characteristic apple-green birefringence (color of a Granny Smith apple).
- Amyloid material can also be demonstrated by various other stains, i.e. thioflavin T, thioflavin S, methyl violet, crystal violet.
- Pretreatment of tissue with potassium permanganate stained by Congo red demonstrates AL amyloid only but not AA amyloid. Congo red stain in renal amyloidosis is shown in Fig. 1.43. Stains used to demonstrate amyloid material is given in Table 1.49.

Immunofluorescence Microscopy

Immunofluorescence microscopy of renal biopsy reveals non-specific trapping of proteins.

Electron Microscopy

Electron microscopy demonstrates fibrils 7.5–10 nm in diameter with randomly dispersed, nonbranching arrangement in mesangial and subendothelial regions of glomeruli.

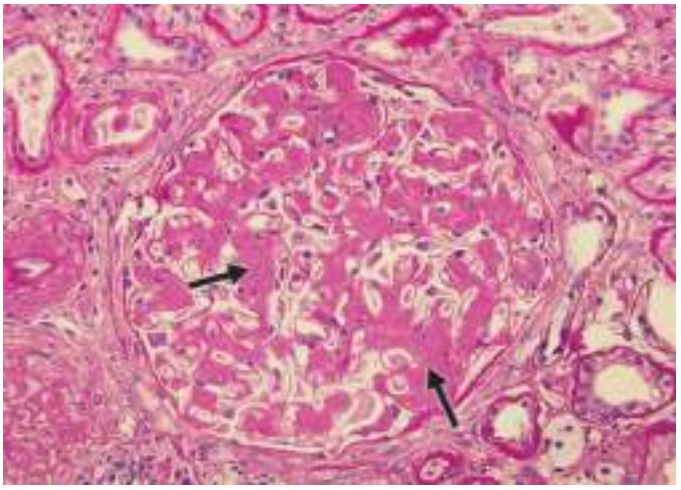


Fig. 1.42: Histology of renal amyloidosis: Initially, amyloid material is deposited in the mesangium and then extending along the inner surface of GBM distorting glomerular lumina. Light microscopy shows amorphous acellular material extending to mesangial matrix and obstructing glomerular lumina (arrows) (400X).

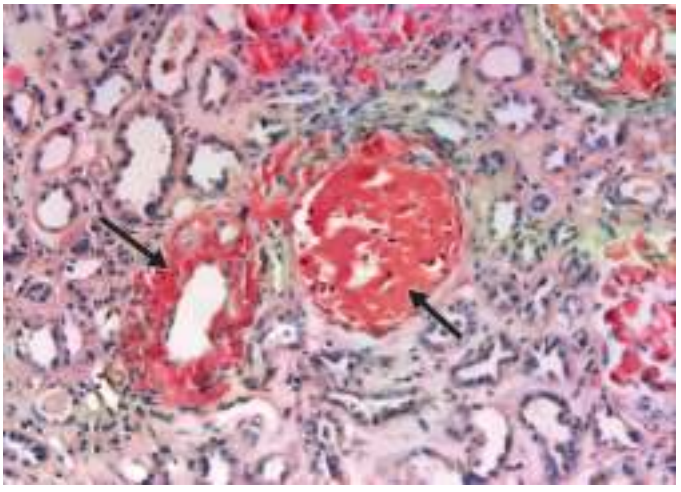


Fig. 1.43: Congo red stain in renal amyloidosis. Amyloid material is stained by Congo red stain. Nodules in diabetic glomerulosclerosis are PAS positive but negative for Congo red stain (arrows) (400X).

Table 1.49 Stains used to demonstrate amyloid material

Amyloid Stain	Color Imparted
Congo red with light microscopy	Rose-pink
Congo red with polarizing microscopy	Apple green birefringence similar to color of Granny Smith apple
Methyl violet (metachromatic stain)	Pink
Crystal violet (metachromatic stain)	Pink
Fluorescent stains: ▪ Thioflavin T ▪ Thioflavin S	Greenish
von Gieson stain	Khaki color
PAS stain	Magenta color

Congo red stain is commonly used to demonstrate amyloid deposits in tissue sections.

SPLEEN AMYLOIDOSIS

Amyloid deposition in the spleen can occur in three major sites: red pulp, white pulp, and blood vessels. Red pulp involvement by amyloid occurs in more than 50% of AL cases but none of the AA cases and produce lardaceous spleen. White pulp amyloid deposition can occur in 70% of the AL and 35% of the AA cases and produce sago spleen. Blood vessels are involved in AL amyloidosis.

- Amyloid involvement of spleen causes moderate to marked splenomegaly weighing 200–800 g, and increases the risk of spontaneous rupture of spleen.
- Amyloid deposit in spleen has two different anatomical patterns such as sago spleen (amyloid deposit in splenic lymphoid follicles with moderate splenomegaly) or lardaceous spleen (amyloid deposit in red pulp with marked splenomegaly).
- In both sago and lardaceous anatomical patterns of spleen due to amyloid deposit, spleen is firm in consistency, and cut surface reveals pale gray, waxy deposits. Comparison between sago and lardaceous spleen in amyloidosis is given in Table 1.50.

Surgical Pathology: Anatomical Patterns of Spleen in Amyloidosis

Sago Spleen

- Amyloid involvement of the spleen occurs in splenic lymphoid follicles resulting in moderate splenomegaly.
- Cut surface of the spleen shows dotted with tapioca-like gray nodules called as 'sago spleen'.
- Sago is an edible starch that is prepared from the pith of an array of tropical palm trees. It is staple food in parts of the tropics.

Lardaceous Spleen

- Alternatively, the amyloid involvement of spleen occurs in the red pulp sparing splenic follicles resulting in marked splenomegaly.
- Gross examination of spleen shows large, firm spleen mottled with waxy discolorations called 'lardaceous spleen'.
- The red pulp of the spleen is composed of connective tissue known as 'cords of Billroth' and many splenic sinusoids that are engorged with blood exhibiting it a red color.
- Primary function of 'cords of Billroth' is to filter circulating blood antigens, microorganisms and defective or worn-out red blood cells. Histology of amyloid deposition in the spleen is shown in Fig. 1.44.

LIVER AMYLOIDOSIS

The liver is almost universally involved in systemic amyloidosis that occurs in myeloma-related (AL) primary amyloidosis and amyloid-associated (AA) secondary or reactive amyloidosis.

- Amyloid deposits occur in the hepatic parenchyma along the sinusoids within the 'space of Disse' or within the walls of blood vessels. As a result of extensive compression of the hepatocytes by amyloid deposits, there may be atrophy of hepatocytes.
- AL amyloid involves the portal vessels as frequently as AA amyloid associated deposit occurs significantly more frequently in the portal stroma, the central vein and the sinusoidal areas.
  - Hepatic dysfunction is usually subclinical and may include hepatomegaly, mild jaundice and rarely severe cholestasis.

Table 1.50 Comparison between sago and lardaceous spleen in amyloidosis

Parameters	Sago Spleen	Lardaceous Spleen
Amyloid deposit	AL (>50%)	AA (70%) and AL (35%)
Amyloid deposit in splenic region	Red pulp	White pulp (lymphoid follicles)
Gross appearance of spleen	Tapioca-like gray nodules	Large firm spleen
Splenomegaly	Marked splenomegaly	Moderate splenomegaly
Consequence	Rupture (more frequent)	Rupture (relatively less frequent)

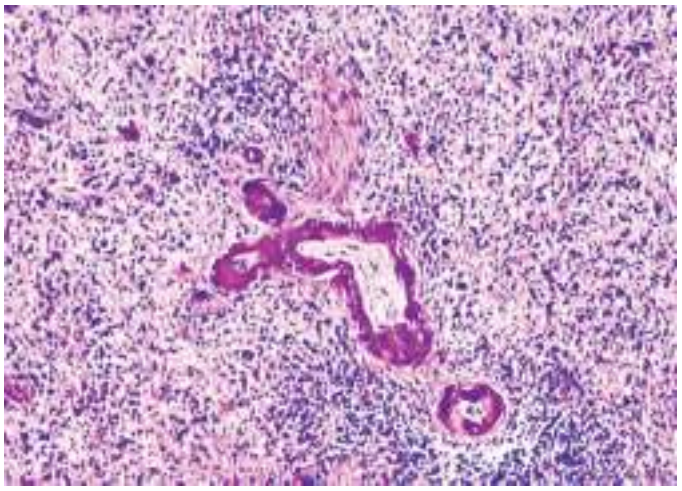


Fig. 1.44: Histology of amyloid deposition in the spleen. Most common types of amyloidosis are AL (primary), AA (secondary), familial ATTR and senile ATTR amyloidosis. Amyloidosis most commonly affects kidneys, liver and spleen. Amyloid deposition in spleen can involve red pulp (AL type), white pulp (AL and AA types) and blood vessels (100X).

- Hepatic amyloidosis can cause portal hypertension, subcapsular hematoma and spontaneous rupture.
- Acute and fulminant hepatic failure have been described as a result of amyloid light (AL) amyloidosis in the setting of multiple myeloma.

Pathologic Features: Liver Amyloidosis

Gross Morphology

The amyloid liver is usually grossly enlarged (as much as 900 gm), pale, and smooth surface, firm consistency. When sectioned, it has sharp rigid edges.

Light Microscopy

- Liver biopsy shows near complete effacement of acinar architecture by sinusoidal and portal deposits extracellular, pale eosinophilic, hyaline amorphous acellular material.
- Amyloid material is initially deposited in the spaces of Disse between the hepatocytes and vascular sinusoids. As more amyloid accumulates, it compresses the hepatic cords and sinusoids.
- The hepatic cords undergo nutritional and pressure atrophy and become displaced or replaced by bands and nodules of amyloid.

FIBRINOID NECROSIS OF ARTERIOLES

Fibrinoid necrosis of arterioles is associated with endothelial damage of small arteries and arterioles characterized by entry and accumulation of serum proteins followed by fibrin polymerization in the blood vessel wall. Accumulated serum proteins and fibrin form an intensely eosinophilic collar that obliterates cellular details of blood vessel. Fibrinoid necrosis lesion is frequent in many acute degenerative and inflammatory disorders of small arteries and arterioles.

HYALINIZATION OF GLOMERULAR BASEMENT MEMBRANE

In long-standing hypertension and diabetes mellitus, the walls of arterioles, especially in the kidney, becomes hyalinized, owing to extravasated plasma protein and deposition of glomerular basement membrane.

DYSGENETIC HYALINIZATION IN SEMINIFEROUS TUBULES

Dysgenetic hyalinization is demonstrated as a diffuse lesion in most seminiferous tubules in the settings of Klinefelter syndrome and cryptorchid testes. Focal hyalinization may be demonstrated in mixed testicular atrophy.

- **Dysgenetic hyalinized seminiferous tubules in Klinefelter syndrome:** During infancy, small seminiferous tubules contain reduced numbers of Sertoli cells and with few or absence of spermatogonia.
  - At puberty dysgenetic Sertoli cells fail to undergo maturation and soon disappear. The seminiferous tubules collapse, exhibiting the appearance of phantom seminiferous tubules. Peritubular cells fail to differentiate resulting in reduction in their numbers, thereby there is no synthesis of elastic fibers and lamina propria.
  - Dysgenesis also involves the interstitium and Leydig cells. The uniform morphology of Leydig cells is altered. Most of Leydig cells contain reduced amounts of lipofuscin granules and lipid droplets. Testosterone secretion is markedly reduced and the resulting hypogonadism is the most salient clinical feature of Klinefelter syndrome.
- **Cryptorchid testis:** In contrast to atrophic collapse observed in Klinefelter syndrome, cross-sections of the dysgenetic hyalinized seminiferous tubules are targetoid in cryptorchid testis. The peritubular cells are arranged into two layers in patients with cryptorchidism. This suggests that atrophic process in cryptorchidism has evolved over a longer period than in Klinefelter syndrome.

PATHOLOGIC CALCIFICATION

Calcification is a process in which calcium builds up in the body tissue, causing the tissue harden. This can be a physiologic or pathologic processes. About 99% of calcium entering the body is deposited in the form of calcium phosphate and calcium carbonates in the bones and teeth. The remaining calcium dissolves in the blood. Dystrophic and metastatic calcification occurs when calcium accumulates in the body cells/tissues, blood vessels or organs. Differences between dystrophic and metastatic calcification are given in Table 1.51.

- Dystrophic calcification is defined as the local deposition of predominantly calcium salts in injured or necrotic tissue that occurs in the setting of pathologic disorders otherwise with normal serum calcium levels. Overtime, ossification may occur at site of dystrophic calcification.
- Metastatic calcification is defined as the local or systemic deposition of predominantly calcium salts in otherwise normal tissues in the setting of hypercalcemia. Lungs are the commonest site for metastatic calcification.
  - Calcification begins in the mitochondria of all organs except kidney. Calcification in kidney begins in the glomerular basement membrane of kidney.
  - The calcium deposits can impair normal functions and cause pain. In hematoxylin and eosin-stained sections, calcium appears as amorphous, basophilic, granular and clumped in the intracellular as well as extracellular sites.
  - Depending on the site involvement, patients can present with skeletal muscle weakness, bone pain, prone to bone fractures, bone spurs, leg new bone deformity such as leg bowing or spine curvature breast lump.
  - Gross morphology of calcified leiomyoma and Monkeberg's medial calcific sclerosis is shown in Fig. 1.45A and B.

DYSTROPHIC CALCIFICATION

Dystrophic calcification occurs in areas of degeneration and necrosis. Some examples of lesions with dystrophic calcification include atherosclerotic plaques, tuberculous lymph node, damaged cardiac valves, hyalinized scars, myositis ossificans, degeneration in leiomyoma, neurocysticercosis and TORCH syndrome.

- TORCH syndrome is characterized by congenital infection with toxoplasmosis, rubella,

Table 1.51 Differences between dystrophic and metastatic calcification

Features	Dystrophic Calcification	Metastatic Calcification
Definition	Calcium deposition in dead or dying tissues	Calcium deposition in in normal tissues.
Calcium metabolism	There is no systemic disturbance of calcium metabolism.	There is systemic disturbance of calcium metabolism
Serum calcium	Normal	Elevated
Organs	Organs affected due to necrosis, i.e. parasitic infections as mentioned and some tumors as mentioned above	Kidneys, lungs, cornea, blood vessels, fundal glands of stomach due to relative alkalinity left after their acid secretions favor it
Causes	▪ Caseous necrosis ▪ Fat necrosis ▪ Cysticercosis (dead parasite) ▪ Filariasis ▪ Hydatid cyst (dead parasite) ▪ Dead fetus (lithopedion) in ruptured tubal pregnancy ▪ Complicated atheromatous plaque ▪ Monkeberg's calcific sclerosis ▪ Venous thrombi (phleboliths) ▪ Damaged cardiac valves ▪ Traumatic myositis ossificans ▪ Hyalinized scar tissue ▪ Leiomyomas undergoing degeneration ▪ Goiter undergoing degeneration ▪ Meningiomas (psammoma bodies) ▪ Serous cystadenoma (psammoma bodies) ▪ Papillary carcinoma thyroid (psammoma bodies) ▪ Pulmonary alveolar microliths	▪ Primary hyperparathyroidism ▪ Secondary hyperparathyroidism secondary to chronic renal disease with phosphate retention ▪ Milk-alkali syndrome ▪ Hypervitaminosis D ▪ Multiple myeloma ▪ Metastatic bone tumors ▪ Prolonged immobilization

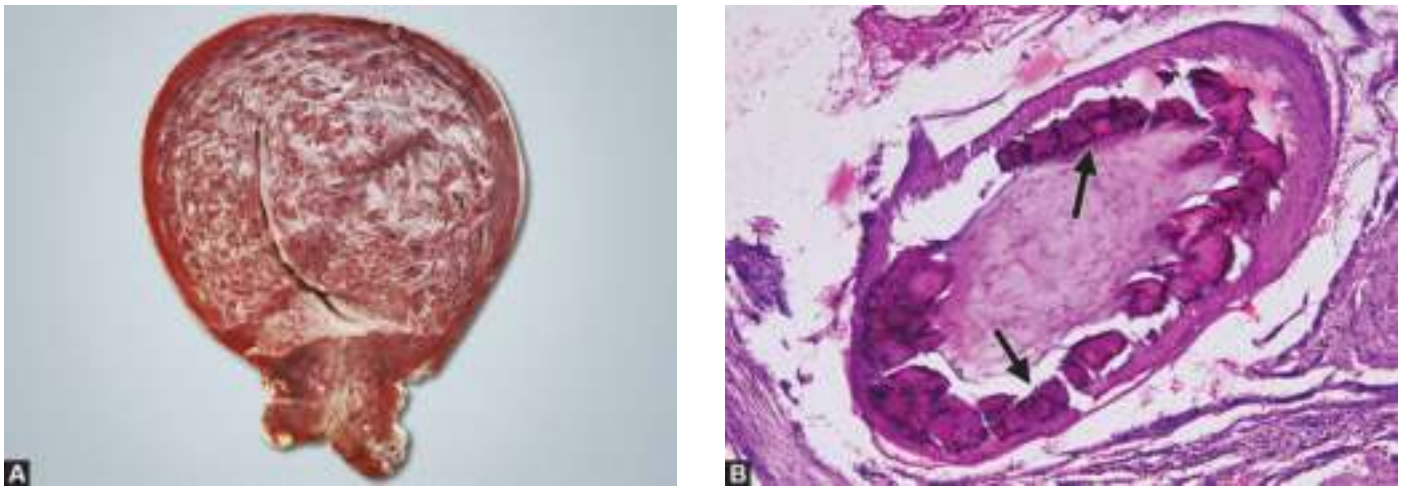


Fig. 1.45: (A) Gross morphology of calcified leiomyoma, (B) Monkeberg's medial calcific sclerosis is a ring-like calcification of the vascular media of small- to medium-sized blood vessels without associated with intimal thickening in the settings of aging, diabetes mellitus and progressive renal failure (arrows) (400X).

cytomegalovirus, herpes simplex and other organisms including syphilis, parvovirus, varicella zoster and Zika virus. TORCH infection can cause spontaneous abortion, premature birth, cerebral calcifications, chorioretinitis and skeletal metaphyseal dystrophy and periostitis.

- Macroscopically, dystrophic calcification appears tiny, white and gritty granules. On histologic examination, calcium is observed as basophilic granules (or sometimes amorphous calcium deposits) either inside or outside of cells.
- Sometimes, single necrotic cells act like grains of sand around which a 'pearl' of calcium is deposited known as psammoma bodies. Psammoma bodies are calcific spherules observed in papillary carcinoma of thyroid, meningioma, and serous cystadenoma, serous cyst borderline tumor and serous cyst adenocarcinoma.
- Disorders associated with dystrophic calcification are given in Table 1.52.

Pathology Pearls: Vitamin D and Vitamin K Role in Calcium Metabolism

- Vitamin D and vitamin K are fat-soluble vitamins and play a central role in calcium metabolism (intestinal and renal calcium absorption).
- Vitamin D promotes the production of vitamin K-dependent proteins, which require vitamin K for carboxylation in order to function properly.

Vitamin D Role in Calcium Metabolism

- Vitamin D, a fat-soluble vitamin, is derived from two sources: dietary foods and synthesis by human skin exposed to sunlight. In the liver, vitamin D is hydroxylated to 25(OH) dihydroxyvitamin D, the main circulating vitamin D metabolite to be analyzed to know vitamin D status.
- Circulating 25(OH)D is further metabolized by kidney into most active form 1,25-dihydroxyvitamin D(1,25[OH]D), also known as calcitriol.
- Vitamin D plays important role in regulating calcium metabolism by increasing intestinal and renal c absorption.

Vitamin K Role in Calcium Metabolism

- Vitamin K regulates calcium metabolism by two mechanisms: (a) it promotes calcification of bones, and (b) it prevents the calcification of blood vessels and kidneys. Vitamin K deficiency can occur due to bile duct obstruction and warfarin therapy (vitamin K antagonist).
- Vitamin K exists in two forms: vitamin K_1 (phylloquinone, mainly found in green leafy vegetables) and vitamin K_2 (menaquinone). Vitamin K stores are limited. Vitamin K_1 is transported to the liver that regulates the production of coagulation factors.
- Vitamin K_2 is transported to extrahepatic tissues such as bone and the blood vessel wall that regulates the activity of matrix Gla protein (MGP) and osteocalcin-vitamin K-dependent proteins. Vitamin K plays important role in carboxylation and functioning of vitamin K-dependent proteins (MGP and osteocalcin).
- When circulating concentrations of vitamin K are insufficient, major proportion of MGP and osteocalcin remain uncarboxylated, which is linked with cardiovascular disease, low bone mineral density (BMD) and osteoporosis.

Pathology Pearls: Mechanism of Dystrophic Calcification

- Dystrophic calcification has its origin in direct cell injury.
- Calcium is deposited in dead or dying tissues.
- Serum calcium concentration is within normal limits. It occurs by two processes: initiation and propagation.

Initiation of Calcification

- The process of dystrophic calcification is thought to start in membrane-bound vesicles within degenerated or necrosed cells.
- When the membrane of a vesicle gets damaged, phosphatase adds phosphate to the calcium forming calcium phosphate that has strong affinity for phospholipids in the cell membrane.
- Now tiny crystals of calcium are released from membrane bound vesicles into the extracellular compartment.

Propagation of Calcification

- Deposition of enough calcium phosphate leads to form clumps, which rearranges itself to generate microcrystals resulting in propagation and more calcium deposition.
- Further deposition of calcium is known as propagation, which depends on level of calcium and phosphate in the extracellular compartment, presence of collagen fibers and other proteins.
- Propagation enhances deposition of calcium. Calcium now binds with osteopontin. Normally, osteopontin participates in mineralization of bone.

METASTATIC CALCIFICATION

The deposition of calcium and phosphate salts in normal tissues is known as metastatic calcification, most often secondary to some derangement in calcium and phosphate metabolism.

- Serum calcium and phosphate concentration is higher than normal range in the settings of advanced stage cancers metastasizing to bones, multiple myeloma, primary/secondary hyperparathyroidism, hypervitaminosis D, milk-alkali syndrome due to excess calcium intake in the form of milk and self-antacid therapy, prolonged immobilization, advanced renal disease with phosphate retention in secondary hyperparathyroidism, and pharmacological agents.
- Most cases of hypercalcemia of malignancy are associated with dysregulation of parathormone (PTH) and PTH-related peptide (PTHrP).
- Under physiologic state, PTH is activated when calcium levels in the blood drop, which leads to increased calcium absorption by renal tubules and the intestine to correct the drop.
- PTHrP, an ectopic hormone, is capable of acting on the same receptors as parathormone. PTHrP is upregulated in breast cancer, lung cancers (small cell lung carcinoma and squamous cell carcinoma), ovarian carcinoma, prostatic carcinoma, hepatocellular carcinoma and adult T cell lymphoma.
- In primary or secondary hyperparathyroidism, the overproduction of parathormone leads to an inevitable increase in serum calcium level as a consequence of calcium released from the bones and increase in absorption of calcium by small intestine under the influence of parathormone.

Table 1.52 Disorders associated with dystrophic calcification

Necrosis
▪ Caseous necrosis ▪ Fat necrosis
Parasitic Disorders
▪ Cysticercosis ▪ Hydatid cyst ▪ Filariasis ▪ Schistosomiasis
Tissue Trauma and Repair
▪ Myositis ossificans (calcium deposition in skeletal muscles after trauma) ▪ Calcium deposition in hyalinized scar
Hamartomas
Hamartomas in the vicinity of the bones
Thyroid Gland Disorders
▪ Multinodular goiter ▪ Papillary thyroid carcinoma (psammoma bodies)
Central Nervous System Disorders
▪ Intrauterine toxoplasma infection of fetus with TORCH syndrome causing acute encephalitis associated with foci of necrosis that become calcified ▪ Neurocysticercosis ▪ Meningioma (psammoma bodies)
Cardiovascular System Disorders
▪ Advanced atheromatous plaque ▪ Venous thrombi ▪ Arteriosclerosis ▪ Scarred cardiac valves
Breast Disorders
▪ Breast carcinoma (calcification demonstrated by mammography) ▪ Breast fat necrosis
Female Genital System Disorders
▪ Degeneration in uterine leiomyoma ▪ Calcium deposition in dead fetus after ruptured tubal pregnancy is called lithopedion ▪ Serous cystadenoma of ovary (psammoma bodies) ▪ Borderline serous tumor of ovary (psammoma bodies) ▪ Serous cystadenocarcinoma of ovary
Metastatic Calcinosis Cutis
Dystrophic calcification under skin in internal malignancy
Other Rare Disorders
▪ Arterial calcification due to CD73 deficiency ▪ Generalized arterial calcification of infancy ▪ Hyperphosphatemic familial tumor calcinosis ▪ Pseudoxanthoma elasticum ▪ Scleroderma ▪ Dermatomyositis ▪ Systemic lupus erythematosus

- The process of metastatic calcification seems to affect renal tubular basement membrane (nephrolithiasis), pulmonary alveolar septa (pulmonary alveolar microlithiasis), cornea, interstitial tissues of the gastric mucosa in fundus region, internal elastic lamina of blood vessels and synovium of joints.
- Metastatic calcification in the lung or kidney may impair functions.
- Adult T cell lymphoma caused by human leukemic virus type 1 synthesizes overproduction of parahormone-related peptides, that has osteoclast overactivity resulting in hypercalcemia in association with bone demineralization.
- Metastatic calcification in kidney causes nephrocalcinosis and renal stones leading to impairment of renal function.
- Metastatic calcification in malignancy is reported in parathyroid carcinoma.
- Etiology of metastatic calcification is given in Table 1.53. Sites of metastatic calcification are given in Table 1.54.

Table 1.53 Etiology of metastatic calcification

Primary hyperparathyroidism
Secondary hyperparathyroidism secondary to chronic renal disease with phosphate retention
Milk-alkali syndrome
Hypervitaminosis D
Multiple myeloma
Metastatic bone tumors
Prolonged immobilization

Table 1.54 Sites of metastatic calcification

Kidney: basement membrane of tubules and tubular lumina
Lung: alveolar cells
Stomach: acid secreting fundal glands
Blood vessels: internal elastic lamina
Eyes: cornea
Joints: synovium causing pain

PULMONARY ALVEOLAR MICROLITHIASIS

Pulmonary alveolar microlithiasis (PAM) is a rare chronic lung disease characterized by deposition of calcium and phosphate in the intra-alveolar region involving bilateral lung parenchyma in the lower and mid zones.

- Exact etiopathogenesis is not fully understood. However, mutation in SLC34A2 gene that codes a sodium phosphate co-transporter in alveolar type 2 pneumocytes resulting in formation and accumulation of microliths rich in calcium phosphate due to impaired clearance is considered the cause of disease.
- Patients with pulmonary alveolar microlithiasis remain asymptomatic till development of hypoxemia and cor pulmonale. In some persons, PAM remains static, while in some persons, PAM progresses to pulmonary fibrosis, respiratory failure and cor pulmonale. Patient presents with dry cough and progressive shortness of breath on exertion.
- Chest radiograph shows dense micronodular opacities giving classical sandstorm appearance. High resolution computed tomography (HRCT) shows microcalcification, subpleural cystic changes and calcified pleura. Lung biopsy shows calcospherites within the alveolar spaces.

CELLULAR BIOLOGY OF AGING

Aging human cells can stop dividing and enter senescence, as a result of cell damage and defective chromosomal telomeres. Cellular aging process is driven at the cellular level by random molecular damage in almost all systems that slowly accumulate with age. Although the cells possess the mechanisms to repair the damage, yet they are not 100% efficient to do so, as their efficiency declines with age.

- Cellular aging is affected by genetic and environmental factors. Genetic abnormalities result in progressive decline in cellular function and viability. Environmental factors induce sublethal injury over years at cellular and molecular level.
- Alternative theories of aging suggest that essential neuroendocrine biological stimuli from brain or endocrine glands are programmed to stop at a certain biological age, resulting in lack of essential trophic factors to maintain cell growth.
- Other proposals suggest inefficient DNA repair, oxygen-derived free radical damage, or failure of protein catabolism as the root cause of aging.
- Cumulative injury theories propose that aging is the result of all cellular impairments sustained throughout life, whether through DNA damage, protein modification, oxygen-derived free radical damage, or disease. Mechanisms of cellular aging is discussed as follows.

Pathology Pearls: Interventions that might Extend Human Life Span

- Clearance of senescent cells
- Elimination of damaged cells
- Telomerase activation
- Activation of chaperones and proteolytic systems
- Dietary restriction, inhibition of mTOR pathway; and activation of AMPK and sirtuin
- Stem cell-based therapies
- Mitohormetics mitophagy
- Anti-inflammatory drugs/blood-borne rejuvenation factors
- Epigenetic drugs

DNA DAMAGE THEORY OF AGING

DNA is a double helix molecule of deoxyribonucleic acid containing a chain of nucleotides winding around a helix axis in a right-handed spiral at 360°. Each helical turn consists of 10 nucleotide base pairs.

- Each nucleotide contains a sugar phosphate backbone and a nitrogen base. Four types of nitrogen bases are (a) purines: adenine (A) and guanine (G); and (b) pyrimidines: thymine (T) and cytosine (C). The order of these nitrogen bases determines DNA instructions or genetic code. The main role of DNA in the cell is the long-term storage of information.
- The DNA damage theory of aging proposes that aging is a consequence of unrepaired accumulation of naturally occurring DNA damage and mitochondrial DNA damage.
- The DNA damage is an alteration in the chemical structure of DNA, such as a break in a strand of DNA, a nucleotide base pair missing from the sugar phosphate backbone of DNA.
- Ultraviolet radiation speeds the natural aging process by breaking down skin's collagen and elastic fibers present in the skin's dermis resulting in early wrinkling.

CELLULAR SENESCENCE

The genetic theory of aging centers on telomeres, which are tandem repeat DNA nucleotide sequences present at the tips of human chromosomes. The number of tandem DNA nucleotide sequences in a telomere determines the maximum life span of a cell, since each time a cell divides, multiple tandem repeat DNA nucleotide sequences are lost.

TELOMERE ATTRITION

Human cells have evolved complex mechanism for regulating cellular life span. Telomerase enzyme, also called terminal transferase, is a ribonucleoprotein that adds a species-dependent telomere repeat sequence (TTAGGG) to the ends of telomeres, which prevent telomere shortening.

- A telomere is a region of repetitive nucleotide sequences of noncoding DNA present at the tips of human chromosomes, that protect the chromosome from damage by preventing their degradation or fusion with neighboring chromosomes thus stabilize the genome and maintain structural integrity during cell division. Each time a cell divides, the telomeres become shorter that the cell can no longer divide. Telomerase is ordinarily inactive in most somatic cells, but can be detected in tumor cells.
- Long telomeres are linked to DNA integrity, normal sperm count/motility and thus fertility occurs in males. Short telomers are linked DNA fragmentation, low sperm count/motility; and thus, infertility occurs in males.
- Telomere length is associated with biological aging, that progressively shortens with each cell division, so that elderly adults have the shortest telomeres. Telomere length can serve as an indication for the biological status of previous cell divisions and DNA damage due to inflammation and oxidative stress.
- Telomeres can also undergo shortening following oxidative stress and chronic diseases, as telomeres are very sensitive to damage by reactive oxygen species (ROS).
- The greatest period of telomere shortening occurs during the first four years of life. Few *in utero* risk factors (i.e. maternal tobacco smoking, large babies for gestational age) accelerate telomeres shortening in infants and young children. In adults, telomere shortening has been associated with development of diabetes mellitus and progression of metabolic syndrome.

ACTIVATION OF TUMOR SUPPRESSOR GENES

Cellular senescence is a stable cell cycle arrest that can be triggered in normal cells in response to many intrinsic and extrinsic stimuli, as well as developmental signals. Cell cycle arrest in senescence is mainly mediated via activation of any one of p53/p21 and p16/pRB tumor suppressor pathways. All these pathways involve many upstream regulators and downstream effectors along with various side branches. Prolonged expressor of any of these four critical components (p53/p21 and p16/pRB) is sufficient to induce cellular senescence.

- Prolonged activation of p53/p21 pathway in response to DNA damage caused by telomere attrition, oxidative stress or oncogenic stress induces cellular senescence.

- The p53, known as 'Guardian of the Genome', plays a central role in inducing cellular senescence. Activation of p53 is dependent on various translation modifications such as phosphorylation, methylation, acetylation, sumoylation and ubiquitination.
- The p21 protein encoded by the CDKN1A gene, is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors (CDKIs) in addition to p27 and p57. The p21 protein is capable of inactivating all CDKIs, thereby inhibiting cell cycle progression.

OXYGEN-DERIVED FREE RADICALS THEORY LINKED TO CELLULAR AGING

Oxygen-derived free radical injury theory proposes that cellular aging is the cumulative result of oxidative damage to biological structures resulting in compromising cellular functions and ultimately short life span. Oxygen-derived free radicals arise primarily as a result of aerobic metabolism. The free radical theory of aging in human is shown in Fig. 1.46.

- Oxygen-derived free radicals such as superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), nitric oxide, peroxynitrite ($ONOO^-$), lipid peroxide radical (RCOO), and hypochlorous acid (HClO) interact with cellular DNA, proteins and lipids resulting in peroxidation of cell membrane, accumulation of misfolded proteins, oxidative damage and cell death.
- Oxygen-derived free radical scavenging system protect the cells and tissues from adverse effects of oxygen-derived free radicals. Intracellular oxidase enzymes, i.e. superoxide dismutase (SOD) in mitochondria converts oxygen to hydrogen peroxide; and glutathione peroxidase converts hydroxyl ion to hydrogen peroxide. Catalase in peroxisome converts hydrogen peroxide to water and oxygen. Exogenous antioxidants such as vitamin E (α-tocopherol), retinoids, vitamin C (ascorbate) and transport proteins (transferrin, ferritin, lactoferritin, ceruloplasmin) either inhibit synthesis or scavenge oxygen-derived free radicals.

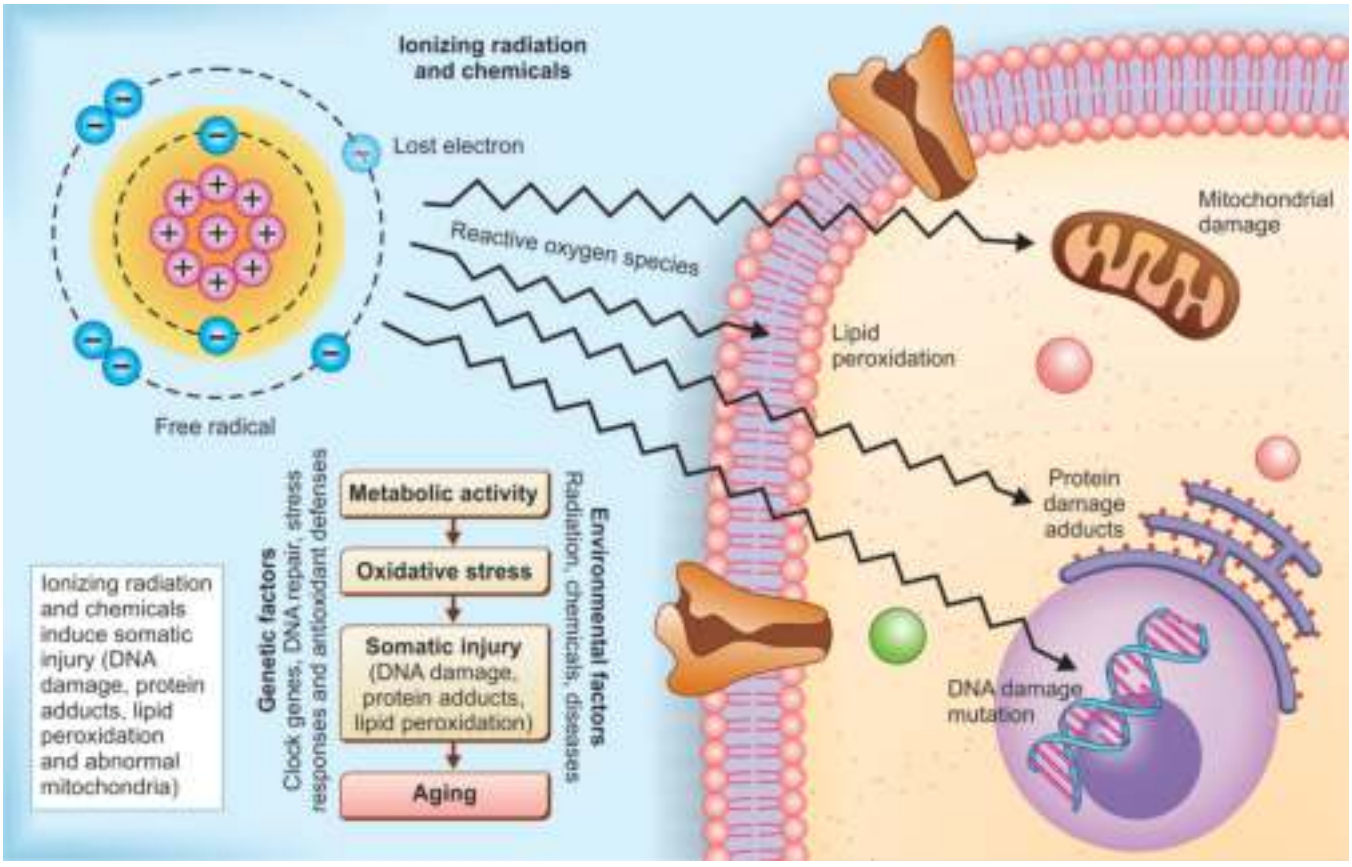


Fig. 1.46: The free radical theory of aging in human. It is caused by accumulation of damage inflicted by reactive oxygen species (ROS). Oxygen-derived free radicals are mainly produced by the mitochondrial respiratory chain as a result of electron transport and reduction of the oxygen molecules. Toxic effects of reactive oxygen species on cellular components lead to accumulation of oxidative damage which causes cellular dysfunction with age.

- Various studies demonstrated that longevity (life span) is enhanced by availability of protective oxygen-derived free radical scavenging system, enhanced expression of antioxidant enzymes and reduced calorie intake, which lead to a decline in the production of reactive oxygen species. On the other hand, decreased oxygen-derived free radical scavenging system, decreased expression of antioxidant enzymes and high calorie intake cause decreased life span.

DEFECTIVE PROTEIN HOMEOSTASIS AND CELLULAR AGING

Three cellular machineries (i.e. translation, folding and clearance of proteins) act in coordinate manner to maintain the stability of the proteome and to ensure its continuous renewal under normal state. Most cytosolic proteins fold spontaneously after synthesis.

- Failure to acquire a proper folding configuration of protein is aided by chaperones and chaperonins, which provide a favorable protein folding environment. Chaperones also assist folding of proteins synthesized in the rough endoplasmic reticulum (ER).
- Misfolded proteins are degraded by the autophagy-lysosome phagocytic system and ubiquitin-proteosome system.
- Altered protein handling is linked to many human diseases known as protein conformational disorders. Recent studies established that both normal folding and degradation of misfolded proteins are implicated in cellular aging.

AGE-RELATED CHANGES IN THE EXTRACELLULAR MATRIX

Extracellular matrix accumulation and/or degeneration due to aging process may alter cellular reparative pathways. The extracellular matrix (ECM) provides a structural framework essential for the functional properties of tissues.

- Each tissue has a three-dimensional organization of the extracellular molecules: proteoglycans, collagen fibers, elastic fibers and structural glycoproteins essential for development and growth. ECM homeostasis is regulated by serine proteases.
- During aging process, interaction of environmental factors and modifications of biosynthesis and degradative processes results in modifications of ECM homeostasis and consequently alterations of tissue functionality. These alterations of tissue functionality are increased during aging process and pathologic processes such as atherosclerosis.
- Intervertebral disc degeneration is driven partially by aging process. During aging, collagen fibers become thicker and less soluble resulting in decline in collagen synthesis. The elastic property of skin is also affected with advancing age, in which elastic fibers become thicker and fragmented.

GENETIC DISORDERS LINKED TO PREMATURE AGING

Premature aging syndromes associated with defective DNA repair system include **Hutchinson-Gilford progeria**, **Werner syndrome** and **Cockayne's syndrome**. In these disorders, skin changes that indicate aging include skin atrophy (skin thinning and loss of elasticity), loss of cutaneous fat and wrinkling.

HUTCHINSON-GILFORD PROGERIA SYNDROME

Hutchinson-Gilford progeria syndrome is an autosomal dominant disorder caused by mutation in LMNA gene encoding protein called lamin A, essential component of nuclear envelope.

- Lamin A protein plays an important role in determining the shape of the nucleus within the cells. Mutation in LMNA is thought to make the nucleus unstable.
- Hutchinson-Gilford progeria syndrome is characterized by rapid appearance of premature aging beginning in childhood.
- Patient presents with early development of cataracts, hair loss, skin atrophy, osteoporosis and atherosclerosis. Patient has a fatal outcome between 13 and 20 years of age.
- Patients are managed with lipid lowering agents and low doses of aspirin to prevent myocardial infarction and cerebral stroke; growth hormone for building height and weight and occupational therapy for stiff joints or hip problems.

WERNER SYNDROME

Werner syndrome, an autosomal recessive disorder, is characterized by the premature aging and cancer predisposition. It occurs due to mutation in the WRN gene, which affects multiple DNA-dependent enzymatic functions, including proteins with ATPase, helicase, and exonuclease activity. Persons develop normally until the end of the first decade. Lack of growth spurt is the first sign during early teen years.

Clinical Features

Patient presents with gray hair, hair loss, voice hoarseness and scleroderma-like skin changes in the 20s; later followed by type 2 diabetes mellitus, bilateral ocular cataracts, hypogonadism, osteoporosis and skin ulcers in the 30s. Myocardial infarction and development of cancer are the most common causes of death in the fifth decade of life with mean age of death in persons with Werner syndrome is 54 years.

Laboratory Diagnosis

The diagnosis of Werner syndrome is established on the basis of following cardinal signs: short stature, bilateral ocular cataracts, premature thinning and/or graying of scalp hair and skin ulcers. Demonstration of biallelic WRN gene mutation on molecular testing establishes the diagnosis of Werner syndrome. Surgical treatment is essential for bilateral ocular cataracts.

Management

In Werner syndrome. it is essential to control type 2 diabetes mellitus, maintenance of normal lipid profile and aggressive treatment of skin ulcers in these patients.

- Malignances should be treated in a standard fashion. Regular exercise, cessation of tobacco smoking and weight control reduce atherosclerosis.
- Annual screening should be performed for type 2 diabetes mellitus, lipid profile analysis, ophthalmologic examination for cataracts, physical examination for malignancies, and history of angina and cerebrovascular disease. It is essential to perform career testing for relatives at-risk and prenatal testing for pregnancies at risk.

COCKAYNE'S SYNDROME

Cockayne's syndrome also known as Weber-Cockayne's syndrome or Neill-Dingwall syndrome is an autosomal recessive disorder characterized by premature aging, failure to thrive, increased photosensitivity, hearing loss, pigmentary retinopathy.

MAMMALIAN SIRTUIN SYSTEM

Mammalian sirtuins are a family of signaling proteins (enzymes) encoded by seven sirtuin genes (SIRT1-SIRT7) implicated a variety of cellular functions ranging from gene silencing over to cell cycle, apoptosis, autophagy and cellular energy homeostasis.

- Homeostasis involves keeping the cells in balance. However, sirtuins can only function in the presence of NAD^+, nicotinamide adenine dinucleotide, a coenzyme found in all living cells.

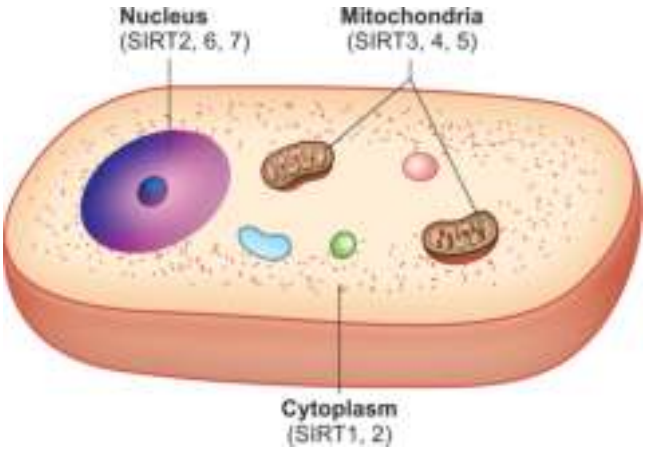


Fig. 1.47: Schematic representation of sirtuins in mammalian cellular compartments. Sirtuins are a family of signaling proteins involved in metabolic regulation.

- Sirtuins are predominantly located in either nucleus (SIRT1, SIRT6 and SIRT7), cytoplasm (SIRT2), or mitochondria (SIRT3, SIRT4 and SIRT5). SIRT1 protein counteracts aging and obesity-related diseases by deacetylating many proteins. SIRT3 deacetylates and regulates many mitochondrial proteins to maintain health.
- Calorie restriction affects the level of specific sirtuins in various tissues. The sirtuin system is involved in mediating the increase in life span by calorie restriction by increasing gluconeogenesis, cholesterol metabolism, fatty acid oxidation, fatty acid mobilization and insulin activity. There is decreased glycolysis and adipogenesis. SIRT3 functions are decreased in aging process and obesity.
- Sirtuins activities and subcellular locations are shown in Fig. 1.47 and Table 1.55.

SIRTUINS IN HEALTH AND DISEASE

SIRT1 protein, the 'master metabolic regulator' is a highly conserved nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylase encoded by SIRT1 gene, which removes acetyl groups from target proteins.

- SIRT1 directly links environmental nutrient signals to cellular metabolic homeostasis. It is expressed in brain, heart, liver, pancreas, spleen, skeletal muscle endothelial tissue and white adipose tissue.
- Activated sirtuins have remarkable property in preventing diseases such as diabetes mellitus, neurodegenerative diseases, and reversing some aspects of aging. Sirtuin is an essential factor, that suppresses

Table 1.55 Subcellular location, enzymatic activity, function, and selected nonhistone target substrates for mammalian sirtuins

Sirtuins Location in Organs	Subcellular Location	Enzymatic Activity	Function
Sirtuin 1 (SIRT1)			
Brain, hypothalamus, heart, kidney, liver, pancreas, spleen, skeletal muscle and white adipose tissue	▪ Nucleus (predominant) ▪ Cytoplasm	Deacetylase	▪ Formation of facultative and constitutive chromatin ▪ Mitochondrial biogenesis ▪ Fatty acid oxidation ▪ Regulation of cholesterol and bile acid homeostasis
Sirtuin 2 (SIRT2)			
Adipose tissue	▪ Cytoplasm ▪ Nucleus (transient)	▪ Deacetylase ▪ Demyristoylase	▪ Promotion of lipolysis in adipocytes ▪ Tumor suppression/promotion ▪ Neurodegeneration
Sirtuin 3 (SIRT3)			
Skeletal muscle, brown adipose tissue, white adipose tissue, heart, kidney, liver	Mitochondria	▪ Deacetylase ▪ ADP-ribosylase	▪ Regulation of mitochondrial activity ▪ Protection against oxidative stress ▪ Tumor suppression
Sirtuin 4 (SIRT4)			
Islets of Langerhans in the pancreas	Mitochondria	▪ ADP ribosylase ▪ Deacetylase ▪ Lipoamidase	▪ Glucose metabolism ▪ Amino acid catabolism ▪ Tumor suppression
Sirtuin 5 (SIRT5)			
Liver	▪ Mitochondria (predominant) ▪ Cytoplasm ▪ Nucleus	▪ Deacetylase ▪ Demanolyase ▪ Desuccinylase ▪ Deglutarylase	▪ Urea cycle ▪ Fatty acid metabolism ▪ Amino acid metabolism
Sirtuin 6 (SIRT6)			
Adipose tissue, skeletal muscle, brain, and heart	Nucleus	▪ ADP ribosylase ▪ Deacetylase ▪ Deacylase	▪ Genomic stability and DNA repair ▪ Glucose metabolism ▪ Lipid metabolism ▪ Inflammation
Sirtuin 7 (SIRT7)			
Adipose tissue and cardiac muscle	Nucleus (nucleolus)	Deacetylase	▪ Ribosome biogenesis ▪ Tumor promotion

cellular senescence-mediated through the age-related telomere attrition, sustaining genomic integrity and promotion of DNA repair. Expression and activity of sirtuins are strongly influenced by dietary, lifestyle or environmental factors.

- Through its deacetylation activity, SIRT1 protein modulates of its downstream pathways by targeting several cellular proteins, such as peroxisome proliferator-activated receptor-γ (PRAR-γ) and its coactivator peroxisome proliferator-activated receptor α (PRAR-α), liver X receptors (LXRs), farnesoid X receptors (FXRs), nuclear factor κ-light-chain enhancer of activated B cells (NF-κB), forkhead transcription factors, protein tyrosine phosphatase (PTP), adenosine monophosphate activated protein kinase (AMPK), CRE-binding protein regulated transcription activator 2 (CRTC2), endothelial nitric oxide synthase (eNOS), myogenic differentiation (MyoD) and transcription factor E2F1.

SIRT1 and Metabolic Diseases

SIRT1 is an important regulator of energy homeostasis in response to nutrient availability. **Liver** is the central regulator of metabolism. Activation of SIRT1 has been shown to be protective against metabolic damage. Hepatic SIRT1 regulates glucose metabolism (gluconeogenesis), lipid metabolism (fat mobilization in white adipose tissue, fatty acid oxidation in the liver and

reverse cholesterol transport), insulin secretion in the pancreas, sensing nutrition availability in hypothalamus, influencing obesity-induced inflammation and modulating activities of cicardian (sleep-wake cycle every 24 hours) clock in metabolic tissues in response to nutritional and hormonal signals.

Glucose Homeostasis

SIRT1 is an important regulator of hepatic glucose metabolism. Hepatic SIRT1 is a key regulator of gluconeogenesis in response to fasting. SIRT1 induces protein gluconeogenic genes and hepatic glucose output through PGC-1α.

- During short-term fasting phase, SIRT1 inhibits TORC2 (also known as CRCT2), a CREB-regulated transcription coactivator that is important for CAMP/CREB-mediated activation of gluconeogenesis genes resulting in reduced gluconeogenesis.
- Prolonged fasting enhances SIRT1-mediated deacetylation and activation of co-activator for numerous transcription factors resulting in increased fatty acid oxidation and improved glucose homeostasis. In addition to TORC2 and PGC-1α, SIRT1 also deacetylates Foxo1 leading to increased gluconeogenesis.

Lipid Homeostasis

Numerous studies demonstrated that hepatic SIRT1 ineracts with a nuclear receptor known as peroxisome proliferator-activated receptor-γ (PRAR-γ) and regulates lipid homeostasis in response to fasting and starvation.

- Hepatic-specific SIRT1 deletion impairs PRAR-γ signaling resulting in decreased fatty acid β-oxidation, whereas hepatic SIRT1 overexpression (upregulation) induces the expression of PRAR-γ targets.
- As hepatic SIRT1 is a regulator of lipid homeostasis, hence pharmacological activation of hepatic SIRT1 may be important for the prevention of obesity-associated metabolic diseases.
- Manipulation of hepatic SIRT1 affects the expression of genes involved in lipid and glucose metabolism.
- Modest hepatic SIRT1 overexpression (upregulation) has protective effect against high fat induced hepatic steatosis and glucose intolerance.

Cholesterol and Bile Acid Homeostasis

SIRT1 regulates hepatic cholesterol and bile acid homeostasis through direct modulation of liver X receptors (LXRs) and farnesoid X receptors (FXRs). LXRs and FXRs are nuclear receptors, that function as important cholesterol and bile acid sensors and regulate cholesterol and bile acid homeostasis.

- Cholesterol homeostasis is vital for proper cellular functions. The cellular cholesterol level reflects the dynamic balance between biosynthesis, uptake, export and esterification—a process in which cholesterol is converted to neutral cholesteryl esters either for fat storage in lipid droplets or for secretion as secretion as constituents of lipoproteins.
- Disturbed cholesterol balance can cause cardiovascular diseases, neurodegenerative diseases and cancers. Since bile acids are detergent-like molecules and toxic to hepatocytes. SIRT1 dysregulation leads to excess of bile acids, which can cause hepatobiliary disease and hepatocellular carcinoma.

Sirtuin 1 and Obesity

Adipose tissue recently emerged as a pivotal organ controlling life span. Obesity is a complex metabolic disorder linked to the development of several diseases associated with insulin resistance, glucose intolerance and hepatic steatosis; in which excess body fat has accumulated as a result of both hypertrophy and hyperplasia of adipocytes.

- Obesity is defined by body mass index (BMI) and further evaluated in terms of fat distribution via the waist–hip ratio and total cardiovascular risk factors. Increased calorie intake causes an expansion of adipocyte size, with subsequent activation of stress pathways leading to metabolic deterioration and decreased insulin sensitivity. The process is associated with increased infiltration by inflammatory cells into the adipose tissue, which contributes to the development of decreased insulin sensitivity.
- SIRT1 has emerged as an important nutrient sensor and regulator of metabolism. Numerous studies have revealed that SIRT1 plays a role in retarding of white adipose tissue (WAT), while stimulating both differentiation and activation of brown adipose tissue as browning of white adipose tissue (WAT), SIRT1 promotes fat mobilization in white adipocytes by repressing the transcription factor 'peroxisome proliferator activated receptor-γ (PPAR-γ). Calorie restriction extends life span of organisms. Upregulation of SIRT1 triggers lipolysis.
- White adipose tissue (WAT) and brown adipose tissue (BAT) have opposite functions.
  - White adipose tissue stores excess energy as triglyceride, and composed of white adipocytes and less capillaries, and white adipocyte which contains a single lipid droplet.
  - Brown adipose tissue is composed of brown adipocytes with more capillaries, and brown adipocyte contains numerous smaller lipid droplets

and much higher number of iron containing mitochondria. BAT activation generates heat from nutrients by non-shivering thermogenesis. BAT is abundant in newborns and helps them to survive cold temperatures. In adults, it has been considered to be absent or at least of no relevance. Recent studies have created interest in adult brown adipose tissue. With increasing as, BAT decreases and body weight increases. Stimulation of BAT appears to be an attractive novel candidate target for the treatment of age-related obesity.

Sirtuins 1 and 6 (SIRT1 and SIRT6) Associated Cardiovascular System in Health and Disease

Oxidative stress represents the common hallmark of cardiovascular disease including atherosclerosis, hypertension, heart failure and other vascular-related diseases.

- SIRT1 and SIRT6 have protective roles against inflammation, and cardiovascular disease including development of atheromatous plaque.
- SIRT1 regulates endothelial homeostasis by modulating endothelial nitric oxide synthase (eNO) activity, p53 angiotensin II type 1 receptor and forkhead box O (FOXO1).
- SIRT2 participates in the hypertension-induced remodeling.
- SIRT3 protects endothelial cells in response to oxidative stress by acting on FOXO3/manganese superoxide dismutase signaling pathway.
- SIRT4 and SIRT7 aggravate cardiac hypertrophy; inhibit proliferation and migration of endothelial–vascular smooth muscle cells.

Sirtuins (SIRT1 and SIRT6) Signalizing in Cellular Redox Status and Oxidative Stress

Cellular redox homeostasis is attained by the regulation of both reactive oxygen species (ROS) and removal from the body. A shift in ROS balance promotes oxidative stress.

- At vascular level, the physiologic functions of SIRT1 and SIRT6 in the control of the cellular redox status are mediated by deacetylation of multiple targets. SIRT1 regulates cellular redox homeostasis mediated by deacetylation of eNOS, FOXOs, CAT, GPx and peroxisome proliferator activated receptor α (PPAR-α) resulting in vascular protection.
- SIRT6 regulates cellular redox homeostasis mediated by nuclear respiratory factor 1 (NRF-1), a transcription activator for gene encoding cytochrome c resulting in vascular protection. Impairment of SIRT1 and SIRT6 results in decreased vascular protection against oxidative stress.

Sirtuins (SIRT1 and SIRT6) Signalizing in Cellular Senescence, Inflammation and Vascular Aging

Oxidative stress and inflammation are main events in the development of vascular aging.

- Changes in arterial vasculature include dilated lumen, endothelial dysfunction, increased endothelial apoptosis, increase in intima—medial thickness, dysregulation of matrix metalloproteinase (MMP), production of inflammatory cytokines and increased reactive oxygen species (ROS).
- Oxidative stress and low-grade inflammation are key features occurring in the arteries with aging process.
- SIRT1 and SIRT6 regulate eNOS via transcriptional and post-transcriptional deacetylation resulting in NO-mediated vascular protection. Impairment of SIRT1 and SIRT6 result in cellular senescence, inflammation and vascular aging.

Sirtuin 6 (SIRT6) Signalizing in Age-related Endothelial Function

SIRT6 plays important role in DNA repair, chromatin compaction, telomerase function and genomic stability resulting in prevention of cellular senescence.

- In endothelial cells, downregulation of SIRT6 occurs during hydrogen peroxide-induced cellular senescence, whereas overexpression of SIRT6 reverses this process.
- Impairment of SIRT6 accelerates cellular senescence via overactive NF-κB signaling. SIRT6 protein silenced by siRNA inhibits endothelial cell replication resulting in endothelial cell senescence. Depletion of SIRT6 causes upregulation of ICAM-1 mRNA, PAI-1 and p21 resulting in decreased eNOS expression and ability to form endothelial cells *in vitro* vessels.

Sirtuin 1 (SIRT1) Signalizing in Glucose Homeostasis and Hyperglycemia

Elevated reactive oxygen species (ROS) and reduced NO bioavailability play important role in the induction and progression of microvasculature and cardiovascular complications during diabetes mellitus.

- The metabolic derangements in diabetes mellitus such as hyperglycemia, dyslipidemia, insulin resistance and hyperinsulinemia induce oxidative stress, apoptosis, vascular permeability and low-grade inflammation through NF-κB signaling pathway.
- Hyperglycemia impairs activity of SIRT1 and SIRT6 resulting in reduction in vascular protection. SIRT1

is a positive regulator of insulin secretion, that triggers glucose uptake and utilization; hence it represents a targeted therapy in diabetes mellitus.
- SIRT1 protects arterial vasculature from hyperglycemia-induced endothelial dysfunction through a mechanism involving the downregulation of PAI-1 and p66 expression.

Sirtuins 1 and 6 (SIRT1 and SIRT6) Signalizing in Preventing Atherosclerosis

SIRT1 protein plays important role in prevention of atherosclerosis. It enhances production of NO.
- SIRT1 reduces oxidized LDL uptake by downregulating Lox1 signaling pathway. SIRT1 prevents foam cell formation by downregulation of LxRs.
- SIRT1 also blocks the NF-κB-mediated inflammatory process, reducing oxidative stress and controlling lysosomal autophagy. SIRT6 prevents synthesis of cholesterol and triglyceride, glycolysis and lipogenesis by downregulating ach3K9 signaling pathway.
- Impairment of SIRT1 and SIRT6 accelerates atherosclerosis by enhancing synthesis of triglyceride and cholesterol, uptake of oxidized LDL, foam cell formation and lysosomal induced autophagy.

Sirtuins 1 and 6 (SIRT1 and SIRT6) Signalizing Networks in the Heart Protection

SIRT1 and SIRT6 protect the heart against cardiac hypertrophy, ischemia/reperfusion injury, oxidative stress, heart failure and autophagy.
- SIRT1 acts by deacetylating NF-κB (nuclear factor κ-light chain-enhancer of activated B cells), FOXO and AKT signaling pathways.
- Similarly, SIRT1 protects cardiomyocytes from oxidative stress of endoplasmic reticulum by deacetylating eukaryotic translation initiator 2α (elf2α) on Lys-141 (K141) and (K143) residues. SIRT6-FOXO3 enhances the transcription of antioxidant genes manganese superoxide dismutase (MnSOD) and CAT.
- SIRT6 protects heart against hypoxic stress by activating AMPK pathway, upregulating BCL-2 and suppressing the activity of NF-κB. SIRT6 inhibits insulin growth factor (IGF)-AKT signaling pathway by targeting c-Jun and deacetylating histone type 3 (H3), acetylated Lys-9 (acH3K9).

Sirtuin 3 (SIRT3) Signalizing in Heart Protection

SIRT3 together with SIRT4 and SIRT5 are located in the mitochondria. SIRT3 preserves mitochondrial metabolic homeostasis via regulation of mitochondrial lysine acetylation, oxidative phosphorylation, boosting antioxidant defense resulting in heart protection.
- SIRT3 also decreases cellular levels of reactive oxygen species. SIRT3 also prevent stress-induced mitochondrial apoptosis of cardiomyocytes.
- Mitochondrial dysfunction plays a central role in various cardiovascular diseases, ranging from hypertrophic cardiopathy, dilated cardiomyopathy, heart failure, pulmonary hypertension, endothelial dysfunction and early development of atherosclerosis.

Sirtuin 7 (SIRT7) Signalizing in Heart Protection

SIRT7 is a master regulator of nuclear-mediated mitochondrial genes. It improves mitochondrial function in cardiac muscle, skeletal muscle, hence protects from cardiomyopathy and skeletal muscle dysfunction. Moreover, SIRT7 gives protection from microvesicular steatosis.

Sirtuins 1, 6 and 7 (SIRT1, SIRT6, SIRT7) and Cancer

Cancer is a leading cause of death worldwide. Hallmarks of cancer include sustained proliferative signaling, evading growth suppressors, resisting apoptotic cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis.
- SIRT1 and SIRT6 have dual role as tumor promotor and tumor suppressor in caner through epigenetic activity, including their involvement in epithelial-to-mesenchymal transition (EMT), energy metabolism program, invasion and metastasis. SIRT6 has both tumor promotor and suppressor functions.
- SIRT6 is upregulated at mRNA and protein level, and acts as oncogene for cancer cell proliferation.
- SIRT7 is involved in cellular pathways such as regulation of cellular metabolism, genomic stability, aging, stress response, transcription, ribosome biogenesis and tumorigenesis. The oncogenic properties of SIRT7 may be due to its interaction with p53 and upregulation of rRNA synthesis determining rapid growth.
- EMT is a reversible transdifferentiation process that transforms an epithelial cell into mesenchymal cell, providing it with the ability to invade, escape apoptosis, and metastasize to distant organs.
- EMT is of three types: (a) In EMT type 1, mesenchymal cell differentiates into epithelial cells, that has role during embryogenesis and organogenesis, (b) in EMT type 2, mesenchymal cell differentiates into fibroblasts, which is implicated in tissue repair after injury; and (c) EMT type 3 occurs in cancer progression and metastasis.
- EMT is activated by numerous transcriptional factors and epigenetic regulators, including sirtuins (SIRTs), members of the class III histone deacetylase family.

During EMT, there is loss of cell-to-cell adhesion E-cadherin protein, that correlates with high rate of metastasis and poor prognosis.

- Normal cells usually switch from aerobic to anerobic status by changing their glucose metabolism. Energy is essential for rapid and uncontrolled growth and proliferation of cancer stem cells by reprograming of cellular energy metabolism. Cancer stem cells demonstrate glycolysis and glutaminolysis, with an increase and accumulation of glycolytic intermediates, as a fuel for macromolecular synthesis resulting in malignant tumor growth.
- Numerous studies have showed that SIRT1 is significantly elevated in human prostatic carcinoma, acute myelogenous leukemia, primary colon carcinoma and hepatocellular carcinoma. Overexpression of SIRT1 has been observed in nonmelanotic skin cancers including basal cell carcinoma, squamous cell carcinoma, Bowen' disease, and actinic keratosis.
- Based on elevated levels of SIRT1, it has been suggested that SIRT1 serves as a tumor promotor. SIRT1 interacts with p53 and attenuates p53-mediated functions through deacetylation of p53. SIRT1 is involved in epigenetic silencing of DNA hypermethylated tumor suppressor genes (TSGs) in cancer stem cells. SIRT1 also interacts with and deacetylates c-Myc leading to decreased c-Myc stability.

Sirtuins 1 and 2 (SIRT1 and SIRT2) and Neurodegeneration Diseases

- Sirtuins 1 and 2 (SIRT1 and SIRT2) are implicated in numerous biological pathways related to stress response, mitochondrial dysfunction, oxidative stress, misfolded protein aggregation and inflammatory processes that are intertwined with age-related neurodegenerative diseases such as Alzheimer disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis.
- SIRT1 targets multiple transcriptional regulators, such as p53, FOXO3 and NF-κB. SIRT1 has a protective role against neurodegenerative diseases (Alzheimer disease, Parkinson's disease, Huntington's disease) via its effects in mitochondria. SIRT2 deletion has a protective role against Parkinson's disease and Huntington's disease.

Sirtuin 1 (SIRT1) and Inflammatory Signalizing in Response to Environmental Stress

SIRT1 gives protection against chronic inflammation by regulating the acetylation of nuclear factor κB (NF-κB), a transcription signaling pathway involved in the innate immune response. Sirtuin 1 (SIRT1) plays a key role in resorting homeostasis during stress responses. Inflammation is linked to altered glycolysis and fatty acid oxidation by NAD^+-dependent function of sirtuins.

Clinical Pearls: Vital Signs Observed in Patients

- **Respiratory rate:** The respiratory rate varies with age. The normal respiratory rate in adults is 12–20 breaths/minute. An elevated respiratory rate >22 breaths is poor indicator of respiratory dysfunction in patients with pneumonia, congestive heart failure and chronic obstructive pulmonary disease (COPD).
- **Blood pressure:** Blood pressure is recorded by two readings—systolic blood pressure (ventricular contraction) and diastolic blood pressure (ventricular filling). Blood pressure 120/80 mm Hg is considered normal. The difference between the systolic and diastolic blood pressure is called the **pulse pressure**. Pulse pressure <25 mm Hg indicates low stroke volume. There is no absolute normal value of blood pressure rather, there is a range of blood pressure.
- **Pulse:** The pulse is the result of physical expansion of the artery. The pulse rate is measured at wrist (radial artery) or ankle (posterior tibial artery) or neck (carotid artery) and behind knee (popliteal artery), and recorded as beats per minute. Pulse can be measured by listening directly to the heart beat using a stethoscope. Pulse rate >100 or <60 per minute is defined as tachycardia or bradycardia respectively.
- **Temperature:** An elevated body temperature (>38° C) is an indicator of illness, when proceeded by chills. Fever will increase the heart rate by 10 beats per minute. Hypothermia should be evaluated since it is an important vital sign of severe illness. The absence of fever does not rule out infection. High spiking fever is observed in patients with blood transfusion reaction or drug reactions.
- **Fifth vital sign**: Fifth vital sign refers to pain, pupil size and reactivity to light or oxygen saturation measurement. A pulse oximetry saturation of 90–92% represents a PaO_2 near 60 mm Hg should be the minimal aim of O_2 supplementation. The 90% of oxygen saturation represents the elbow of the hemoglobin saturation curve, and below this number (<90% of oxygen saturation) indicates rapid hemoglobin desaturation. Above this number (>90% of oxygen saturation) indicates little gain in oxygen carrying capacity of hemoglobin.

Clinical Pearls: How to Approach Clinical Problems

- There are many key questions essential for application of basic science information to the clinical settings. The scientific aspect of medicine seeks to gather data in an objective manner for understanding pathophysiologic processes in the light of scientific information and propose rational explanations. A skilled clinician must be able to correlate basic sciences and clinical sciences.

- The history is the most important tool in obtaining diagnosis. All physical examination findings, laboratory findings, imaging studies are first obtained, and then interpreted in the light of the pertinent history.
- In history taking, basic information is gathered related to age, gender, ethnicity, chief complaints, past history, family history, and social history.
- Physical examination includes general appearance, vital signs (blood pressure, heart rate, respiratory rate, height, weight, pain, pupil size and reactivity to light or oxygen saturation measurement) and body systems examination.
  - The presence of specific physical findings can be useful in making differential diagnosis and guiding therapy.
  - Inspiratory crackles may indicate pulmonary edema. Cardiac murmur indicates valvular insufficiency.
  - An S3 gallop indicates left ventricular dysfunction.
  - A pleural or pericardial rub can point to pleuritis or pericarditis. It is important to remember that a normal physical examination does not exclude potentially serious causes of chest pain.
- Laboratory investigations should be carried out, which include complete blood count, chemical panel, electrocardiogram, arterial gas analysis, lipid panel, urine analysis, and Gram staining and blood culture for isolation of bacteria.
- Common types of imaging include: X-rays, CT (computed tomography), MRI (magnetic resonance imaging), ultrasonography and positron emission tomography (PET). Children with infections, trauma and congenital abnormalities require imaging. Brain tumors are detected in children by CT scan and MRI. MRI is the study of choice, although non-contrast CT scan is used initially if intracranial hemorrhage or recent trauma is suspected.
  - Chest radiography is extremely useful in assessing cardiac size and contour, chamber enlargement, pulmonary vasculature and infiltrates the presence of pleural effusions.
  - Ultrasonography is noninvasive and helpful in evaluating biliary tree looking for ureteral obstruction and evaluating vascular strictures, but has limited utility in obese patients. Ultrasonography evaluates fetal and infant brain as long as the fontanelles remain open. It is also done to evaluate lateral ventricles, choroid plexus, thalamus, temporal lobes, and posterior fossa. Ultrasonography is done to assess ventricular enlargement and suspected brain hemorrhage.
  - Computed tomography (CT) is helpful in possible intracranial bleeding, abdominal and/or pelvic masses, and pulmonary processes, it may help delineate the lymph nodes and retroperitoneal disorders. Generally, CT requires radiocontrast dye, which can be nephrotoxic.
  - Magnetic resonance imaging (MRI) identifies soft tissue planes very well and provides the best imaging of the brain parenchyma. When used with gadolinium contrast (which is not nephrotoxic agent) magnetic resonance angiography is useful in delineating vascular structures.
- Cardiac procedures include echocardiography, angiography, and stress treadmill test. Two-dimensional colored echocardiography enables a quantification of shunts, cardiac output and provides a noninvasive assessment of concomitant valvular disease. The presence and extent of ischemic heart disease is determined by monitoring segmental wall motion.
- There are typically four distinct steps to the systemic solving of clinical problems: (a) gathering information with a differential diagnosis and looking for discriminating features to narrow the differential diagnosis and establishing the diagnosis, (b) assessing the severity of the disease (stage), (c) rendering a treatment based on the stage of disease, and (d) following the patient's response to the treatment. Hemodynamic instability disorders include myocardial infarction, cardiogenic shock, tamponade, acute mitral regurgitation, ventricular septum rupture, hypovolemic shock, and septic shock.
- Clinician needs to identify the objectives of therapy, symptom relief, prevention of complications or reduction in mortality.

CHAPTER

2

Inflammation and Tissue Repair

Vinay Kamal, Anubhav and Vigyat

LEARNING OBJECTIVES

INFLAMMATION

- General features of inflammation
- Causes of inflammation
- Type of inflammatory responses
  - Acute inflammatory response
  - Chronic inflammatory response

ACUTE INFLAMMATION

- Vascular events in acute inflammation
  - Hemodynamic changes
  - Increased vascular permeability of postcapillary venules
  - Exudate formation
- Cellular events: leukocytes recruitment to the sites of inflammation
  - Margination of leukocytes
  - Rolling of leukocytes
  - Firm adhesion of leukocytes
  - Transmigration of leukocytes
  - Chemotaxis of leukocytes
  - Activation of leukocytes
- Phagocytosis and clearance of the injurious agents
  - Intracellular microbial killing
  - Leukocyte-induced injury
- Termination of acute inflammatory response
- Chemical mediators of inflammation
  - Plasma protein-derived chemical mediators
  - Cell-derived chemical mediators
- Morphologic patterns of acute inflammation
  - Serous inflammation
  - Fibrinous inflammation
  - Purulent (suppurative) inflammation
  - Pseudomembranous inflammation
  - Hemorrhagic inflammation
  - Gangrenous inflammation
  - Catarrhal inflammation
- Outcome of acute inflammation
  - Complete resolution
  - Tissue destruction
  - Progression to chronic inflammation
  - Healing by fibrosis
- Anti-inflammatory therapeutic agents

CHRONIC INFLAMMATION

- Common causes of chronic inflammation
- Essential morphologic features of chronic inflammation
  - Mononuclear cells infiltrate
  - Tissue destruction
  - Tissue healing by fibrosis
- Cells involved in chronic inflammation
  - Monocytes/macrophages
  - Lymphocytes
  - Other cells involved in chronic inflammation
- Chronic nonspecific inflammation
- Granulomatous inflammation
  - Type of granulomas
  - Multinucleated giant cells
- Systemic effects of inflammation
  - Acute-phase reactants synthesis
  - Clinical manifestations in inflammation
  - Systemic inflammatory response
  - Metabolic alterations in inflammation
  - Release of chemical substances in inflammation
  - Hematologic alterations in inflammation

TISSUE REPAIR

- Normal cell proliferation and tissue growth regulation
  - Tissue proliferative activity
  - Stem cells in tissue homeostasis
- Cell cycle in tissue homeostasis
  - Cell cycle phases
  - Cell cycle regulation
- Cell regeneration: regulation
  - Growth factors and cytokines involved in cell regeneration
  - Signaling mechanisms of cell growth
- Extracellular matrix
  - Structural fibrous proteins
  - Amorphous ground substance
  - Cell adhesion molecules
- Wound healing and tissue repair
  - Hemostasis phase
  - Inflammatory phase
  - Proliferative phase
  - Remodeling phase
- Cutaneous wound healing and tissue repair
  - Cutaneous wound healing and tissue repair by primary intention
  - Cutaneous wound healing and tissue repair by secondary intention
- Factors affecting wound healing and tissue repair
  - Local factors affecting wound healing
  - Systemic factors affecting wound healing
- Complications of wound healing and tissue repair
  - Inadequate granulation tissue
  - Excessive fibrosis
- Healing of bone fracture
  - Type of bone fractures
  - Stages of bone fracture healing
  - Clinical significance
- Regeneration of specific organs
  - Regeneration of liver
  - Regeneration of nervous system

INFLAMMATION

GENERAL FEATURES OF INFLAMMATION

Inflammation is a tissue response to cellular injury in living cells, that results in vascular changes which allow fluid and leukocytes into extravascular tissue. It involves both innate and adaptive immune responses. It localizes and eliminates the causative injurious agent, limits tissue injury and restores tissue to its normal state.

- Within minutes of tissue injury, histamine, nitric oxide and leukotrienes are synthesized. Within hours other chemical mediators (e.g. IL-1, tumor necrosis factor and interferon-gamma (IFN-γ) are synthesized.
- Neutrophil is the predominant cell type of acute inflammation, which reach the injured tissues by margination, rolling, adhesion along vascular endothelium and transmigration. Neutrophils production occurs in bone marrow, which circulates in peripheral blood and migrates in tissues. **Hematopoietic stem cells (HSCs)** produce neutrophils in the bone marrow and undergo maturation in about 1–2 weeks. Majority of neutrophils are released into peripheral blood circulation in the form of segmented forms. Increased demand results in release of immature form of neutrophils. About 50% of the neutrophils circulate in the peripheral blood circulation in the marginating pool; and rest 50% of the neutrophils circulate in the circulating pool. Neutrophils spend <10 hours in the peripheral blood before marginating and reaching tissues, where they spend 1–5 days. Schematic representation of neutrophils production in bone marrow and circulation in peripheral blood migration in tissues is shown in Fig. 2.1.
- Macrophages, lymphocytes and plasma cells are demonstrated in chronic inflammation. Chronic inflammation is nonspecific in majority of cases. There is specific cause of chronic inflammation in tuberculosis, leprosy and syphilis.
- Tissue injury may induce fibrinous inflammation of coelomic cavities, tissue necrosis, and wall thickening of hollow organs. Distinguishing features of neutrophil and macrophage are given in Table 2.1.

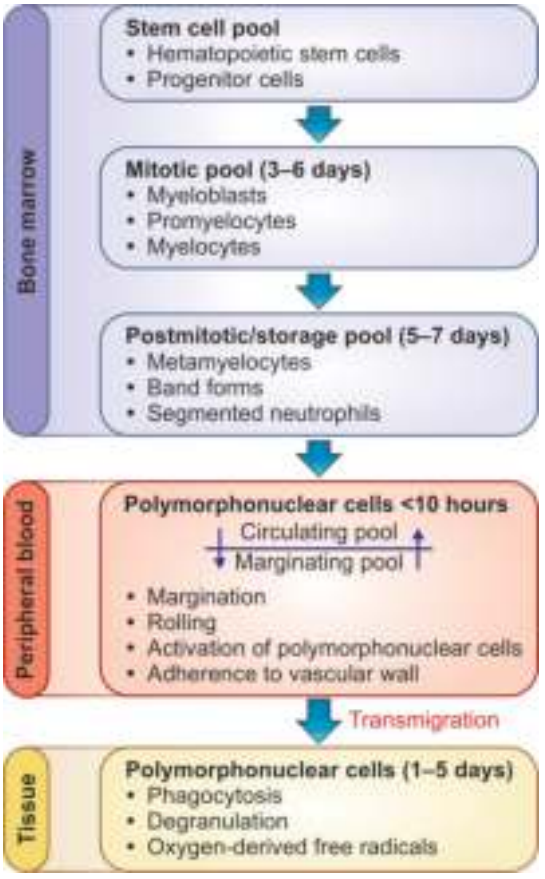


Fig. 2.1: Schematic representation of neutrophils production in bone marrow and circulation in peripheral blood migration in tissues. Hematopoietic stem cells produce neutrophils in the bone marrow and undergo maturation in about 1–2 weeks. Majority of neutrophils are released into peripheral blood circulation in the form of segmented forms. Increased demand results in release of immature form of neutrophils. About 50% of the neutrophils circulate in the peripheral blood circulation in the marginating pool; and rest 50% of the neutrophils circulate in the circulating pool. Neutrophils spend <10 hours in the peripheral blood before marginating and reaching tissues, where they spend 1–5 days.

Table 2.1 Distinguishing features of neutrophil and macrophage

Characteristics	Polymorphonuclear Cell	Macrophage
Cell morphology	Granular leukocyte comprising nucleus with three to five lobes	Macrophage is agranular leukocyte comprising large round nucleus
Antigen presenting function	Neutrophils are phagocytes without antigen presenting capability	Macrophages are antigen-presenting phagocytes to T cells or can act antigen-presenting cells
Immune response	Neutrophils are primarily involved in innate (non-specific) immune response with its phagocytic capability	Macrophages play key roles in innate (non-specific) and adaptive (specific) response by recruitment of lymphocytes

Contd...

Table 2.1 Distinguishing features of neutrophil and macrophage (*Contd...*)

Characteristics	Polymorphonuclear Cell	Macrophage
Site of production	Hematopoietic stem cells (HSCs) in bone marrow	Hematopoietic stem cells (HSCs) in bone marrow inflammation, stem cells in yolk sac or fetal liver during embryogenesis
Maturation of cell	Bone marrow	Tissues
Presence of cell	Neutrophils are normally found in the blood circulation and recruited to the site of infection or injury	Macrophages are found in all tissues
Duration in blood circulation	7–10 hours	20–40 hours (monocytes in blood circulation)
Average life span	Several days (1–-2 days)	▪ Macrophages in inflammation (days or weeks) ▪ Tissue macrophages (months to years)
Numbers in blood circulation	(2.5 × 7.5) × 10^9/L constituting 50–70% of circulating leukocytes	(0.2–0.8 × 7.5) ×10^9/L constituting 2–8% of leukocytes
Numbers in tissues	Mainly present in blood circulation but transient presence in tissues	Present in large numbers in tissues
Receptors on cell membrane	Ly6G, and MPO+ receptors	EMR1+, CD107b+ (Mac-3+) and CD68
Phagocytic function	First to kill bacteria at the site of infection by phagocytosis	Engulfs large pathogens, apoptotic cells, tissue debris, foreign particles
Principal killing mechanisms	Oxidative and nonoxidative mechanisms	Oxidative, nitric oxide, cytokines
Reactive oxygen species	Neutrophils are rapidly induced by assembly of phagocyte oxidase (respiratory burst)	Less prominent
Nitrogen oxide synthesis	Low level or none	Induced following transcriptional activation of iNOS
Cytokine synthesis level	Cytokine low level per neutrophil	Cytokine high level per macrophage, which requires transcriptional activation of cytokine genes
Secretion of lysosomal enzymes	Prominent	Less prominent
Cell activated by products	TNF-α, IFN-γ, GM-CSF, microbial products activate neutrophils	TNF-α, IFN-γ, GM-CSF, microbial products (i.e. LPS) activate macrophages
Important deficiencies	Chronic granulomatous disease, myeloperoxidase deficiency, chemotactic deficiency, and Chédiak-Higashi disease	Lipid storage diseases
Major secretory products	Lysozyme	Over 80; lysozyme, cytokines (TNF-α, IL-1)
Neutrophil extracellular trap (NET)	▪ Neutrophil extracellular traps primarily composed of DNA from neutrophils, which bind pathogens ▪ Neutrophil extracellular traps are rapidly induced by extrusion of nuclear contents	Neutrophil extracellular traps (NET) absent in macrophage
Fate of cell	Neutrophils die after phagocytosis of pathogen and immediately taken by macrophages	Macrophages are also able to engulf apoptotic neutrophils and make use of antimicrobial molecules present in their granule

GM-CSF: Granulocyte-macrophage colony-stimulating factor; IFN: Interferon; TNF: Tumor necrosis factor; LPS: Liposaccharide.

CAUSES OF INFLAMMATION

Each of the injurious stimuli may induce reactions with some distinctive characteristics, but all inflammatory reactions have the same features. When a deficit of water, oxygen, or nutrients occurs or if constant temperature and adequate waste disposal are not maintained, cellular synthesis does not take place. A lack of just one of these basic requirements can cause cell disruption or cell death.

- **Infectious agents:** Bacteria, viruses, fungi, and parasites can cause cell injury or death. Mammal's cells possess family of 'toll-like receptors' and several cytoplasmic receptors, which can detect pathogens

resulting in synthesis of chemical mediators of inflammation. These infectious organisms adversely affect cell integrity, usually by interfering with cell synthesis, producing mutant cells. Human immunodeficiency virus alters the cell, when virus is replicated in the cells.

- **Physical agents:** Mechanical injury (trauma or surgery) includes blunt or penetrating injuries (cutaneous laceration, osseous fracture). Physical injury disrupts cell's organelles (such as mitochondria, nuclei, lysosomes, and ribosomes). Thermal injury includes burns, or frostbite and irradiation.
- **Toxic agents:** Endogenous and exogenous toxic agents cause cell injury. Endogenous toxic agents include genetically determined metabolic errors, gross malformations, and hypersensitivity reactions. Exogenous toxic agents such as alcohol, lead, carbon monoxide (toxic gas), acids, alkali, drugs, chemotherapeutic agents or immunosuppressive drugs alter cellular function.
- **Foreign bodies:** Foreign bodies (sutures, splinters, dirt) elicit inflammation, because foreign bodies cause tissue injuries.
- **Immunologic reactions:** Immunologic reactions induce: (a) hypersensitivity reactions on exposure to environmental antigens such as bee sting or self-antigens, and (b) autoimmune disorders. Since these stimuli cannot be eliminated, hence such immunologic reactions tend to be persistent, and often have features of chronic inflammation, which are important cause of morbidity and mortality.
- **Endogenous causes:** Circulatory system disorders causing acute inflammation include thrombosis, acute myocardial infarction, and hemorrhage. Activation of pancreatic enzymes results in acute pancreatitis. Metabolic products such as uric acid may cause inflammatory response.

TYPE OF INFLAMMATORY RESPONSES

Inflammatory response is expressed through vascular response (arterioles, capillaries and venules) and cellular response coordinated by chemical mediators.

- Increased vascular permeability due to widened intercellular junctions and contraction of endothelial cells by chemical mediators (e.g. histamine, bradykinin and vascular endothelial growth factor) lead to extravasation of proteins, inflammatory cells and red blood cells resulting in formation of exudate. Transudate is poor in protein that occurs due to increased hydrostatic pressure.
- Macrophages and lymphocytes participate in tissue repair either by complete resolution and return of tissue to normal state or formation of scar tissue.
- Septic inflammation caused by pathogens has a protective character. Aseptic inflammation due to sterile chemical agents, radiation has a reparative character.
- Depending on the persistence and extent of tissue injury, clinical symptoms, and the nature of inflammatory response, inflammation may be acute (seconds, minutes to <48 hours), subacute (2–6 weeks) or chronic (several months to years).

ACUTE INFLAMMATORY RESPONSE

Acute inflammatory response is a short-term transient and rapid process occurring in response to tissue injury, usually appearing within seconds, minutes to <48 hours. It dilutes, neutralizes and eliminates the

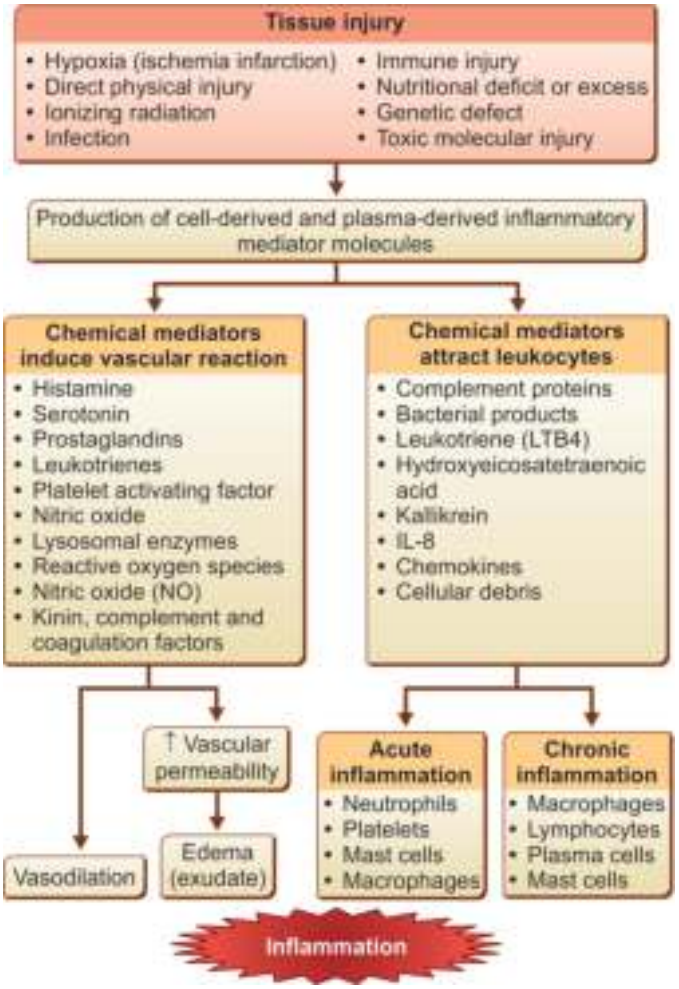


Fig. 2.2: Acute and chronic inflammatory responses. Inflammation is a normal response of the body to protect tissues from infection, injury or disease. Acute inflammation is the initial response of the body to harmful stimuli and achieved by increased movement of plasma and leukocytes (polymorphonuclear cells) from the blood into the injured tissues. Chronic inflammation is characterized by mononuclear cells infiltration, tissue destruction and healing of the tissues from inflammatory response.

injurious agents by exudation of plasma proteins, fluid along with neutrophils. Neutrophils are the dominant players of acute inflammation.

- Neutrophils clear debris and begin the process of wound healing. During mild tissue injury, removal of inflammatory exudate results in restoration of normal tissue architecture (resolution). Transition of acute inflammation to chronic inflammation occurs due to persistent injury.
- Acute inflammatory response is characterized by five **cardinal signs**: **pain**, **redness**, **immobility** (loss of function), **swelling** and **heat**.

CHRONIC INFLAMMATORY RESPONSE

Chronic inflammatory response has the potential to cause tissue destruction, fibrosis and disease, which lasts for days, weeks or years. It is associated with influx of macrophages, lymphocytes and plasma cells, proliferation of blood vessels (angiogenesis), fibrosis (role of fibroblasts, macrophages); and tissue necrosis (organ damage).

- Chronic inflammation is mediated by reactive oxygen species (ROS), hydrolytic enzymes, IFN-γ, other cytokines and growth factors.
- Macrophages and fibroblasts regulate scar tissue formation. Examples of chronic inflammation include tuberculosis, chronic sinusitis, chronic hepatitis, autoimmune diseases (i.e. ulcerative colitis, Crohn's disease).
- Acute and chronic inflammatory responses are shown in Fig. 2.2. Differences between acute and chronic inflammation are given in Table 2.2.

Table 2.2 Differences between acute and chronic inflammation

Parameters	Acute Inflammation	Chronic Inflammation
Pathogenesis of inflammation	Pathogens, trauma, burns	Progression of persistent acute inflammation, foreign bodies, tuberculosis, autoimmune diseases
Onset of inflammation	Immediate (rapid onset within seconds)	Delayed (slow onset)
Cells involved in inflammation	PMN cells	Lymphocytes and macrophages
Chemical mediators	Histamine, prostaglandins, thromboxane A_2 (TXA_2), prostacyclin, leukotrienes, lipoxins	Cytokines (IL-1), growth factors
Immunoglobulin	IgM	IgG
Duration of inflammation	Shorter (lasting for few hours to days)	Longer duration (lasting for weeks, months, or years)
Exudation in inflammation	Present	Absent
Tissue necrosis	Present	Less prominent
Tissue fibrosis	Absent	Present
Angiogenesis	Absent	Present
Outcome of inflammation	Complete resolution, tissue destruction (abscess), progression to chronic inflammation	Scar tissue formation, secondary amyloidosis
Peripheral blood leukocytic response	Neutrophilic leukocytosis	Lymphocytosis or monocytosis

ACUTE INFLAMMATION

Acute inflammation is short-term transient, rapid response to living tissue injury. It is characterized by hyperemia (rubor), pain (dolor), heat (color) and edema/swelling. Predominant cells in acute inflammation are polymorphonuclear cells. Macrophages and mast cells also play important role in acute inflammation. Acute inflammation is a series of processes initiated to limit tissue damage.

- The main features of acute inflammation are vasodilatation, increased vascular permeability and exudation into extravascular tissues, intravascular stasis and leukocyte margination, intravascular activation of polymorphonuclear cells, and activation of platelets.
- During inflammation, central stream of cells (e.g. leukocytes and red blood cells) move to peripheral

cell-free layer of plasma close to vessel wall. This phenomenon is known as margination. Neutrophils of the central column come close to the vessel wall; the phenomenon is known as **pavementing**. Peripherally marginated neutrophils slowly roll over (rolling phase).
  - Transient bond between neutrophils and endothelial cells becomes firmer (adhesion phase). Selectins, integrins and immunoglobulin superfamily adhesion molecules bring about rolling and adhesion of neutrophils over endothelium.
  - Escape of neutrophils with the help of pseudopodia across basement membrane into the extravascular space is known as emigration.
  - After extravasation from the blood, neutrophils migrate toward sites of infection by chemotactic molecules, phenomenon is known as chemotaxis.
- Vasodilatation and increased permeability in acute inflammation are caused by inflammatory chemical mediators. These vascular events participate in delivery of leukocytes (PMNs cells) and plasma proteins to the site of injury.
- Acute inflammation is mediated by chemical mediators such as histamine, nitric oxide, bradykinin, interleukin-1, tumor necrosis factor and interferon-γ, which act on nearby blood vessels.
  - Vasoactive amines (histamine and serotonin) are the first chemical mediators to be released in acute inflammation because they are present in preformed stores in mast cells, basophils, and/or platelets.
  - Resident tissue macrophages recognize inflammatory inducers such as bacterial products, immune complexes, toxins, physical injury, or dead cells and attempt to eliminate them. Macrophages synthesize master cytokines such as IL-1 and TNF-α.
- Chemical mediators are derived from plasma proteins, tissue macrophages, mast cells, platelets and endothelial cells, local injured tissue, and bacterial products. These chemical mediators increase the vascular permeability, with exudation of water, salts and plasma proteins including fibrinogen along with numerous polymorphonuclear cells to eliminate injurious agent.
- Inflammatory inducers, i.e. bacterial products immune complexes, toxins, physical injury activate tissue macrophages resulting in synthesis of cytokines (IL-1 and TNF-α), which activate neutrophils and venular endothelium.
- Glucocorticoids are broad spectrum of powerful anti-inflammatory agents, which downregulate expression of genes encoding proinflammatory cytokines (IL-1 and TNF-α) and nitric oxide synthase. Long-term use of corticosteroids is associated with many side effects including reduced bone densities (osteoporosis), diabetes mellitus, hypertension and cataracts. The prolonged administration of corticosteroids is, therefore, carefully regulated the lowest possible effective dose is prescribed.
- During acute inflammation, neutrophils are recruited at injured site, which eliminate injurious agent, removes cellular debris from injured area. Inflammatory exudates dilute or neutralize the injurious agent. Acute inflammation prevents the spread of infection followed by resolution. Without inflammation, infections would go unchecked and wound would never heal.
- Tissue damage occurs due to liberation of lysosomal enzymes into the extracellular tissue, especially due to prolonged unregulated inflammatory response in chronic inflammation and hypersensitivity reactions. Tissue damage may also occur in space occupying lesions in brain compressing vital structures.
- Acute inflammation has two major components: vascular and cellular responses. Vascular and cellular events in acute inflammation are shown in Fig. 2.3.
  - **Vascular response:** Vascular response depends on nature and severity of inflammatory stimulus, leading to alterations in vascular blood flow and structural changes in the microvasculature (vasodilatation and increased vascular permeability). These vascular changes result in extravasation of plasma fluid and proteins (edema) along with leukocytes to the site of injury.
  - **Cellular response:** In cellular response, inflammatory cells are recruited from circulation, which migrates to the injurious tissue by margination, rolling, adhesion, transmigration to initiate inflammation and eliminate injurious stimulus. Chemotaxis occurs due to endogenous signaling molecules such as lymphokines and exogenous toxins. Neutrophils are dominant cells in acute inflammation during 1–2 days, which phagocytose and degrade injurious agent by lysosomal enzymes, oxygen-derived free radicals and oxidative burst. Macrophages and mast cells play important role in acute inflammation.

VASCULAR EVENTS IN ACUTE INFLAMMATION

Alteration in the microvasculature is the hallmark of acute inflammation that occurs in two phases: hemodynamic changes and alterations in vascular permeability.
- Normally, most of the capillary bed is closed down by precapillary sphincters. In acute inflammation,

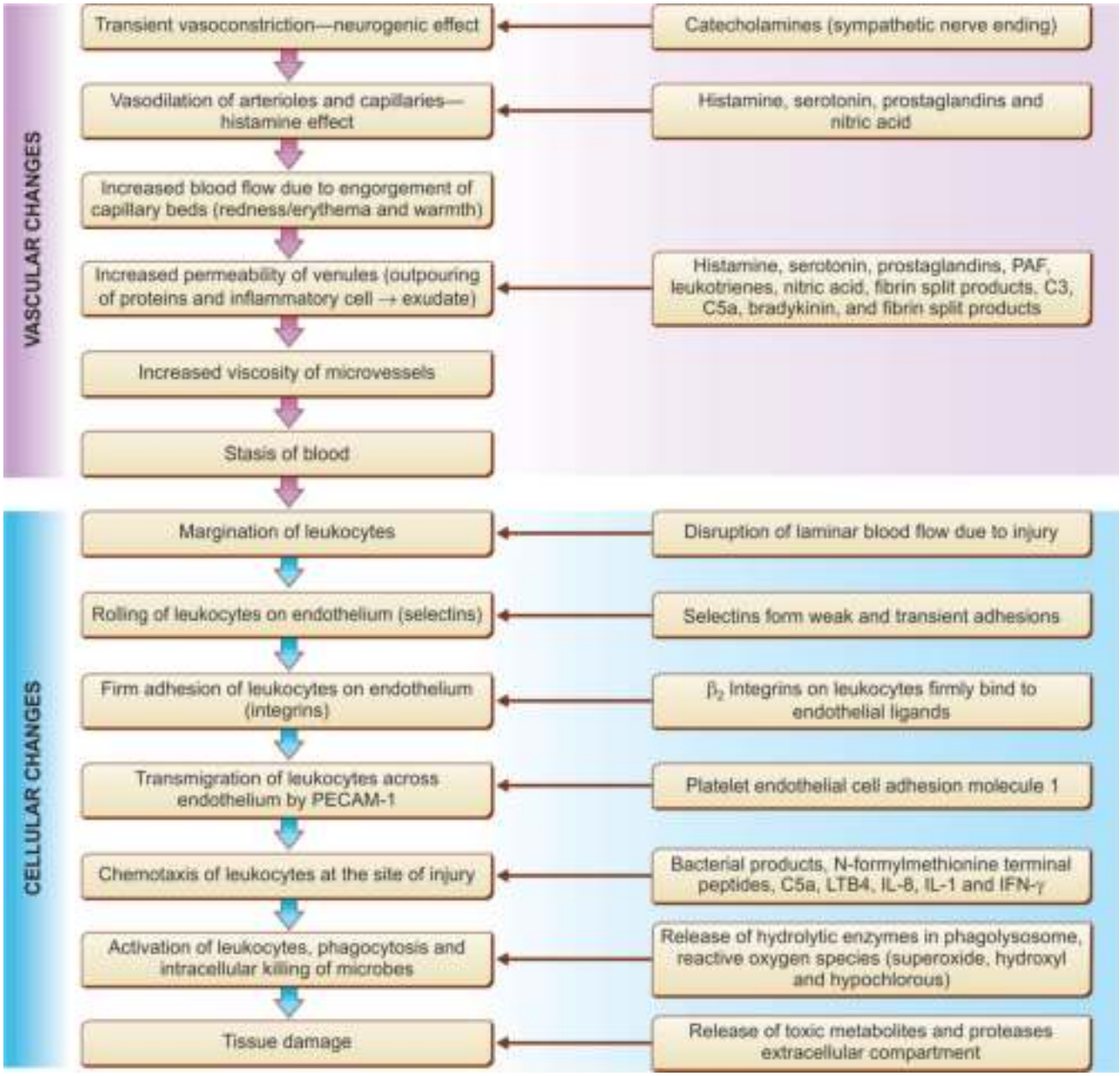


Fig. 2.3: Vascular and cellular events in acute inflammation. Vascular phase is characterized by vasodilatation and increased vascular permeability of the vascular barrier and the process is regulated by chemical mediators. Cellular phase is characterized by neutrophil margination, rolling, adhesion, and emigration to the injured site.

precapillary sphincters open, which cause blood to flow through all capillaries. During acute inflammation, vascular changes start with a transient neurogenic-mediated vasoconstriction of arterioles, which is shortly followed by vasodilatation of arterioles, capillaries, and post-capillary venules leading to increased blood flow to the injured area, which becomes red and warm.

- Under optimal conditions, the inflammatory response remains confined to a localized area (i.e. redness, swelling, heat, pain and loss of function). The four cardinal signs of acute inflammation were described nearly 2000 years ago by Celsius, which include rubor (redness), calor (heat), dolor (pain), and tumor (swelling). Virchow added functio laesa (loss of function) sign.
- Rubor, calor and tumor are mediated by histamine. These cardinal signs result from vascular changes, neutrophilic recruitment to the site of injury, synthesis of chemical mediators, and leukocyte-induced tissue injury. Acute inflammation subsides in <48 hours after elimination of injurious agents.
  - **Rubor (redness):** Inflamed area becomes red due to histamine induced vasodilatation of arterioles

and increased blood flow through all capillaries to the inflamed area in acute inflammation.

- **Calor (heat):** Inflamed area becomes hot due to increased blood flow in the injured area to skin surface, mediated by chemical mediators such as histamine, serotonin, C3a, C5a, prostaglandins (PGI_2, PGD_2, PGE_2, and PGF_2).
- **Dolor (pain):** Certain chemical mediators stimulate sensory nerve endings giving pain. Nerves are also stimulated by stretching due to edema. The inflamed area becomes painful, when touched. Pain occurs due to pressure on nerve endings from swelling; and direct effect of prostaglandin E_2 (PGE_2) and bradykinin effect on nerve endings.
- **Tumor (swelling):** Increased vascular permeability induces tissue swelling due to accumulation of blood and damaged tissue cells. Chemical mediators such as histamine, bradykinin, and prostaglandins increase vascular permeability leading to extravasation of inflammatory exudate into the interstitial tissue and edema formation.
- **Functio laesa (loss of function):** Loss of function occurs due to increased pain and swelling. When swelling and pain are marked, there is partial or complete loss of function of the inflamed structure. This clinical sign was added by Virchow in 1800s.

HEMODYNAMIC CHANGES

Vascular component in acute inflammation comes into play when tissue is damaged by infectious agents, trauma, toxic agents, endogenous product (uric acid), immunological reactions and foreign bodies (sutures, splinters, dirt). Vascular response in acute inflammation includes immediate (e.g. immediate transient and sustained) and late response. Vascular response in acute inflammation is given in Table 2.3.

Transient Vasoconstriction of Arterioles

Initially, rapid transient vasoconstriction of arterioles occurs after minor tissue injury within 30 seconds and is mediated by neurologic and liberation of catecholamines from sympathetic nerve endings. Blood flow is decreased to the site of injury and followed by vasodilatation of microvasculature.

Persistent Progressive Vasodilation of Arterioles and Capillaries

Persistent progressive vasodilation of arterioles and capillaries increases blood flow to injured site within half an hour referred to as active hyperemia responsible for redness (rubor), increased local temperature (heat = calor) and removal of toxins. It is immediate sustained microvasculature response after tissue injury. It starts 2–3 hours after injury and lasts for about 8 hours. It is mediated by chemical mediators synthesized by damaged tissue and mast cells such as histamine, serotonin, and sustained by prostaglandins (PGI_2, PGD_2, PGE_2, $PGF_{2\alpha}$) and nitric oxide.

Progressive Vasodilation of Arteriols and Capillaries and Elevated Local Hydrostatic Pressure and Transudate Formation

Permeability of vascular endothelium is usually normal, but alterations in hydrostatic pressure or oncotic pressure leading to formation of transudate. Normal fluid exchange between blood and extracellular fluid is shown in Fig. 2.4.

- A transudate is clear extravascular ultrafiltrate of plasma with water, dissolved eletrolytes, low protein, low cellularity fluid content mostly albumin and specific gravity <1.012. Formation of transudate due to fluid exchanges between blood and extracellular fluid is shown in Fig. 2.5.

Table 2.3 Vascular response in acute inflammation

Vascular Response Mediated by Neurologic/ Chemical Mediators	Net Effect
Transient vasoconstriction of microvasculature	
Transient vasoconstriction of microvasculature by neurologic mechanism by liberation of catecholamines from sympathetic nerve endings	Blood flow decreased
Vasodilatation of arterioles and capillaries	
▪ Histamine ▪ Serotonin ▪ Vasodilatation is sustained by prostaglandins (PGI_2, PGD_2, PGE_2, $PGF_{2\alpha}$) and nitric oxide	Blood flow increased (redness/erythema and warmth)
Increased vascular permeability of venules	
▪ Histamine ▪ Serotonin ▪ Bradykinin ▪ C3a, C5a ▪ Fibrin split products ▪ Prostaglandins ▪ Leukotrienes ▪ Platelet-activating factor (PAF) ▪ Nitric oxide (NO)	Inflammatory exudate and increased viscosity of blood

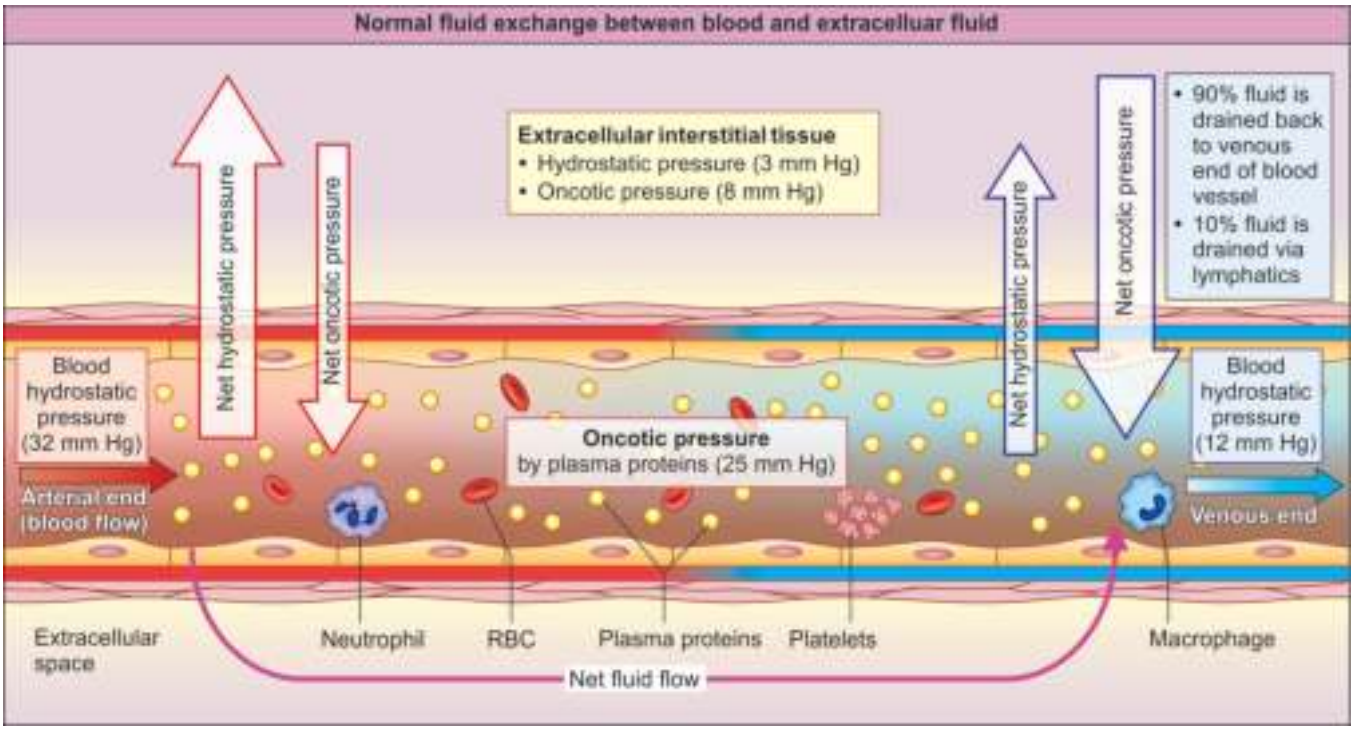


Fig. 2.4: Normal fluid exchange between blood and extracellular fluid. Normal hydrostatic pressure at arterial end of capillary is 32 mm Hg and 12 mm at venous end therefore fluid leaves blood vessel. Oncotic pressure exerted by plasma proteins is 25 mm Hg. At venous end, hydrostatic pressure is less than plasma oncotic pressure, so fluid enters blood vessel. Approximately 90% of fluid is drained back to capillary, while 10% of excess interstitial fluid is drained by lymphatic channels to venous circulation.

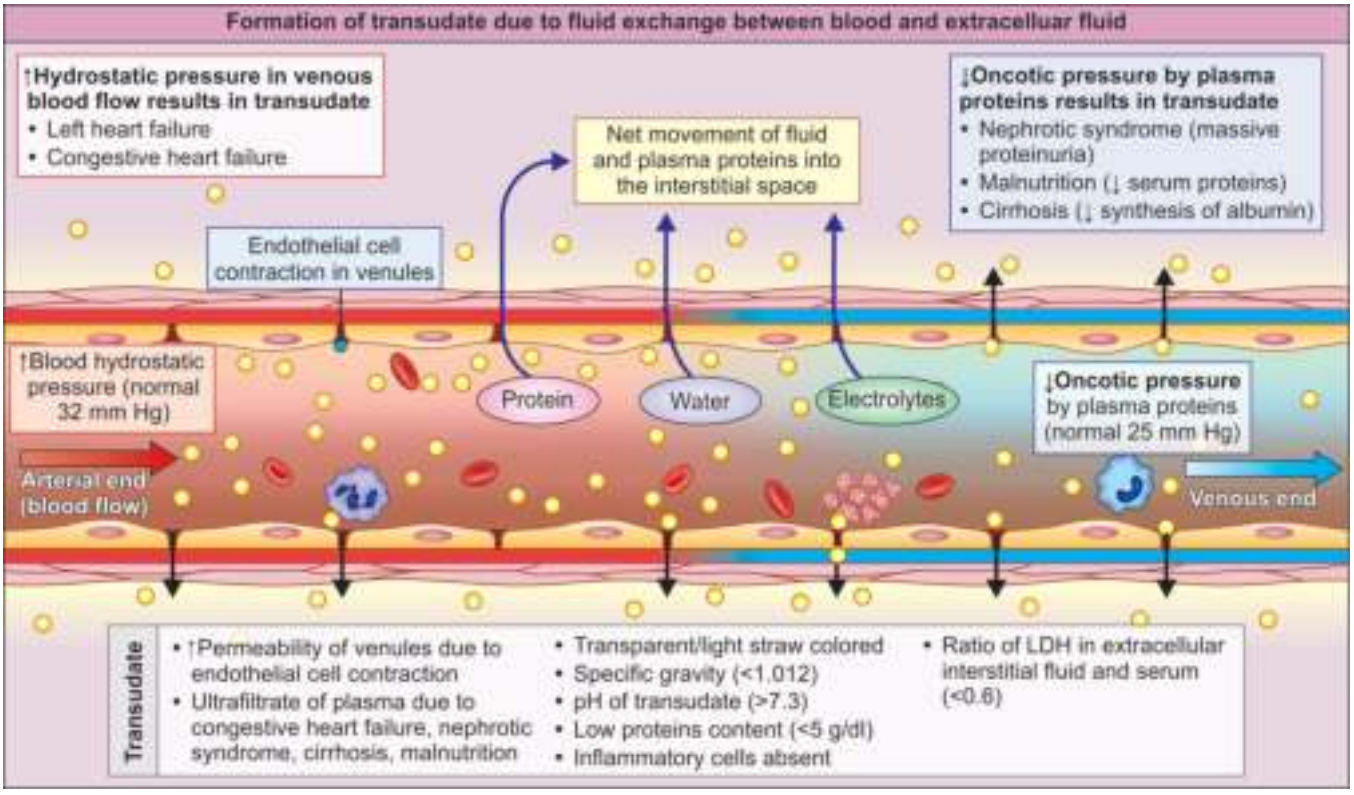


Fig. 2.5: Formation of transudate due to fluid exchanges between blood and extracellular fluid. Transudate is formed as a result of increased hydrostatic pressure or decreased plasma oncotic pressure. Therefore, fluid leaves microvasculature along whole length with no reabsorption. There is low protein loss, so this is a transudate.

- Transudate implies a problem due to alteration of hydrostatic pressure. Transudate is formed when fluid escapes from the normal endothelium of microvasculature. It occurs in non-inflammatory conditions.
- Transudate formation occurs either due to increased hydrostatic pressure (congestive heart failure) or decreased oncotic pressure (decreased protein synthesis in liver diseases, malnutrition, proteins loss in kidney diseases such as nephritic or nephrotic syndrome), and lymphatics obstruction (filariasis). Woman with breast cancer develops edema over arm, which has undergone mastectomy along with removal of lymph nodes and lymphatic channels.

Slowing or Stasis of Blood and Increased Blood Viscosity

Escape of fluid from microvasculature into the interstitial tissue increases the concentration of the red blood cells and blood viscosity leading to slowing of blood flow (stasis). The small vessels are dilated and congested. Leukocytes principally neutrophils now begin to accumulate along the endothelial surface, a process is called 'margination'.

INCREASED VASCULAR PERMEABILITY OF POSTCAPILLARY VENULES

Increased vascular permeability is the hallmark of acute inflammation that affects postcapillary venules. Increased vascular permeability is caused by chemical mediators, bacterial toxins, chemical agents, physical agents, severe burns, thermal injuries, X-rays, ultraviolet rays, leukocyte-induced endothelial injury, widening of venular interendothelial cells and leakage from newly formed vessels.

- Under physiologic state, normal venules are sealed by tight junctions between adjacent endothelial cells. Fluid leaving and entering blood vessels is in equilibrium.
- Increased vascular permeability occurs due to various chemical mediators derived from plasma (fibrin split products, bradykinin, C3a, C5a) and cells (histamine, serotonin, prostaglandins, leukotrienes, platelet-activating factor, nitric oxide).
- Due to widening of interendothelial junctions of postcapillary venules, there will be outside movement of the blood fluid rich in plasma proteins and inflammatory cells from the blood vessels to the site of injury and exudate formation.

Mechanism of Increased Vascular Permeability of Postcapillary Venules

Increased vascular permeability of postcapillary venules is mediated by chemical mediators, bacterial toxins, chemical agents, physical agents, severe burns, thermal injury, X-rays and ultraviolet rays, leukocytes induced, vascular endothelial growth factor and leakage from newly formed blood vessels. Chemical mediators responsible for increased vascular permeability are given in Table 2.4.

Table 2.4 Chemical mediators responsible for increased vascular permeability

Plasma and Cell-derived Components	Chemical Mediators
Plasma-derived chemical mediators	
Coagulation/fibrinolytic systems	Fibrin split products
Kallikrein-kinin system	Kinins (bradykinin)
Complement system	C3a, C5a
Cell-derived chemical mediators	
Mast cells, platelets	Histamine
Platelets	Serotonin
Inflammatory cells	Prostaglandins, leukotrienes, platelet-activating factor
Endothelium	Prostaglandins, nitric oxide, platelet-activating factor

- **Chemical mediators:** Histamine (mast cells, platelets, basophils), serotonin, prostaglandins, complement (C3a, C5a), fibrin split products, prostaglandins, leukotrienes, platelet-activating factor and nitric oxide increase permeability of postcapillary venules resulting in formation of exudate and increased viscosity of blood. Action of chemical mediators on postcapillary venules lasts for 15–20 minutes. Histamine binds and induces intracellular signaling pathway by phosphorylation of contractile myosin cytoskeleton proteins leading to increased vascular permeability.
- **Bacterial toxins, chemical agents, physical agents, and severe burns:** These injurious agents cause necrosis of endothelium of microvasculature. It leads to prolonged vascular leakage of fluid elements into interstitial tissue persisting for several hours to days until the damaged endothelium is thrombosed or repaired.
- **Thermal injury, X-rays and ultraviolet rays:** Delayed prolonged vascular leakage starts 2–12 hours after injury and lasts for several hours or days due to mild to moderate thermal injury, X-rays, and ultraviolet rays.
- **Leukocyte-dependent endothelial injury:** In acute inflammation, leukocytes liberate oxygen-derived free radicals and proteolytic enzymes, which cause detachment of venular endothelial cells leading

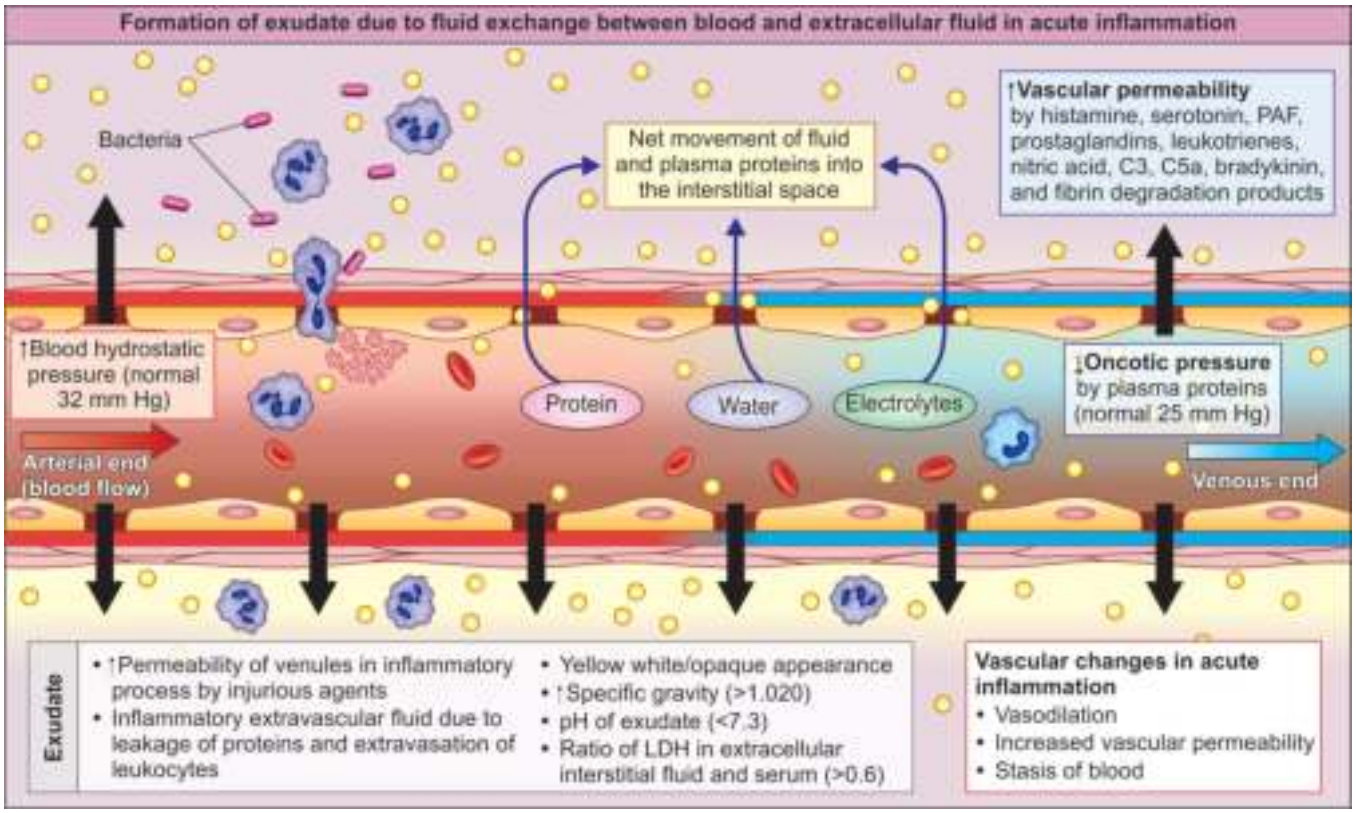


Fig. 2.6: Formation of exudate due to fluid exchange between blood and extracellular fluid in acute inflammation. An exudate is formed due to increased vascular permeability. Protein leaks out of microvasculature, so that there is no osmotic pressure difference between plasma and tissue (plasma oncotic pressure=0), therefore fluid leaves microvasculature. Fluid contains proteins, so this is an exudate.

to increased permeability. It is a delayed vascular response, which persists for hours. Glomeruli and pulmonary capillaries are also injured by this mechanism.

- **Vascular endothelial growth factor (VEGF) related increased vascular permeability:** Vascular endothelial growth factor acts on vesiculovacuolar organelles located close to intercellular junction of venules increase the size and number of these channels leading to increased vascular permeability.
- **Leakage from newly formed vessels:** New vessels formed are responsible for persistent leakage of fluid. Increased expression of receptors for histamine and VEGF further increases vascular permeability.

EXUDATE FORMATION

Exudate formation is a consequence of altered endothelial permeability of postcapillary venules that results in extravasation of plasma proteins and neutrophils into interstitial space. Formation of exudate due to fluid exchange between blood and extracellular fluid in acute inflammation is shown in Fig. 2.6. Acute inflammatory exudate in tissue is shown in Fig. 2.7.

- Starling forces are altered so that there is increased flow of fluid from the vascular system into the interstitium. There are four pressures involved in Starling's law: hydrostatic pressure in the capillary, hydrostatic pressure in the interstitium, oncotic pressure in the capillary and oncotic pressure in the interstitium.

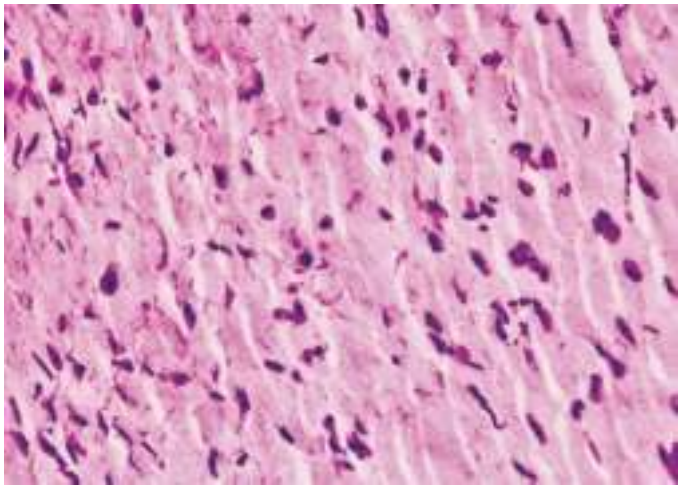


Fig. 2.7: Acute inflammatory exudate in tissue. Exudate is inflammatory in nature that occurs due to vascular permeability caused by the release of inflammatory chemical mediators from the inflamed tissue. Neutrophils are predominant cell type in most exudates, along with some macrophages (400X).

- Normal hydrostatic pressure in capillaries at arterial end is 32 mm Hg and venous end is 12 mm Hg. Osmotic pressure exerted by plasma proteins is 25 mm Hg in capillaries. Due to pressure gradient, hydrostatic blood pressure forces water out of capillaries at the arterial end. At the venous end, 90% of extravasated fluid is drained back in capillaries at venous end due to oncotic pressure exerted by plasma albumin. Remaining 10% fluid in interstitial tissue is drained via lymphatic channels into circulation. When the balance of starling forces is altered, the net result is accumulation of fluid in the interstitial spaces (i.e. edema).
- During acute inflammation, lymph flow is increased, which helps to remove edema fluid from extravascular fluid along with cell debris and microbes. Severe inflammation results in inflammation of lymphatic channels and lymph nodes. There is presence of red streaks near a skin wound due to lymphangitis.

Mechanism of Exudation

Exudation occurs as a result of several mechanisms: endothelial contraction, cytoskeleton reorganization, direct injury to blood vessels, leukocyte-dependent injury and increased transcytosis.

- **Endothelial contraction:** Endothelial cell contraction develops temporary gaps in venules and vascular leakiness, which is mediated by vasoactive chemical mediators (histamine, leukotrienes). These vascular changes occur within 15–30 minutes immediately after injury and persists for short duration.
- **Cytoskeleton reorganization in endothelium:** Reversible endothelial cell retraction occurs at the intercellular junctions due to structural reorganization of cytoskeleton of endothelial cells. The process is mediated by cytokines IL-1 and TNF-α.
- **Direct injury to endothelium**: Chemical agents, severe bacterial infections, radiation and thermal injury induce necrosis of endothelial cells in arterioles, capillaries and venules. Direct injury to endothelium initiates thrombus formation. The process appears immediately after injury and persists for hours to days.
- **Leukocyte-dependent endothelial injury:** In bacterial infections, leukocytes adhere to the endothelium at the site of inflammation. Activation of the leukocytes releases proteolytic enzymes and reactive oxygen species, which cause endothelial injury and increased vascular leakiness. The process mostly affects venules and pulmonary capillaries. Injury to endothelium initiates thrombus formation. The process persists for several hours to 2–12 hours.
- **Increased transcytosis (channels across endothelial cytoplasm):** It is mediated by vascular endothelial growth factor (VEGF) at the site of angiogenesis. The vessels become leaky, which allows plasma proteins, such as fibrin, to be delivered directly to the injury site.

Composition of Exudate

An exudate has a high protein content due to increased vascular permeability of postcapillary venules during acute inflammatory process. Exudate is rich in fibrinogen/fibrin, immunoglobulins, salts, water and neutrophils. Specific gravity is >1020 in exudate. Because the metabolically active leukocytes consume glucose, the glucose content in blood is often greatly reduced.

Exudation and Clinical Correlation

Acute inflammation brings chemical mediators of inflammation to injured site, which act on nearby blood vessels. It is responsible for swelling (edema), pain, and impaired function. Exudate formed in severe burns is life-threatening. Fibrin formation in the injured area aids in localizing the spread of infectious microorganisms. Exudate dilutes bacterial toxins and provide opsonins (IgG, C3b) to assist in phagocytosis. Differences between exudate and transudate are given in Table 2.5.

CELLULAR EVENTS: LEUKOCYTES RECRUITMENT TO THE SITES OF INFLAMMATION

The cellular response in acute inflammation is a multistep process, marked by recruitment of phagocytic cells (e.g. neutrophils and monocytes/macrophages) into extravascular tissue to the site of injury. Steps of leukocyte extravasation include: margination, rolling, and adhesion to endothelium, transmigration across endothelium (leukocyte diapedesis) and migration toward a chemotactic stimulus in tissues. Cellular changes in acute inflammation are given in Table 2.6.

- Neutrophils are the primary leukocytes in acute inflammation, i.e. *Staphylococcus aureus* infection. Macrophages and mast cells also play important role in acute inflammation. On the other hand, macrophages, lymphocytes and plasma cells participate in chronic inflammation.
- The leukocytes first roll, then become activated and adhere to endothelium, then transmigrate across the endothelium, pierce the basement membrane, and migrate to site of injury by chemotaxis. **PECAM-1 (platelet endothelial cell adhesion molecule 1)** synthesized by interendothelial junctions and platelets participate in transmigration of leukocytes.

Table 2.5 Differences between exudate and transudate

Characteristics	Exudate	Transudate
Mechanism	Increased permeability of postcapillary venules in inflammation	Increased hydrostatic pressure or decreased oncotic pressure
Causes	Acute inflammation	Congestive heart failure, hypoproteinemia (malnutrition), nephrotic syndrome (protein loss)
Endothelial permeability	Altered endothelial permeability	Normal endothelial permeability
Fluid nature	Inflammatory extravascular fluid	Ultrafiltrate of blood plasma
Composition	Mainly protein and cellular debris	Mainly water and dissolved electrolytes
Color	Yellowish-white opaque appearance	Transparent/light straw colored
pH	<7.3	>7.3
Specific gravity	>1.020 (due to high proteins content in exudate)	<1.012
Proteins content	High protein content (which raises specific gravity)	Low protein fluid (<5 g/dl)
Glucose concentration	Glucose concentration is low in infections and cancer	Glucose concentration remains same as that of blood
Ratio of LDH levels in fluids and serum	High ratio (>0.6)	Low ratio (<0.6)
Light microscopy	PMN cells in acute inflammation, macrophages and lymphocytes in chronic inflammation	Inflammatory cells absent

Table 2.6 Cellular changes in acute inflammation

Multistep Process	Role
Margination of leukocytes	
Localization of leukocytes from central axial column along the vascular endothelium	Disruption of laminar blood flow due to injury
Rolling of leukocytes	
Rolling (or tumbling) along the vascular endothelium	Selectins form weak and transient adhesions
Firm adhesion of leukocytes	
Leukocytes bind firmly to endothelium	β_2 Integrin on leukocytes firmly bind to endothelial ligands
Transmigration (diapedesis) of leukocytes	
Movement of leukocytes across venular endothelium	PECAM-1 (platelet endothelial cell adhesion molecule 1)
Chemotaxis of leukocytes	
Recruitment of leukocytes at the site of tissue injury site known as chemotaxis	C5a, bacterial products, N-formyl methionine terminal peptides, leukotrienes (LTB4,) hydroxyeicosatetraenoic acid, kallikrein and IL-8, IL-1 and IFN-γ; and cellular debris
Phagocytosis by leukocytes	
Phagocytes (neutrophils and macrophages) eliminate microbes and cellular debris by process of phagocytosis	Opsonization, binding, engulfment, phagolysosome formation, release of hydrolytic enzymes in phagolysosome
Intracellular killing of microbes of leukocytes	
Killing and degradation of microbes in neutrophils	▪ Oxygen-dependent [superoxide, highly reactive hydroxyl molecule, hypochlorous (HOCl)] free radical ▪ Oxygen-independent system (lactoferrin, defensins, bactericidal permeability increasing protein, lysosomal enzyme and major basic proteins)

- Chemoattractants, i.e. bacterial products (peptides with N-formyl methionine termini), plasma proteins (C5a), cells (IL8, LTB4), and cellular debris attract neutrophils to the site of tissue injury. Neutrophils play important role in elimination of injurious agents.
- Selectins are sugar-binding glycoproteins that mediate the initial loose transient adhesion of leukocytes to endothelial cells in acute inflammation. These are found at the cell surface and are not part of the extracellular matrix.

- Selectin family is comprising E-selectins (endothelial cells), P-selectins (platelets and endothelial cells) and L-selectins (leukocytes).
- Leukocytes with the help of their Sialyl-Lewis X (oligosaccharides) bind to E- and P-selectins expressed on vascular endothelial cells that mediate the initial adhesion of leukocytes (primarily neutrophils) to endothelial cells in acute inflammation. P-selectin is a cell adhesion molecule that mediates the margination of neutrophils during acute inflammation.

■ Integrins are a family of transmembrane cell surface receptors that mediate interactions with extracellular matrix components and with other cells. These cell surface glycoproteins transmit mechanical and chemical signals, thereby regulating cellular survival, proliferation, differentiation, and migration.
- The motility of epithelial cells is also regulated by integrin receptors. During inflammation, β_2 integrins form firm adhesion of leukocytes to immunoglobulin family adhesion proteins expressed on vascular endothelium, which are involved in leukocyte recruitment to the injury site in acute inflammation.
- The locomotion of leukocytes is powered by membrane extensions called lamellipodia. It is worth mentioning that slower moving cells, such as fibroblasts, extend finger-like membrane protrusions called filopodia.
- Integrins expressed on various inflammatory cells include lymphocyte function-associated antigen 1 (LFA-1), macrophage antigen 1 (MAC-1), and very late antigen 4 (VLA-4). These bind to Ig family adhesion proteins expressed on endothelium.

■ Emigration is escape of inflammatory leukocytes between endothelial cells into surrounding interstitial tissue, which occurs by margination, rolling, firm adhesion, and transmigration processes. Mechanism of leukocyte emigration is shown in Fig. 2.8.

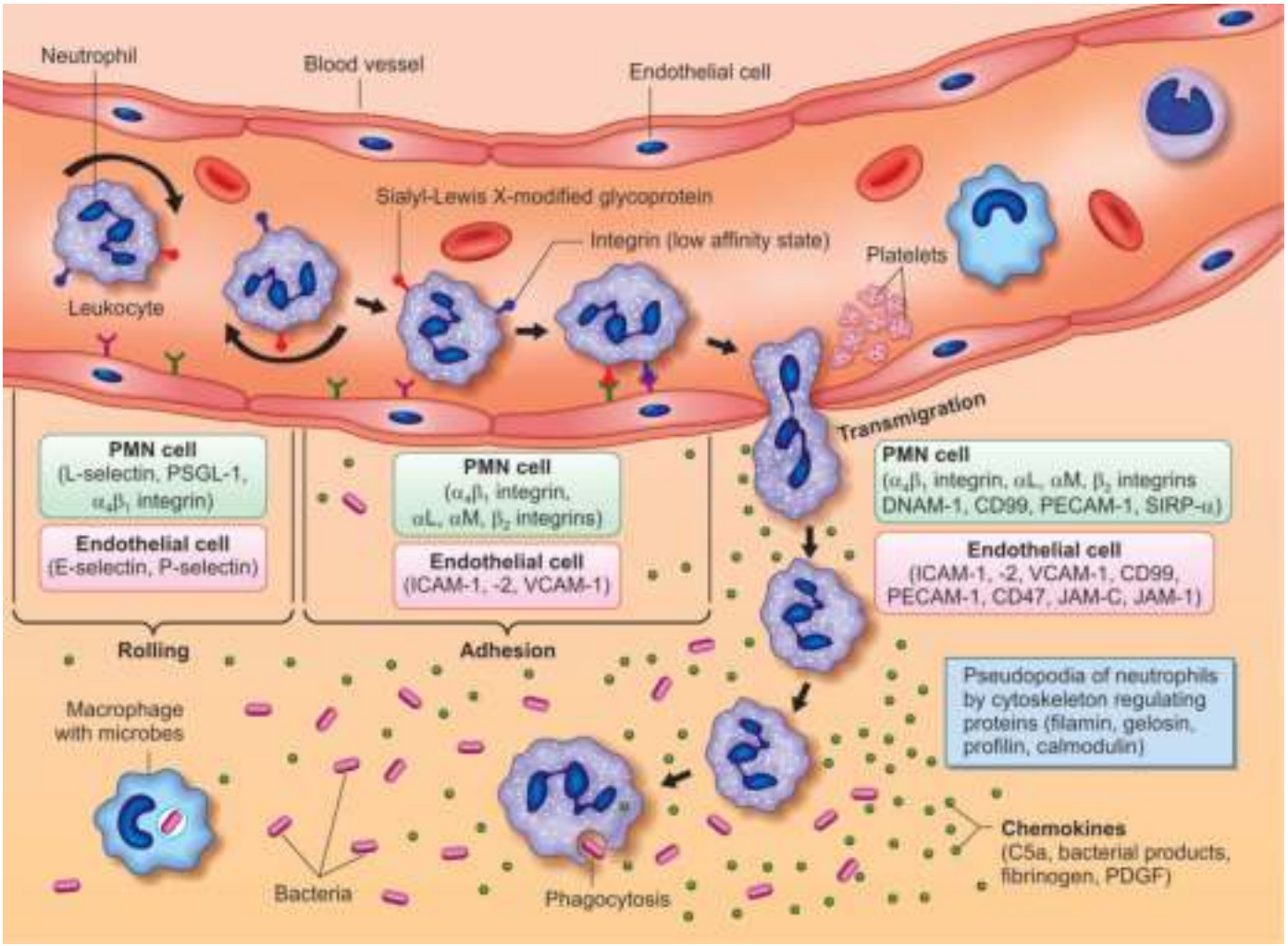


Fig. 2.8: Mechanism of leukocyte emigration. It involves rolling, adhesion, transmigration, chemotaxis and phagocytosis of injurious agent. Rolling is mediated by selectins, firm adhesion by β integrin, and transmigration.

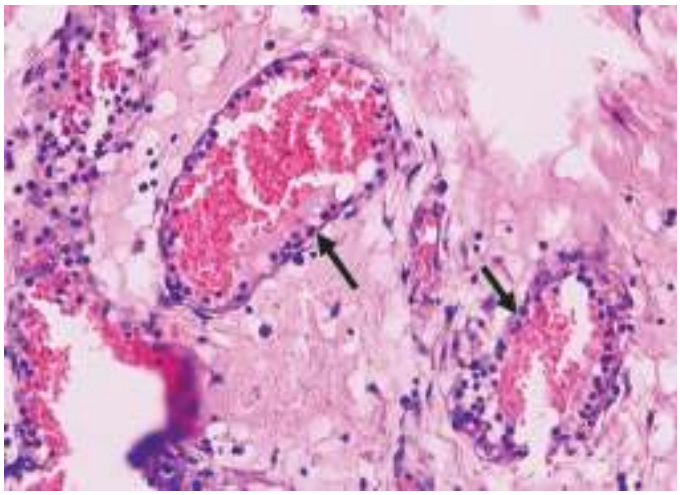


Fig. 2.9: Margination of leukocytes in microvessel. Leukocytes, in contrast to red blood cells fall out of the axial stream in the blood vessel and their attachment to the endothelial cells in the peripheral stream is under the influence of selectins. Margination is the first step in the firm adhesion of leukocytes to the endothelium (arrows) (100X).

MARGINATION OF LEUKOCYTES

Margination occurs due to localization of leukocytes from central axial column along the vascular endothelium. Normally, 50% of neutrophils travel in laminar flow, while remaining 50% are circulating along venular endothelial cells (marginating zone). Red blood cells being smaller in size move faster than large sized white blood cells.

- During acute inflammation, release of chemical mediators (i.e. histamine, leukotrienes and kinins) and cytokines act on endothelial cells of capillaries and leukocytes leading to increased expression of adhesion molecules and slowing down of leukocytes in blood circulation.
- Margination (pavementing) occurs as all leukocytes line the endothelial surface, due to activating and deactivating neutrophilic adhesive molecules. RBCs also aggregate into rouleaux formation ("stacks of coins") in venules. Margination of leukocytes in microvessel is shown in Fig. 2.9.

ROLLING OF LEUKOCYTES

After margination, the leukocytes start rolling (or tumbling) along the vascular endothelium by forming weak and transient adhesions mediated by 'selectins' family of adhesive molecules. Selectin family consists of three types of structurally related selectins.

- E-selectin is found on endothelial cells. P-selectin is expressed on platelets and endothelial cells. L-selectin is present on leukocytes except T cells.
- E-selectin and P-selectin expressed on endothelial cells bind to oligosaccharides such as Sialyl-Lewis X on the surface of leukocytes.
- Selectins mediate the initial adhesion of leukocytes (primarily neutrophils) to endothelial cells in acute inflammation.

Pathology Pearls: Regulation of Selectin Family
E-Selectin (CD62E)
▪ E-selectin (CD62E) is stored in Weibel-Palade bodies of resting endothelial cells. ▪ E-selectin is redistributed along the luminal surface of the endothelial cells, where they mediate the initial adhesion and rolling of leukocytes.
P-Selectin (CD62P)
P-selectin (CD62P) is stored in endothelial Weibel-Palade bodies and platelet α-granules; relocate to the plasma membrane after stimulation by mediators such as histamine and thrombin.
L-Selectin (CD62L)
L-selectin (CD62L) is expressed on all leukocytes except T cells, that bind to endothelial mucin-like molecules such as GlyCam-1.

FIRM ADHESION OF LEUKOCYTES

During inflammation, β_2 integrins expressed over leukocytes firmly bind to ligands expressed on venular endothelium. Normally, β_2 integrins (LFA-1, MAC-1, and VLA-4) are expressed in low concentration on leukocytes. Hence, leukocytes do not adhere firmly to their appropriate ligands endothelial surface, unless activated by chemokines (C5a, LTB_4 and bacterial endotoxins) during acute inflammation. Leukocytic activation leads to increased concentration and conformational changes of β_2 integrins on their surface. Corticosteroids, lithium and catecholamines inhibit activation of adhesion molecules.

- Chemical mediators (chemokines, IL-1 and TNF-α) increase the expression of ligands ICAM (intercellular adhesion molecule) and VCAM (vascular cell adhesive molecule) over vascular endothelium. VCAM also participates in adhesion of eosinophils, monocytes, lymphocytes to venular endothelium.
- Leukocyte β_2 integrin receptor binds to ligands expressed over venular endothelium. During acute inflammation, binding of integrin to heparin sulfate glycosaminoglycans inhibits rolling of leukocytes. Net result is formation of firm adhesion of leukocytes to endothelial surface.
- Endothelial–leukocyte adhesion molecules are shown in Fig. 2.10. Major leukocyte–endothelial cell adhesion molecules are given in Table 2.7. Endothelial/leukocyte adhesion molecules and their major roles are given in Table 2.8.

Clinical Pearls: Defects in Firm Adhesion of Leukocytes to Venular Endothelium

Leukocytic adhesion to venular endothelium is impaired in leukocyte adhesion (LAD1, LAD-2, LAD3) deficiency, diabetes mellitus and patient on steroid therapy and hemodialysis.

Leukocyte Adhesion Type 1 (LAD-1) Deficiency

- Leukocyte adhesion type 1 deficiency occurs due to mutations of ITGB2 gene that causes deficiency of β_2 integrins (LFA-1 and MAC-1) expressed on leukocytes. It impairs adhesion of leukocytes to venular endothelium.
- Patients suffer from recurrent severe bacterial and fungal infections of skin and mucous membranes, gingivitis, peritonitis, tooth loss, delayed wound healing and delayed detachment of umbilical cord in newborns along with infection of the umbilical cord stump (omphalitis).

Leukocyte Adhesion Type 2 (LAD-2) Deficiency

- Leukocyte adhesion type 2 deficiency occurs due to mutations of SLC35C1 gene that causes mild disorder. Infants with LAD-2 develop pneumonia, recurrent bacterial infections such as otitis media, peritonitis, cellulitis. The infections are not life-threatening.
- As affected infant grows older, severe peritonitis is the main infectious complication. Unlike LAD type 1, deficiency infants with LAD type 2 deficiency do not experience a delay in the detachment of the umbilical cord. LAD type 2 deficiency can develop a unique Bombay blood group.
- LAD type 2 deficiency can develop additional features such as diminished muscle tone resulting in hypotonia, distinctive facial features, severe mental retardation and short stature.
- LAD type 2 deficiency may also be known as congenital disorder of glycosylation type 2C due to basic defect in fucose metabolism.

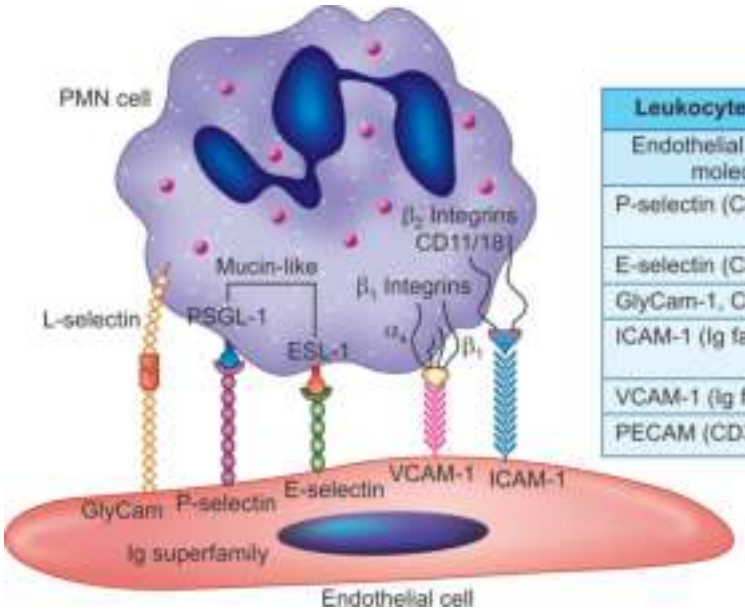
Leukocyte Adhesion Type 3 (LAD-3) Deficiency

- Leukocyte adhesion type 3 (LAD-3) deficiency occurs due to gene mutation in Kindlin-3 encoding a protein essential for all integrins activation. Patients with LAD type 3 deficiency develop recurrent bacterial and fungal infections similar to seen in LAD type 1 deficiency.
- LAD type 3 deficiency can develop life-threatening bleeding complication that resembles a platelet disorder, Glanzmann thrombasthenia. Patient presents with profuse bleeding tendencies especially after surgical procedures, epistaxis, gingival bleeding and /or red- or purple-colored spots on skin. The bleeding tendency usually starts at birth.

TRANSMIGRATION OF LEUKOCYTES

Transmigration is the movement of leukocytes across venular endothelium. It is also known as diapedesis. Transmigration is an essential step in acute inflammation and thus in host defense and healing process.

- Transmigration of leukocytes occurs in venules in all tissues except lung where it occurs via capillaries. Interendothelial junctions of postcapillary venules and platelets synthesize a chemokine known as 'platelet endothelial cell adhesion molecule 1' (PECAM-1).
- PECAM-1 stimulates adherent neutrophils to produce collagenase, elastase, and metalloproteinases leading to focal digestion of venular basement membrane.



Leukocyte and endothelial adhesion molecules

Endothelial adhesion molecule	Leukocyte adhesion molecule
P-selectin (CD62P)	Sialyl-Lewis X PSGL-1
E-selectin (CD62E)	Sialyl-Lewis X
GlyCam-1, CD4	L-selectin (CD62L)
ICAM-1 (Ig family)	Integrins (LFA-1, Mac-1) CD11/CD18
VCAM-1 (Ig family)	VLA-4 integrin
PECAM (CD31)	CD31

Fig. 2.10: Endothelial–leukocyte adhesion molecules. The adhesion of leukocytes to vascular endothelium is the hallmark of the inflammatory process. Endothelial cells express immunoglobulin-like molecules such as ICAM-1, ICAM-2, VCAM-1 and PECAM-1. Which engage leukocyte counter receptor to mediate rolling (L-selectin on leukocytes, E-selectin and P-selectin on endothelium), adhesion mediated by integrin (CD11/CD18, VLA4 on leukocytes) and/or transendothelial migration of neutrophils that occur for several hours after the initiation of inflammatory response.

- Neutrophils start inserting pseudopodia; migrate across resting endothelium into the interstitial tissue spaces towards chemotactic stimulus. Red blood cells also transmigrate along with neutrophils towards site of injury.

CHEMOTAXIS OF LEUKOCYTES

Leukocyte chemotaxis is a critical feature of innate immune response in which movement of neutrophils and macrophages from one site in the body to another site of tissue injury to provide effector functions in response to concentration gradients of extracellular signals.

- Common chemotactic agents are bacterial products, complement (especially C5a), leukotrienes and cytokines (IL-8), which cause leukocyte movement.
- Chemotaxis occurs under the influence of chemotactic mediators derived from various sources: (a) complement proteins (C5a), (b) bacteria and mitochondrial products, particularly of low-molecular weight N-formyl methionine terminal peptides, (c) cell-derived arachidonic acid metabolites (LTB4, hydroeicosatetraenoic acid, kallikrein and IL-8), and (d) chemokines (e.g. IL-1 and IFN-γ); and (e) cellular debris.

- **Binding of chemokines on leukocytes:** Chemotactic chemical mediators bind to seven transmembrane G protein-coupled receptors (GPCRs) family on leukocytes. G protein-coupled receptors mediated signal transduction lead to generation of lipid second messengers and increased cytosolic calcium, which ultimately induce cytoskeleton polymerization and contraction.
- **Enhancement of neutrophilic motility:** Calcium causes contraction of 'cytoskeleton actin regulating proteins' (filamin, gelsolin, profilin and calmodulin), which are necessary for pseudopodia formation that anchor to the ECM. Cytoskeleton actin regulating proteins pull the leukocytes in the direction toward injury site.
- **Recruitment of leukocytes:** Neutrophils predominate in the acute inflammatory infiltrate during first 6–24 hours, which are short lived. Cellular infiltrate is dominated by continuously recruited neutrophils for several days in Pseudomonas infection.
  - Neutrophils undergo apoptosis and disappear after 24–48 hours which are replaced by monocytes/macrophages in 24–48 hours, which survive longer. Inflammation lasting more than a few days attracts macrophages and lymphocytes.

Table 2.7 Major leukocyte–endothelial cell adhesion molecules

Molecules	Distribution	Ligand/Molecule on Cell Type
Selectin family		
P-selectin glycoprotein ligand 1 (CD62P)	Endothelium activated by histamine and thrombin	Sialyl-Lewis on X P-selectin glycoprotein ligand 1 (PSGL-1) and other glycoproteins; neutrophils, monocytes, T cells (memory, effector)
E-selectin (CD62E)	Endothelium activated by IL-1 and tumor necrosis factor (TNF)	Sialyl-Lewis (e.g. cutaneous lymphocyte antigen-1; CLA-1) on glycoproteins; neutrophils, monocytes, T cells (memory, effector)
L-selectin (CD62L)	Neutrophils, monocytes, T cells (naïve), B cells (naïve) and central memory cells	Sialyl-Lewis X/PNAd on GlyCam-1 (glycan bearing cell adhesion molecule 1), CD34, MadCAM-1, others; endothelium (HEV)
Integrin family		
Leukocyte function antigen 1 (leukocyte function-associated antigen 1; LFA-1) (CD11a/CD18)	Neutrophils, monocytes, T cells (naïve), B cells (naïve) and central memory cells	Intercellular adhesion molecule 1 (ICAM-1) (CD54), ICAM-2 (CD102); endothelium cell immunoglobulin family (upregulated when cytokine activated), ICAM-3 (intercellular adhesion molecule 3) (CD50) neutrophil immunoglobulin family
Macrophage antigen 1 (MAC-1) (CD11bCD18)	Neutrophils, monocytes, dendritic cells	Intercellular adhesion molecule 1 (ICAM-1) (CD54), ICAM-2 (CD102); endothelium cell immunoglobulin family (upregulated when cytokine activated), ICAM-3 (intercellular adhesion molecule 3) (CD50) neutrophil immunoglobulin family
VLA-4 (very late antigen 4) (CD49a, CD29)	Monocytes, T cells (naïve, effector, memory)	Vascular cell adhesion molecule 1 (VCAM-1)(CD106); endothelium cell immunoglobulin family (upregulated when cytokine activated)
$\alpha_4\beta_7$ (CD49d, CD29)	Monocytes, T cells (gut, naïve, memory) B cell (gut homing)	Vascular cell adhesion molecule 1 (VCAM-1) (CD106); mucosal adhesion cell adhesion molecule 1 (MadCAM-1); endothelium cell immunoglobulin family in gut and gut-associated lymphoid tissue

PECAM-1 (platelet endothelial cell adhesion molecule 1) (CD31).

Table 2.8 Endothelial–leukocyte adhesion molecules and their major roles

Endothelial Molecule	Leukocyte Receptor	Major Roles
P-selectin (CD62P)	▪ Sialyl-Lewis X ▪ PSGL-1	Rolling of polymorphonuclear (PMN) cells, monocytes, lymphocytes
E-selectin (CD62E)	Sialyl-Lewis X	Rolling and adhesion of leukocytes
GlyCam-1, CD4	L-selectin (CD62L)	Rolling of PMN cells and monocytes
ICAM-1 (Ig family)	Integrins (LFA-1, Mac-1) CD11/CD18	Adhesion, arrest and transmigration of PMN cells, monocytes and lymphocytes
VCAM-1 (Ig family)	VLA-4 integrin	Adhesion of PMN cells, monocytes and lymphocytes
PECAM (CD31)	CD31	Transmigration of all leukocytes through endothelium

- Macrophages participate in clearance of pus, cellular debris, damaged tissue and dead neutrophils. In acute viral infections lymphocytes may be the first cells to arrive. In hypersensitivity reactions, eosinophils may be the main cell type.

- **Impaired chemotaxis:** Chemotaxis is impaired in diabetes mellitus, cancer, sepsis, immunodeficiency disorders and thermal injuries. Patients develop increased susceptibility to infections due to defects in complement proteins, pathologic activation and deficiency of regulatory proteins. Chemotactic factors for various cells in inflammation are given in Table 2.9.

ACTIVATION OF LEUKOCYTES

Leukocytes express on their surface different kinds of receptors that recognize different stimuli. After recruitment of leukocytes to the injured site, microbes, products of necrotic cells and chemical mediators activate the leukocytes. Activated leukocytes participate in phagocytosis and degradation of injurious agents. Chemical mediators amplify the innate and adaptative inflammatory immune responses.

- **Pathogen-associated molecular patterns:** Pathogens contain pathogen-associated molecular patterns (PAMPs) in gram-negative bacteria (e.g. lipopolysaccharides, endotoxin), gram-positive bacteria (peptidoglycan, e.g. lipoprotein, lipoteichoic acid), bacteria with flagella (e.g. flagellin), viruses (e.g. single stranded, double-stranded RNA molecules) and fungi (e.g. zymosan). Pathogen-associated molecular patterns (PAMPs) are shown in Fig. 2.11. Major pathogen-associated molecular patterns (PAMPs) in microbes are given in Table 2.10.
- **Damage-associated molecular patterns:** Necrotic cells/tissues release damage-associated molecular patterns (DAMPs), whereas apoptotic cells typically do not release DAMPs.
  - Stimuli that induce necrosis or severe cellular damage, which results in rapid cell rupture with consequent release of intracellular DAMPs to the extracellular space. DAMPs can then engage cells of immune system and promote inflammation.

Table 2.9 Chemotactic factors for various cells in inflammation

Chemotactic Factor	Source	Chemotaxis for Cells
Formylated peptides	Bacterial products of *Escherichia coli*	Neutrophils
C5a	Activated complement component	Neutrophils
Kallikrein	Product of factor XIIa-mediated conversion of prekallikrein	Neutrophils
Fibrinogen	Plasma protein	Neutrophils
Acetyl-glyceryl-ether-phosphorylcholine (AGEPC), a potent platelet-activating factor (PAF)	▪ Basophils ▪ Mast cells	Eosinophils
PDGF	▪ Platelets ▪ Monocytes ▪ Macrophages ▪ Smooth muscle cells ▪ Endothelial cells	▪ Neutrophils ▪ Macrophages
TGF-β	▪ Platelets ▪ Neutrophils ▪ Macrophages ▪ Lymphocytes ▪ Fibroblasts	▪ Macrophages ▪ Fibroblasts
Fibronectin	Extracellular matrix protein	▪ Fibroblasts ▪ Endothelial cells

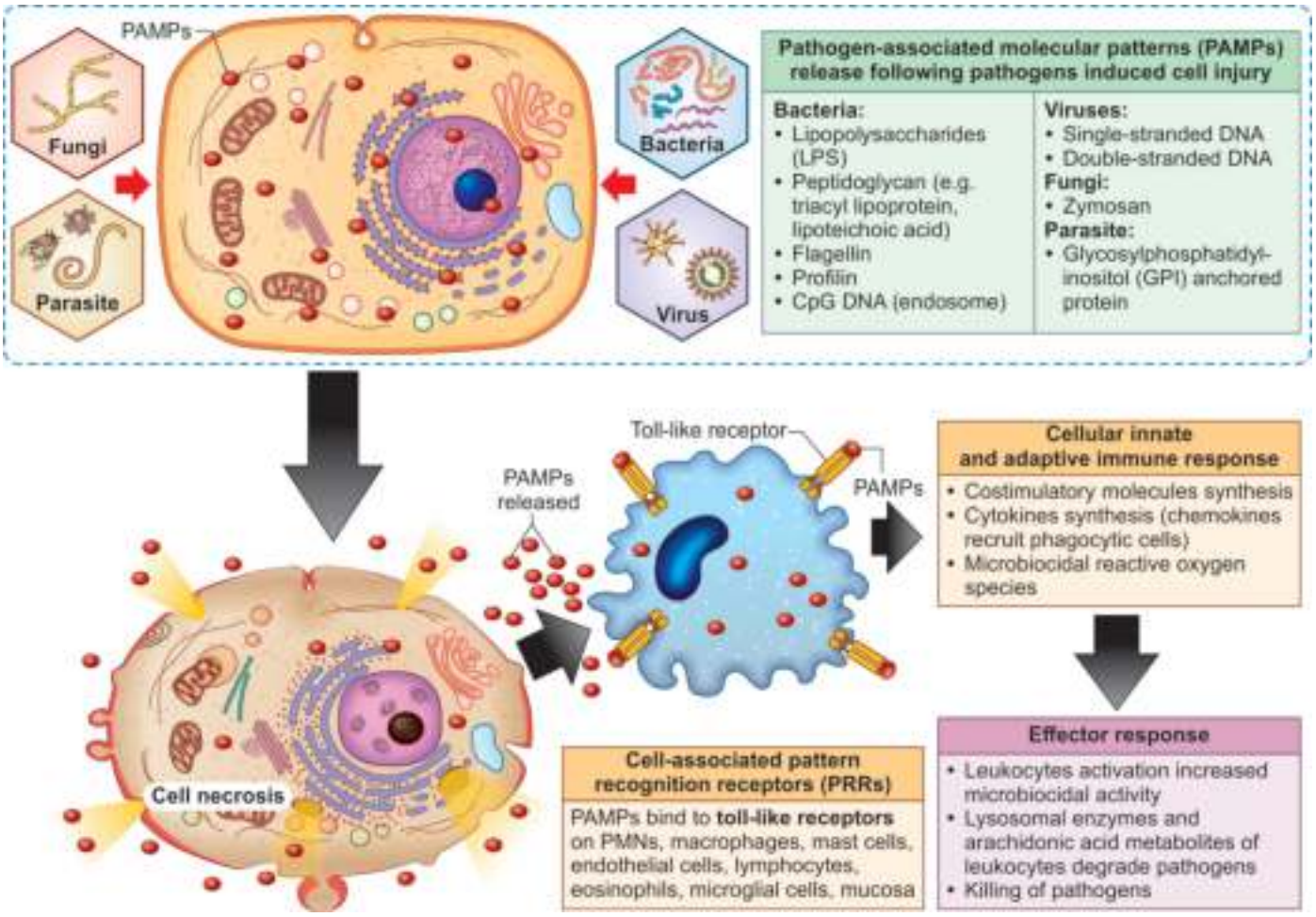


Fig. 2.11: Pathogen-associated molecular patterns. Microbes release PAMPs that bind to the family of pattern recognition receptors (PRRs), i.e. toll-like receptors and mediate innate and adaptive immune responses. Activation of toll-like receptors by specific ligands induces cytokine release and costimulatory molecules that instruct the type on immune response and direct antimicrobial response and tissue injury.

Table 2.10 Major pathogen-associated molecular patterns (PAMPs) in microbes

Pathogens	Pathogen-associated Molecular Patterns (PAMPs)	Tolle-like Receptors
Bacteria		
Gram-negative bacilli	Lipopolysaccharides (LPS), endotoxin	TLR-4
Gram-positive cocci	Peptidoglycan (e.g. triacyl lipoprotein, lipoteichoic acid)	TLR-1, TLR-2, TLR-6
Bacterial flagella	Flagellin	TLR-5
Bacterial profilin	Profilin	TLR-1
Endosome	CpG DNA (immunostimulatory cytosine-guanosine rich DNA sequence ends of DNA)	TLR-2, TLR9
Viruses		
Nucleus	▪ Single-stranded DNA ▪ Double-stranded DNA	▪ TLR-3, TLR-7, TLR-9 ▪ TLR-3
Yeast		
Fungi	Zymosan	TLR-2
Parasite		
Parasite component	Glycosylphosphatidylinositol (GPI) anchored protein	TLR-2

- On the other hand, because mild stimuli that initiate apoptosis (regulated cell death) do not undergo cell rupture and their removal is coordinated by macrophages and surrounding other cells of innate immune system, before release of DAMPs can occur. For this reason, apoptosis is not associated with activation of the immune system. Damage-associated molecular patterns (DAMPs) are shown in Fig. 2.12.

- **Binding of PAMPs and DAMPs to receptors:** PAMPs and DAMPs bind to receptors belonging to the family of pattern recognition receptors (PRRs) and initiate cell signaling leading to enhanced activation of cytokines and reactive oxygen species (ROS). These inflammatory signals can lead to further release of DAMPs, which initiate innate and adaptive inflammatory immune response. Major damage-associated molecular patterns in microbes are given in Table 2.11. Various receptors expressed on leukocytes (macrophages and neutrophils) are discussed below.

Toll-like Receptors on Leukocytes

Toll-like receptors (TLRs) play essential role in the innate immune response against invasion by microorganisms by recognizing pathogen-associated molecular patterns (PAMPs) derived from various microbes.

- Toll-like receptors are expressed on the surface of macrophages, neutrophils, dendritic cells, microglial cells, eosinophils, mast cells, endothelial cells,

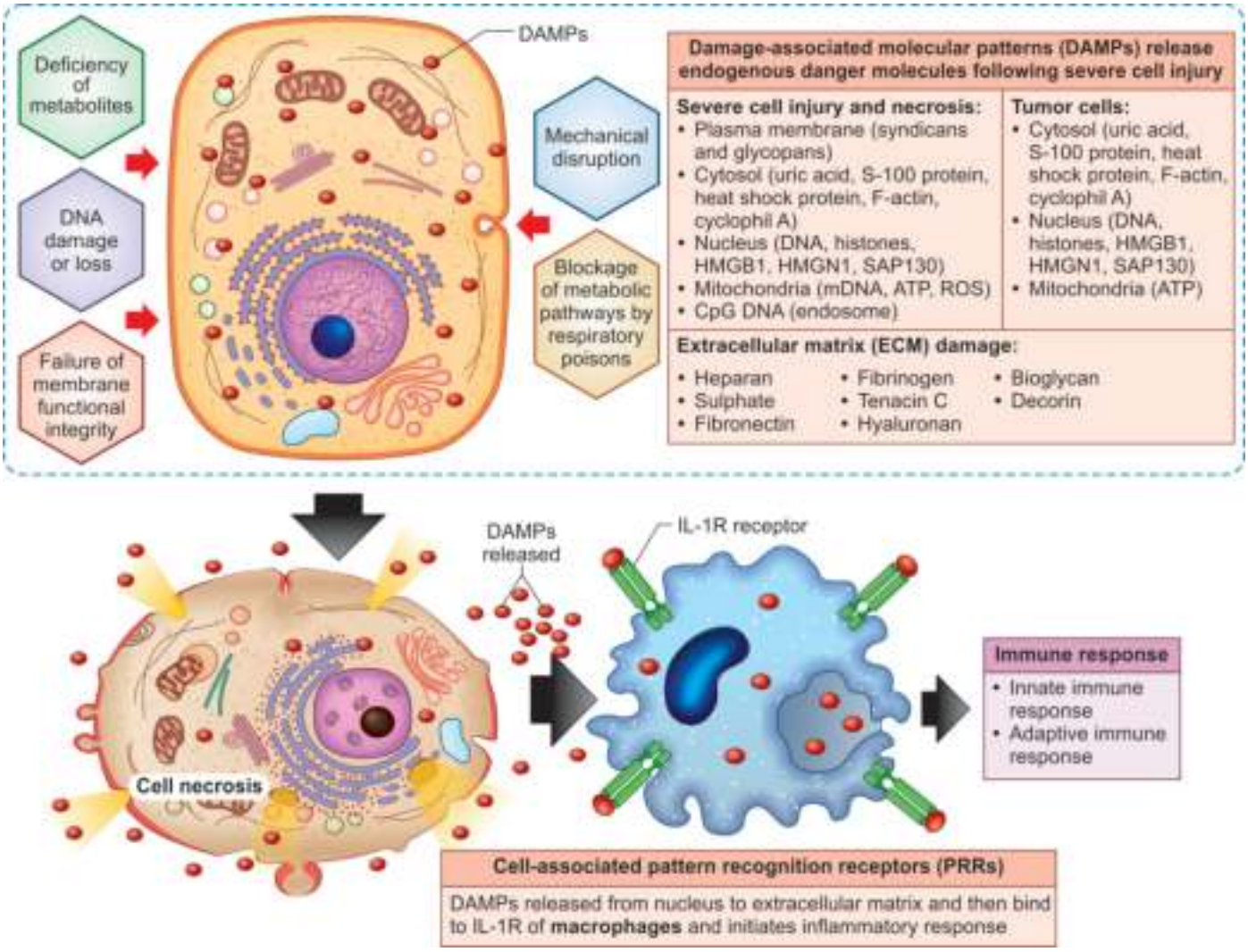


Fig. 2.12: Damage-associated molecular patterns. Necrotic cells release damage-associated molecular patterns (DAMPs), i.e. endogenous danger molecules from damage cells, whereas mild cell injury apoptosis induced cells do not. Stimuli that induce cell necrosis frequently cause severe cellular damage, which leads to rapid cell rupture with consequent release of intracellular DAMPs. DAMPs can then engage cells of the immune system and promote inflammation. On the hand, because stimuli that initiate apoptosis are physiological and relatively mild, apoptotic cells do not rupture and their removal is coordinated by macrophages and other cells of the innate immune system, before release of DAMPs can occur. For this reason, apoptosis is not typically associated with activation of the immune system.

Table 2.11 Major damage-associated molecular patterns (DAMPs) in microbes

Damage-associated Molecular Patterns (DAMPs)	Receptors
Extracellular matrix (ECM) damage	
Heparan sulfate	TLR-4
Fibronectin	TLR-4
Fibrinogen	TLR-4
Tenacin C	TLR-4
Hyaluronan	TLR-2, TLR-4, NLRP-3
Decorin (proteoglycan)	TLR-2, TLR-4
Bioglycan	TLR-2, TLR-4
Plasma membrane damage	
Syndicans	TLR-4
Glycopans	TLR-4
Nucleus damage	
DNA	TLR-9, AIM-2
Histones	TLR-2, TLR-4,
HMGB1	TLR-2, TLR-4, RAGE
HMGN1	TLR-4
SAP130	Mincle
Cytosol damage	
Uric acid	NLRP3, P2X7
S-100 protein	TLR-2, TLR-4, RAGE
Heat shock protein	TLR-2, TLR-4, CD91
ATP	P2X7, P2Y2
F-actin	DAGR-1
Cyclophil A	CD147
Mitochondrial damage	
Mitochondrial DNA	TLR-9
Mitochondrial reactive oxygen species	NLRP-3

mucosal cells and lymphocytes. Each TLR recognizes and binds distinct microbial molecules called pathogen-associated molecular patterns (PAMPs).

- Pathogen-associated molecular patterns present in pathogens include gram-positive cocci (lipoprotein, lipoteichoic acid), gram-negative cell wall lipopolysaccharides (LPS), bacteria with flagella (flagellin) viruses (single-stranded or double-stranded RNA molecules) and fungi (zymosan).
- Toll-like receptor binds and transmits a signal to the nucleus by recruiting intracellular proteins such as MyD88, IRAK-M and TRAF6 to activate the MAP3 kinase/NF-κB signaling pathway and promote the expression of proinflammatory genes leading to production of proinflammatory cytokines.
- Nuclear factor κ-light chain-enhancer of activated B cells (NF-κB) stimulates synthesis of cytokines and increases expression of adhesion molecules. Net result is recruitment and activation of leukocytes. Production of microbiocidal reactive oxygen species (ROS) and lysosomal enzymes kill the microbes. Interferon regulatory factors (IRFs) stimulate antiviral cytokines. TLRs increase baseline inflammatory activity and promote atherosclerosis.
- Toll-like receptors and their actions are shown in Fig. 2.13. Four family of pattern recognition receptors (PRRs), their location and actions are given in Table 2.12.

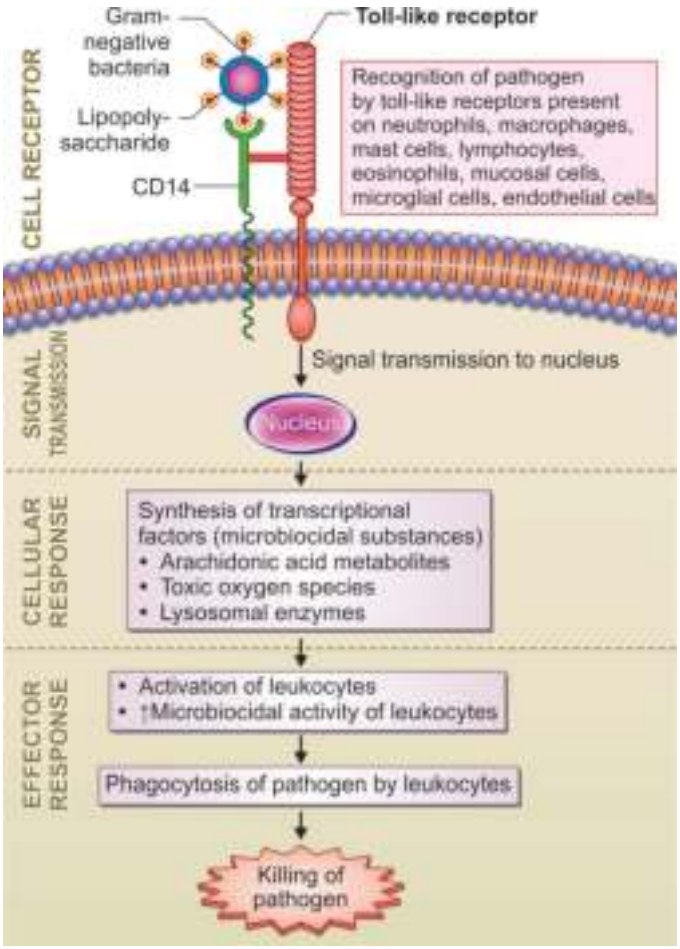


Fig. 2.13: Toll-like receptors and their actions. Toll-like receptors (TLRs) constitute the first line of defense system against microbes. These can recognize both invading pathogen-associated molecular patterns (PAMPs) and endogenous danger molecules released from the damaged cells/tissues (damage-associated molecular patterns—DAMPs). Toll-like receptors play key role in linking innate and adaptive immune response.

Table 2.12 Four family of pattern recognition receptors (PRRs), their location and actions

Toll-like Receptors
TLR1 (macrophages and neutrophils): Lipid and carbohydrates from gram-positive bacteria
TLR2 (macrophages, neutrophils and basophils): Gram-positive bacteria and fungi
TLR3 (macrophages): Nucleic acid derivatives (viral DNA)
TLR4 (macrophages, neutrophils and basophils): Lipopolysaccharides from gram-negative bacteria
TLR5 (macrophages, neutrophils and basophils): Bacterial flagellin
TLR6 (macrophages and neutrophils): Lipid and carbohydrates from gram-positive bacteria
TLR7 (macrophages and neutrophils): Nucleic acid derivatives (viral DNA)
TLR8 (macrophages and neutrophils): Nucleic acid derivatives (viral DNA)
TLR9 (macrophages and neutrophils): Nucleic acid derivatives (viral DNA) and bacterial DNA containing unmethylated CpG motifs
TLR10 (macrophages and neutrophils): Ligand unknown
TLR11 (macrophages and neutrophils): Bacterial profilin
C-type Lectin Receptors
C-type lectin receptors are expressed on the plasma membrane of macrophages and dendritic cells. These receptors detect fungi and elicit inflammatory response
NOD-like Receptors (NLRs) Located in Cytoplasm
NLRs are present in cytoplasm of cell. These recognize microbial products and products of necrotic cells (uric acid)
NLRs signal via cytosolic inflammasome protein, which activates caspase 1. This enzyme cleaves precursor IL-1 to form biologically active form
NLRs inflammatory pathway may participate in pathogenesis of atherosclerosis, gout and obesity associated type 2 diabetes mellitus
RIG-like Receptors (RLRs) for Viral Nucleic Acid
RLRs are located in the cytoplasm of the cell
RLRs detect nucleic acids of viruses that are replicating in the cytoplasm of infected cells
RLRs stimulate the synthesis of antiviral cytokines

Seven α-Helical Transmembrane G Protein-coupled Receptors

Certain bacterial N-formylmethionyl peptides, lipid mediators, chemokines, C5a, platelet-activating factor, prostaglandin E and leukotrienes bind to seven α-helical transmembrane G protein coupled receptors on leukocytes. Cellular changes in leukocytes lead to cytoskeleton changes by signal transduction, increased expression of integrin adhesion molecules resulting in adhesion of leukocytes to endothelium, transmigration and chemotaxis of leukocytes to site of tissue injury by activation of the respiratory burst. Seven α-helical transmembrane G protein-coupled receptors and their actions are shown in Fig. 2.14.

Phagocytic Receptors

Microbes bind to phagocytic receptors leading to killing of bacteria by production of microbiocidal reactive oxygen species, release of lysosomal enzymes, leukocytic activation and killing the microbes. Phagocytic receptors on phagocytes and their actions are shown in Fig. 2.15.

Opsonin Receptors on Leukocytes

Macrophages express opsonin receptors (FcR1, CR1 and C1q), scavenger receptors, macrophage integrin receptors MAC1 (CD11 and CD18). FcR1, CR1, C1q receptors recognize microbes coated by IgG, C3 (classical and alternate pathways) and plasma proteins derived mannose binding lectin (MBL). Scavenger receptors bind microbes and modified low-density lipoprotein (LDL). Opsonin receptors on leukocytes and their actions are shown in Fig. 2.16.

Cytokine Receptors

Cytokine IFN-γ synthesized by NK cells and CD8+ cytotoxic T cells bind to cytokine receptor superfamily on leukocytes. It leads to activation of leukocytes resulting

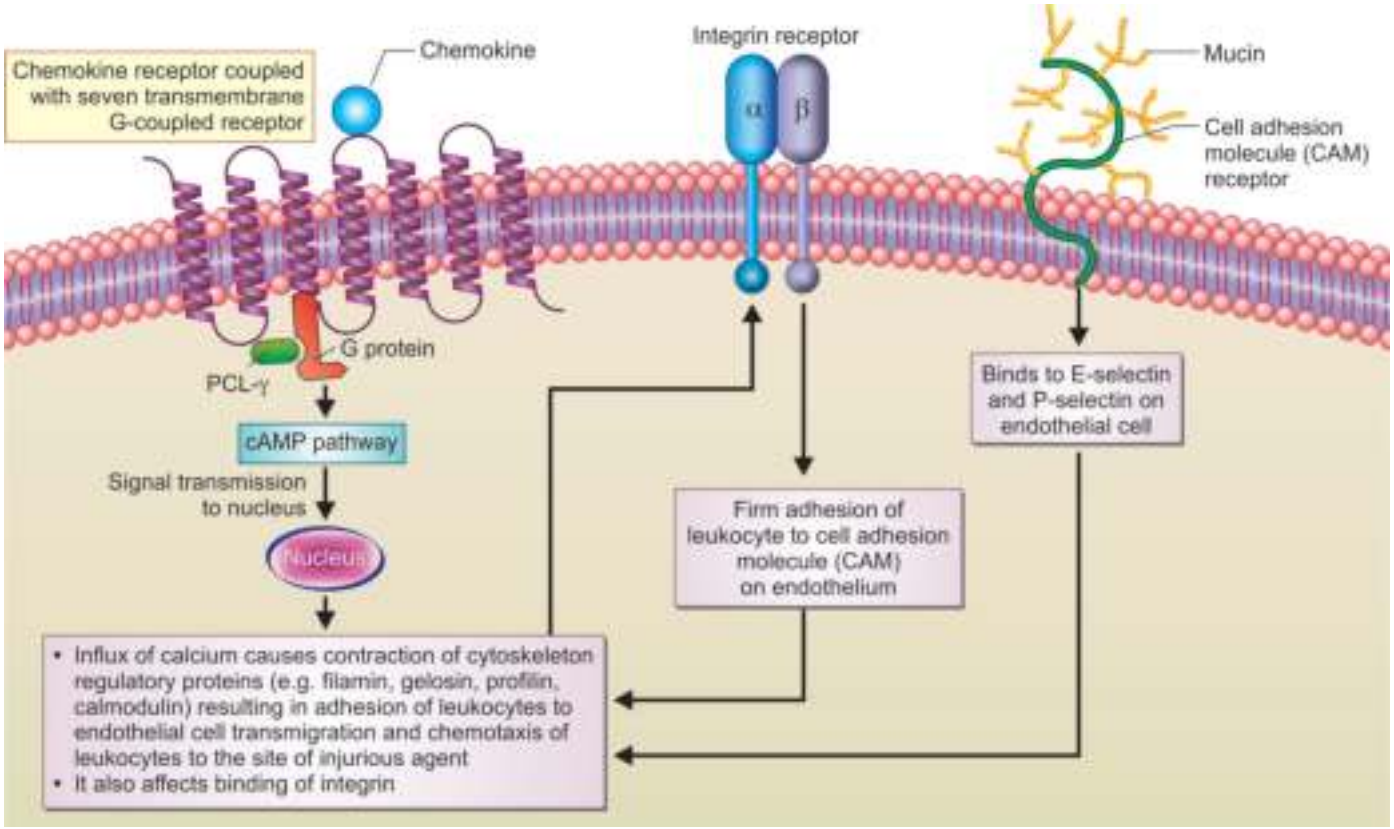


Fig. 2.14: Seven α-helical transmembrane G protein-coupled receptors and their actions. These form the largest superfamily of cell-surface receptors. They respond to wide range of stimulants including light energy, peptides, lipids, sugars and proteins. Increased cytosolic calcium stimulates integrin receptors, which induces firm adhesion of leukocytes to vascular endothelium leading cross-linkage with effector molecules of seven α-helical transmembrane G protein-coupled receptors.

in production of reactive oxygen species, release of lysosomal enzymes and ultimately killing of microbes.

- Signaling by cytokine receptors depends upon their association with the Janus kinases (JAKs), which couple ligand binding to tyrosine phosphorylation of signaling proteins recruited to cytokine receptor complex.
- Among these signaling proteins are unique family of transcription factors such as signal transducers and activators of transcription (STAT). The receptors and their corresponding cytokines have been divided into several families based on their structure and activities. Cytokine receptors on phagocytes and their actions are shown in Fig. 2.17.

PHAGOCYTOSIS AND CLEARANCE OF THE INJURIOUS AGENTS

The process of engulfment of solid particulate material by phagocytic cells (neutrophils and macrophages) is known as phagocytosis. Phagocytes eliminate microbes and cellular debris by process of phagocytosis.

- Activated phagocytes recognize, internalize, release proteolytic enzymes (i.e. lysozyme, protease, collagenase, elastase, lipase, proteinase, gelatinase and hydrolases) and degrade the injurious agent and dead cells by releasing reactive active species (ROS), nitrogen and oxygen species (NO) and lysosomal enzymes in phagolysosomes.
- Phagocytosis involves distinct steps, i.e. recognition, opsonization, binding, engulfment, phagolysosome formation, intracellular killing and degradation of microorganisms by lysosomal enzymes, and reactive oxygen species (ROS). Sequential steps in phagocytosis are shown in Fig. 2.18. Phagocytosis in tissue is shown in Fig. 2.19.
- Enzymes or molecules involved in O_2-dependent phagocytosis are nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase, H_2O_2 activity, superoxide radical and NADPH oxygenase.
- Enzymes or molecules involved in O_2-independent phagocytosis include bactericidal permeability increasing (BPI) protein, lactoferrin, lysozyme, major basic proteins and defensins. A young infant deficient in NADPH enzyme is susceptible to bacterial infections due to impaired phagocytosis.
  - **Opsonization of injurious agent:** Phagocytosis is initiated by the expression of surface receptors

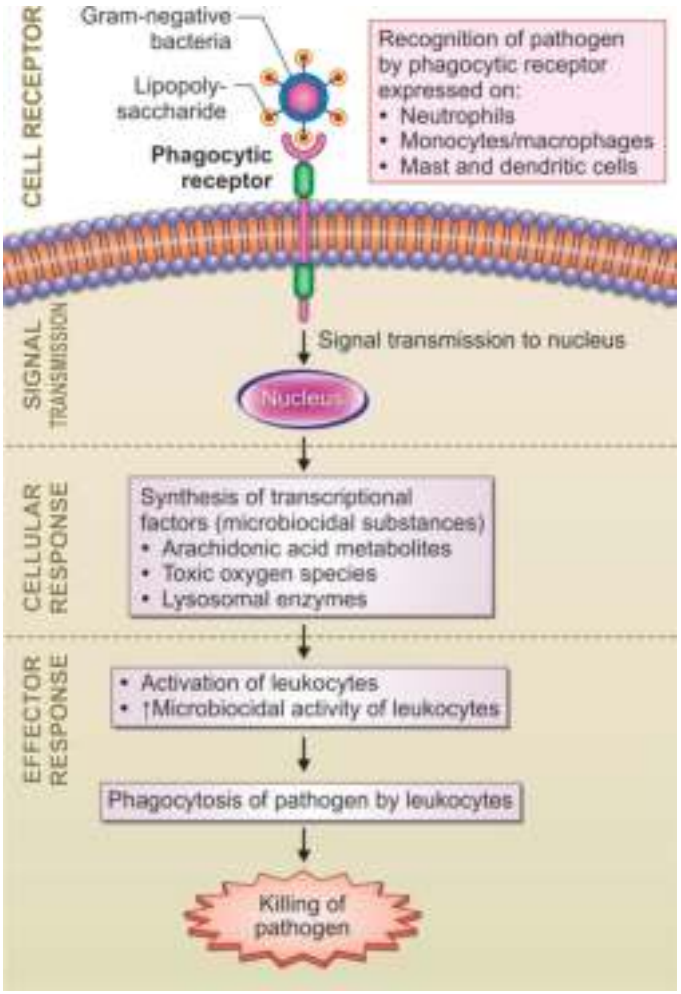


Fig. 2.15: Phagocytic receptors on phagocytes and their actions. Phagocytic receptors are expressed on professional phagocytes such as neutrophils, monocytes/macrophages, and dendritic cells. These professional phagocytes activate signaling pathways resulting in phagocytosis and elimination of pathogens.

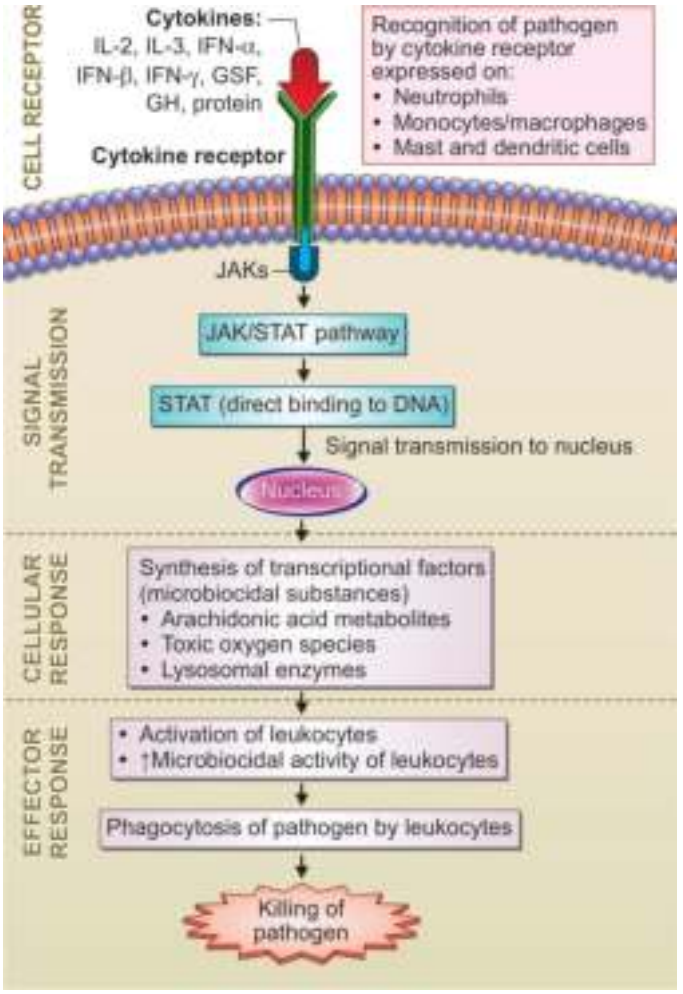


Fig. 2.17: Cytokine receptors on phagocytes and their actions. Professional phagocytes (i.e. neutrophils, monocytes/macrophages and dendritic cells) play a key role innate immunity. The signaling induced by stimuli such as bacterial products, cytokines and inflammatory mediators leads to stimulation of molecules involved in phagocytosis.

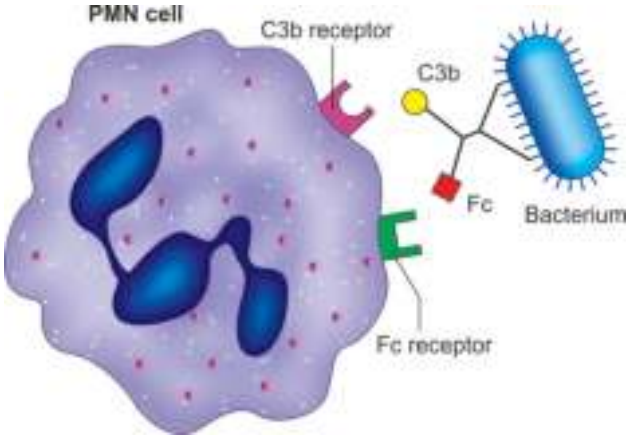


Fig. 2.16: Opsonin receptors on leukocytes and their actions. Function of opsonins is to react with bacteria and make them susceptible to ingestion by phagocytes. Phagocytes express receptors for Fc end of IgG molecule and for the C3a fragment of complement, which recognize and ingest IgG or C3b coated bacteria. Facilitation of phagocytosis is called opsonization. IgG and C3b are called opsonins.

on neutrophils and macrophages. Phagocytosis is further enhanced when the microorganisms are coated with specific proteins, opsonins. Opsonization is a process by which injurious stimulus coated by opsonins, i.e. IgG, C3b and complement mannose-binding lectin (MBL). Opsonization enhances binding of injurious agent to membrane receptors (e.g. FcR1, CR1 and MBL) expressed on activated neutrophils. Defects in opsonization results in 'Bruton's agamma-globulinemia'.

- **Binding opsonized injurious agent to cellular receptors of phagocytes:** Microbes are coated (opsonized) by immunoglobulin (IgG), complement (C3b) and mannose-binding lectin. Opsonized microbes bind to cell-surface receptors of leukocytes. Fc portion of the IgG binds to FcR1 and C3b to FcR1 of leukocytes. Bacterial polysaccharide binds

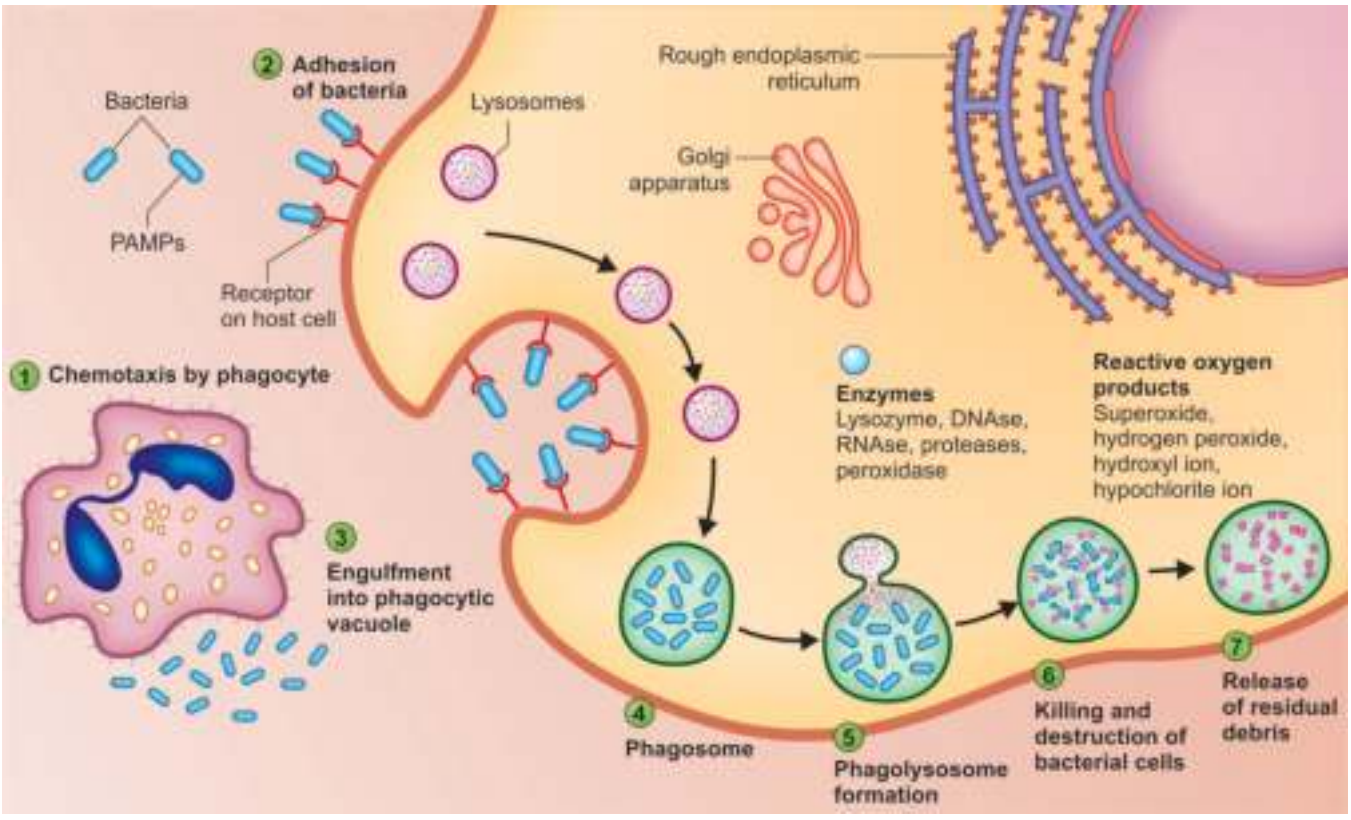


Fig. 2.18: Sequential steps in phagocytosis. The process of phagocytosis involves recognition of injurious agent, adhesion, engulfment, formation of phagosome, maturation of phagosome to transform into a phagolysosome, killing of pathogens by lysosomal enzyme, and reactive oxygen species.

to mannose-binding lectin (MBL) receptors on leukocytes.

- **Engulfment of injurious agent:** Neutrophils push out pseudopodia to surround the injurious agent completely, forming an endocytic phagocytic vacuole (phagosome), driven by polymerization (assembly and disassembly) of actin filaments. Special proteins probably allow final sealing of the leukocytic membrane.
- **Phagolysosome formation:** Phagocytic vesicle fuses with neutrophilic membrane of lysosome to form phagolysosome. In 'Chédiak-Higashi syndrome' a defect in microtubule function prevents phagolysosome formation. Impaired membrane fusion of lysosomes with melanosomes in melanocytes causes 'albinism'.
- **Release of lysosomal hydrolytic enzymes into phagolysosome:** Neutrophils release lysosomal hydrolytic enzymes into the phagolysosome and degrade the pathogens. Products of azurophil and specific granules of neutrophils participate in vascular permeability, chemotaxis and tissue damage in acute inflammation.
- **Degradation of injurious agent:** Degradation of microorganism occurs by intracellular and extracellular organisms. Intracellular mechanism to degrade microorganisms includes oxidative bactericidal mechanism by oxygen-derived free radicals (myeloperoxidase dependent and myeloperoxidase independent), oxidative bactericidal mechanism by lysosomal granules, and nonoxidative bactericidal mechanisms. Extracellular mechanism to degrade microorganisms is mediated by lysosomal granules and immune mechanism.

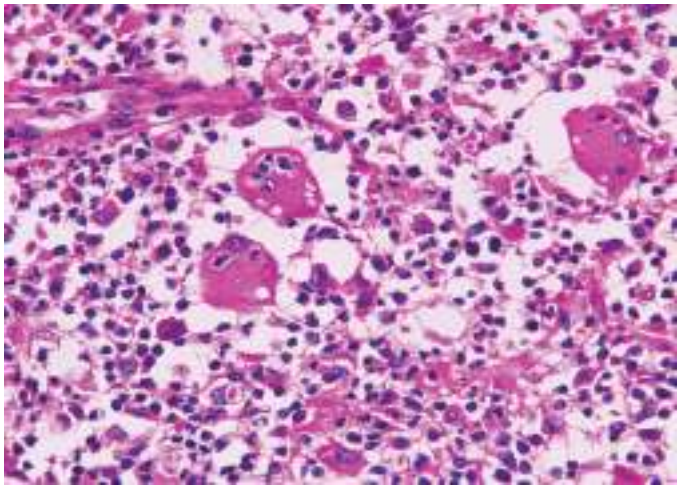


Fig. 2.19: Phagocytosis in tissue. Phagocytosis is a cellular process for ingesting and eliminating particles larger than 0.5 μm in diameter, including microorganisms, foreign substances and apoptotic cells. Phagocytes are showing ingested injurious agent (arrows) (400X).

Pathology Pearls: Defects in Opsonization, Phagocytosis and Phagolysosome Formation, Defective Degranulation and Clinical Correlation

Phagocytosis is impaired due to defects in opsonization and phagolysosome formation, in the settings of leukemias, diabetes mellitus, malnutrition and neonates.

Defects in Opsonization

- Opsonization is a process by which injurious stimulus coated by opsonins, i.e. IgG, C3b and complement mannose-binding lectin (MBL). Opsonization enhances binding of injurious agent to membrane receptors (e.g. FcR1, CR1 and MBL) expressed on activated neutrophils.
- Defects in opsonization results in 'Bruton's agammaglobulinemia', an X-linked inherited agammaglobulinemia is characterized by the absence of mature B cells which in turn leads to severe immunoglobulin deficiency and recurrent infections.

Defective Phagolysosome Formation and Defective Degranulation of Neutrophils

- Chédiak-Higashi syndrome occurs due to mutation in LYST gene located on chromosome 1q42 that encodes a protein essential for assembly of microtubules in the cytoplasm that results in defective fusion of phagosome with lysosome (phagolysosome) and defective degranulation in neutrophils.
- Chédiak-Higashi syndrome is characterized by defective degranulation of neutrophils, impaired microbial killing, and recurrent bacterial infections (*Staphylococcus aureus*) forming soft tissue abscess. Impaired membrane fusion of lysosomes with melanosomes in melanocytes causes 'albinism'.
- Neutrophils contain giant granules due to aberrant organelles. Chédiak-Higashi syndrome also affects platelets (bleeding), melanocytes (albinism), Schwann cells (neuropathy), natural killer cells, and cytotoxic T cells (aggressive lymphoproliferative disorder).

INTRACELLULAR MICROBIAL KILLING

Neutrophils participate in phagocytosis and destruction of microorganisms. When coated with opsonins (e.g. complement and/or immunoglobulin), microorganisms bind to the specific receptors on the surface of the neutrophil and invagination of the cell membrane occurs with the incorporation of the microorganisms into an intracellular phagosome.

- There follows a burst of oxygen consumption and extra oxygen consumed is converted to highly reactive oxygen species (ROS). In addition, cytoplasmic lysosomal enzymes discharge their contents into the phagosome leading to degradation of the ingested microorganisms.
- Bactericidal activity is carried out either via myeloperoxidase (MPO)-dependent enzyme (most effective) or myeloperoxidase (MPO)-independent (less effective) mechanisms.
- Myeloperoxidase is a heme peroxidase expressed mainly in neutrophils and to a lesser degree in monocytes/macrophages.
- In the presence of hydrogen peroxide and halides, myeloperoxidase catalyzes the formation of reactive oxygen species intermediates, including hypochlorous acid (HOCl).
- Oxygen-independent intracellular killing uses preformed cytoplasmic granules containing antimicrobial agents. Superoxide is subsequently converted into hydrogen peroxide (H_2O_2). Antibacterial compounds in the phagolysosome are given in Table 2.13.
  - **Myeloperoxidase dependent bactericidal mechanism:** Activated neutrophils use enzyme myeloperoxidase to generate an array of potent toxic oxidants in the phagosome. During degranulation, hydrogen peroxide is formed by the respiratory burst and a halide particularly chloride. The initial product of MPO-H_2O_2-chloride system in HOCl

Table 2.13 Antibacterial compounds in the phagolysosome

Compounds	Functions
Oxygen-dependent antibacterial compounds	
Superoxide, hydroxyl molecule hypochlorous acid ($HOCl^-$)	▪ Kill intracellular microbes in neutrophils and (not by macrophages) ▪ Deficiency results in recurrent infections
Oxygen-independent antibacterial compounds	
Lactoferrin	Lactoferrin binds iron in neutrophils leading to inhibition of bacterial reproduction
Defensins	Cytotoxic to microbes
Bactericidal permeability increasing protein	Bactericidal permeability increasing protein activates phospholipase, which degrades phospholipids of bacterial cell wall resulting in killing of microbes
Lysosomal enzyme	Lysosomal enzyme hydrolyzes the muramic acid-N-acetylglucosamine bond of bacterial glycopeptides resulting killing of microbes
Major basic proteins	Major basic proteins, cationic protein in eosinophilic granules cytotoxic to helminths

and subsequent formation of chlorine, chloramines, hydroxyl radicals, singlet oxygen and ozone has been proposed.

- **Myeloperoxidase independent bactericidal mechanism:** Mature macrophages lack myeloperoxidase enzyme. Bactericidal activity of mature macrophages is mediated by producing hydroxyl ions and superoxide singlet oxygen. Fenton and Haber and Weiss reactions are a source of oxidative stress. The generation of oxygen-derived free radicals occurs first with the reduction of ferric to ferrous ion and then by Fenton reaction with ferrous iron catalyzing the breakdown of hydrogen peroxide to hydroxyl radicals. Haber-Weiss reaction generates hydroxyl radicals from hydrogen peroxide (H_2O_2) and superoxide catalyzed by iron ions.

Oxygen-dependent Microbial Killing by Generation of Oxygen-derived Free Radicals

Phagocytic cells such as neutrophils, eosinophils monocytes and macrophages phagocytose and kill internalized pathogens by respiratory or oxidative burst. During respiratory burst, phagocytes require energy and oxygen for the generation of a variety of cytotoxic reactive oxygen species. Generation of reactive oxygen species (ROS) in polymorphonuclear cell is shown in Fig. 2.20.

- The phagocytes contain NADPH dependent oxidase enzyme, which transfers one electron to an oxygen molecule to generate oxygen-derived free radicals such as superoxide anion, highly reactive hydroxyl molecule and hypochlorous acid (bleach). Superoxide dismutase reduces the superoxide radical to H_2O_2.
- The superoxide anion dismutases spontaneously in the presence of superoxide dismutase to hydrogen peroxide that serves as the parent product for a variety of other highly reactive metabolites such as hydroxyl radical and hypochlorous acid; which enter phagosome and together with enzymes participate in the killing of phagocytosed pathogens.

Superoxide Anion Free Radical

In resting neutrophils, NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase) is present in the plasma membrane as well as cytoplasm. In acute inflammation, NADPH oxidase in the phagolysosome membrane reduces oxygen by the addition of electrons to form superoxide anion.

- **Chronic granulomatous disease of childhood:** Defect in NADPH oxidase activity in neutrophils causes X-linked chronic granulomatous disease of childhood. Disease is characterized by phagocytic cells that ingest but unable to kill certain microorganisms.

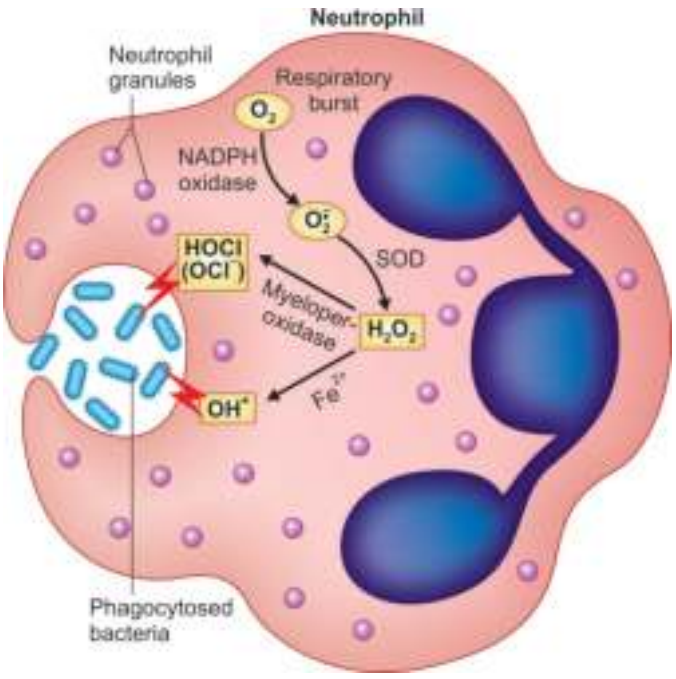


Fig. 2.20: Generation of reactive oxygen species (ROS) in polymorphonuclear cell. Reactive oxygen species are generated in polymorphonuclear cell as a result of engulfment of bacteria during mitochondrial electron transport system. The sequential reduction of oxygen through the addition of electrons leads to formation of a number of ROS including superoxide, hydrogen peroxide, hydroxyl radical ion, and nitric oxide.

- **Nitroblue tetrazolium slide test to detect defect in NADPH oxidase:** Nitroblue tetrazolium (NBT) slide test is used for screening defects in NADPH oxidase. The number of neutrophils with dark cytoplasmic granules of reaction product is counted. Normally, >95% of the granulocytes containing NADPH oxidase are positive for nitroblue tetrazolium. In the abnormal NBT slide test in chronic granulomatous disease, less than 5% of neutrophils are stained.

Highly Reactive Hydroxyl Free Radical

During phagocytosis, superoxide dismutase converts superoxide radical to hydrogen peroxide (H_2O_2). This reaction is neutralized by glutathione peroxidase. Hydrogen peroxide dissociates to generate highly reactive hydroxyl molecule.

Hypochlorous ($HOCl^-$) Free Radical

Myeloperoxidase (MPO) enzyme in phagocytic vacuole combines with H_2O_2 and chloride ions (Cl^-) to form hypochlorous free radicals (HOCl), which kills bacteria by halogenation, in which halide is bound covalently to cellular constituents or by oxidation of proteins and lipids (lipid peroxidation). Patients deficient in myeloperoxidase cannot produce hypochlorous acid, which leads to increased susceptibility to Candida infections. It is rarely associated with recurrent bacterial infections.

Oxygen-independent Microbial Killing

Oxygen-independent microbial killing is much less effective than oxygen-dependent microbial killing.

- **Lactoferrin:** Normally, bacteria require iron for bacterial reproduction. Binding of lactoferrin present in neutrophilic granules to iron leads to inhibition of bacterial reproduction.
- **Defensins:** Defensins are cationic arginine rich granule peptides in neutrophils, which are cytotoxic to microbes.
- **Bactericidal permeability increasing protein:** Bactericidal permeability increasing protein activates phospholipase, which degrades phospholipids of bacterial cell wall leading to killing of microbes.
- **Lysosomal enzyme:** Lysosomal enzyme present in azurophilic granules hydrolyzes the muramic acid-N-acetylglucosamine bond of bacterial glycopeptides leading to killing of microbes.
- **Major basic proteins:** Major basic proteins, cationic proteins in eosinophilic granules are cytotoxic to helminths.

LEUKOCYTE-INDUCED INJURY

Inflammation plays important role to eliminate the initial cause of cell injury as well as the necrotic cells and tissues, but it is itself capable of causing tissue damage by liberation of products by neutrophils and macrophages. During phagocytosis, leukocytes release microbiocidal and other products (e.g. lysosomal enzymes, reactive oxygen species, arachidonic acid products, prostaglandins and leukotrienes) within phagolysosomes, but also into extracellular space causing tissue damage and may amplify the effects of injurious stimulus.

Mechanism of Tissue Damage by Leukocytes

Leukocyte adhesion and emigration are involved in host defense and phagocytosis and thus serve a beneficial role during the mounting of a well-contained inflammatory response. However, in certain situations, leukocytes turn against the host and contribute in tissue damage and organ dysfunction. Leukocyte-induced injury linked inflammatory disorders are given in Table 2.14.

- **Digestion of basement membranes:** During transmigration, leukocytes release elastase and metalloproteinases, which digest the basement membrane.
- **Regurgitation before complete closure of the phagolysosome:** Regurgitation may occur if the phagocytic vacuole remains transiently open to outside before complete closure of the phagolysosomes.
- **Frustrated (regurgitated) phagocytosis:** Deposition of immune complex on the flat surface of glomerular basement membrane activates complement system and recruitment of leukocytes. Leukocytes fail to engulf these immune complexes and release lysosomal hydrolytic enzymes and O_2-derived free radicals leading to glomerular basement membrane injury, increased permeability and proteinuria. After phagocytosis, potentially cytotoxic substances such as urate crystals are released, which damage the membrane of phagolysosomes. Leukocyte-induced injury mechanism is known as 'frustrated (regurgitated) phagocytosis'. In addition, there is some evidence that substances in secondary granules of neutrophils may be directly secreted by exocytosis. After phagocytosis, neutrophils rapidly undergo apoptotic cell death and are digested by macrophages.
- **Chemical mediators induced tissue damage:** Inflammatory chemical mediators produced by phagocytes, such as prostaglandins and leukotrienes play key role

Table 2.14 Leukocyte-induced injury linked inflammatory disorders

Inflammatory Disorders	Cells and Molecules Involved in Injury
Acute inflammatory disorders	
Acute respiratory distress syndrome	Neutrophils
Bronchial asthma	Eosinophils, IgE antibodies
Acute transplant rejection	Lymphocytes, antibodies and complement
Vasculitis	Antibodies, complement, neutrophils
Glomerulonephritis	Antibodies, complement, neutrophils, monocytes
Septic shock	Cytokines
Chronic inflammatory disorders	
Chronic transplant rejection	Lymphocytes, cytokines
Arthritis	Lymphocytes, macrophages and antibodies
Atherosclerosis	Macrophage, lymphocyte?
Pulmonary fibrosis	Macrophages, fibroblasts

in tissue damage. Prolonged severe injury causes necrosis of tissue. Leukocyte-induced injury may cause acute injury (e.g. glomerulonephritis, acute transplant rejection, bronchial asthma, acute adult respiratory syndrome, septic shock, vasculitis) and chronic injury (e.g. chronic graft rejection, atherosclerosis, arthritis and chronic lung disease).

TERMINATION OF ACUTE INFLAMMATORY RESPONSE

Acute inflammatory response is triggered by changes in intracellular chemical mediators that activate stress-sensitive kinases and transcription factors, which regulate the synthesis of proinflammatory and anti-inflammatory cytokines.

- **Gene silencing and reprogramming:** Acute inflammation is associated with gene silencing and reprogramming that induces various effects: (a) downregulation of proinflammatory cytokines (cytokines (IL-1β, IL-8, TNF-α and IFN-γ), (b) upregulation of anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-IRA), TNF-α receptors, IL-6 IL-10, IL-11, IL-12 and LI-13, and (c) resolution of inflammation.
- **Role of interleukins:** Several interleukins such as IL-6 IL-10, IL-11, IL-12 and LI-13 limit acute inflammation by reducing production of TNF-α. This process may occur by preservation of nuclear factor κB (NF-κB), thus blocking cell activation and release of proinflammatory cytokines.
- **Apoptosis of neutrophils:** Neutrophils have a short half-life in tissues, which undergo apoptosis within a few hours after leaving the blood circulation. Anti-inflammatory lipoxins transmit signal to macrophages, which phagocytose apoptotic bodies of neutrophils by programmed death.
- **Downregulation of leukotrienes and upregulation of lipoxins:** Leukotrienes and lipoxins are synthesized by cell–cell interactions (neutrophils-platelets). After an inflammatory response, neutrophils 'switch off' chemical mediators of inflammation such as proinflammatory leukotrienes (LTC_4, LTD_4 and LTE_4), and '**switch on**' actions of anti-inflammatory lipoxins (LXA_4, LXB_4).
  - **Leukotrienes actions:** There is downregulation of proinflammatory leukotrienes (LTC_4, LTD_4 and LTE_4) synthesized by leukocytes and mast cells. Leukotrienes cause vasodilation and increased vascular permeability. These also activate PMNs leukocytes leading to adhesion on venular endothelium, chemotaxis, and generation of oxygen-derived free radicals and release of lysosomal enzymes.
  - **Lipoxins actions:** Lipoxins (LXA_4, LXB_4) are potent anti-inflammatory cytokines that terminate acute inflammation in the first few hours of injurious stimulus by inhibiting adhesion of leukocytes to endothelium as well as chemotaxis. Lipoxins also cause vasodilatation. Lipoxins transmit signal to macrophages, which phagocytose apoptotic bodies of neutrophils by programmed cell death (apoptosis).
- **Glucocorticoids:** Glucocorticoids inhibit many inflammation-associated molecules such as cytokines, chemokines, arachidonic acid metabolites and adhesion molecules. Glucocorticoids suppress vasodilation and inhibit vascular permeability; and decrease emigration of leukocytes into inflammation sites.
- **Kininases:** Kininases in blood circulation degrade the potent proinflammatory mediator bradykinin.
- **Phosphatase:** A phosphatase is an enzyme that removes a phosphate group from a protein and thus acts to modulate the activities of the proteins in a cell, often in response to external stimuli. Protein phosphatase activation attenuates acute inflammation.
- **Transforming growth factor-β (TGF-β):** Apoptotic neutrophils induce TGF-β expression, that suppresses proinflammatory leukotrienes (LTC_4, LTD_4, and LTE_4) and chemokines. TGF-β upregulates synthesis of anti-inflammatory lipoxins (LXA_4, LXB_4) leading to clearance of apoptotic neutrophil debris by macrophages.

CHEMICAL MEDIATORS OF INFLAMMATION

Chemical mediators of inflammation mediate specific response by acting on the blood vessels, inflammatory cells or other cells in tissues. These regulate inflammation and immunity. These are synthesized either at injury site (local synthesis by cells) or plasma derived (distant synthesis).

- **Synthesis of chemical mediators:** Chemical mediators are derived from various sources: (a) plasma (complement system, kinins, and clotting factors), (b) cells (neutrophils, tissue macrophages, endothelial cells of blood vessels, mast cells and platelets), (c) bacterial products, and (d) fibroblasts of damaged tissues in response to tissue injurious stimulus.
- **Properties of chemical mediators:** Chemical mediators have short life span (e.g. seconds to minutes), which possess enzymatic or toxic activity. These have potential to cause harmful effects.
- **Mechanism of actions of chemical mediators:** Chemical mediators bind to specific receptors on single or multiple target cells, and stimulate them to release secondary molecules, which may have direct enzymatic effects and/or systemic toxic effects.

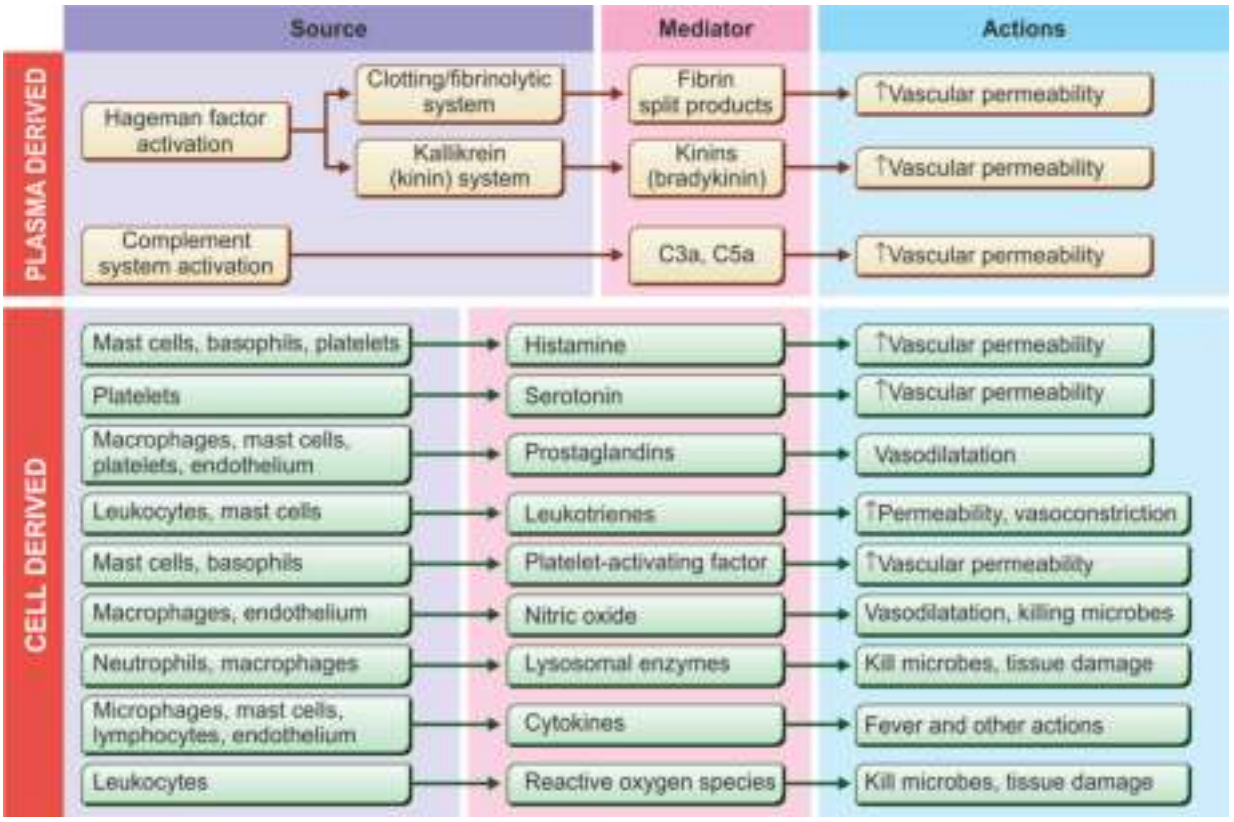


Fig. 2.21: Plasma- and cell-derived chemical mediators and their functions. In response to the inflammatory process, plasma- and cell-derived chemical mediators mediate the inflammatory process by preventing further tissue damage and ultimately resulting in healing and restoration of tissue function.

- **Plasma- and cell-derived chemical mediators—categories:** Chemical mediators are derived from plasma proteins or inflammatory cells. Plasma- and cell-derived chemical mediators and their functions are shown in Fig. 2.21.
  - **Plasma-derived chemical mediators:** These include kinin system, fibrinolytic system, coagulation system, and complement system. These are synthesized by liver.
  - **Cells-derived chemical mediators:** These are synthesized by monocytes, macrophages, lymphocytes, fibroblasts, mast cells, platelets, and endothelial cells of blood vessels. Chemical mediators may be preformed or newly synthesized.
- **Functional categories of chemical mediators:** Chemical mediators produce vascular and cellular changes in inflammation. Based on functions, chemical mediators are classified into following categories: vasoactive, chemotactic and combined chemotactic as well as vasoactive chemical mediators.

Pathology Pearls: Functional Categories of Chemical Mediators

Chemical mediators are categorized according to their actions described as under. Functional categories of chemical mediators and their inflammatory responses are shown in Fig. 2.22 and Table 2.15.

Chemical Mediators with Vasoactive Actions

- **Vasodilation of arterioles and capillaries:** Chemical mediators such as histamine, serotonin, nitric oxide, bradykinin, platelet-activating factor cause vasodilation of microvasculature and sustained by prostaglandins (PGI_2, PGD_2, PGE_2, $PGF_{2\alpha}$) resulting to increased blood flow to injury site. It is responsible for redness (rubor) and increased local temperature (heat—calor).
- **Increased permeability of venules:** Chemical mediators such as histamine, serotonin, bradykinin, anaphylatoxins (C3a, C5a), prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$), leukotrienes (LTC_4, LTD_4, LTE_4), platelet-activating factor (PAF) and neuropeptide (substance P) increase the permeability of venules results to inflammatory exudates.

Chemical Mediators with Chemotactic Actions

Chemotactic agents include such as C5a, chemokines, leukotrienes (LTB_4, LTC_4), collagen, fibrin and bacterial peptides (pathogen-associated molecular patterns: PAMPs).

Chemical Mediators with Combined Vasoactive and Chemotactic Actions

Complement system components, cytokines (interferon and interleukins), products of arachidonic acid metabolism, and platelet-activating factor produce vasoactive and chemotactic effects.

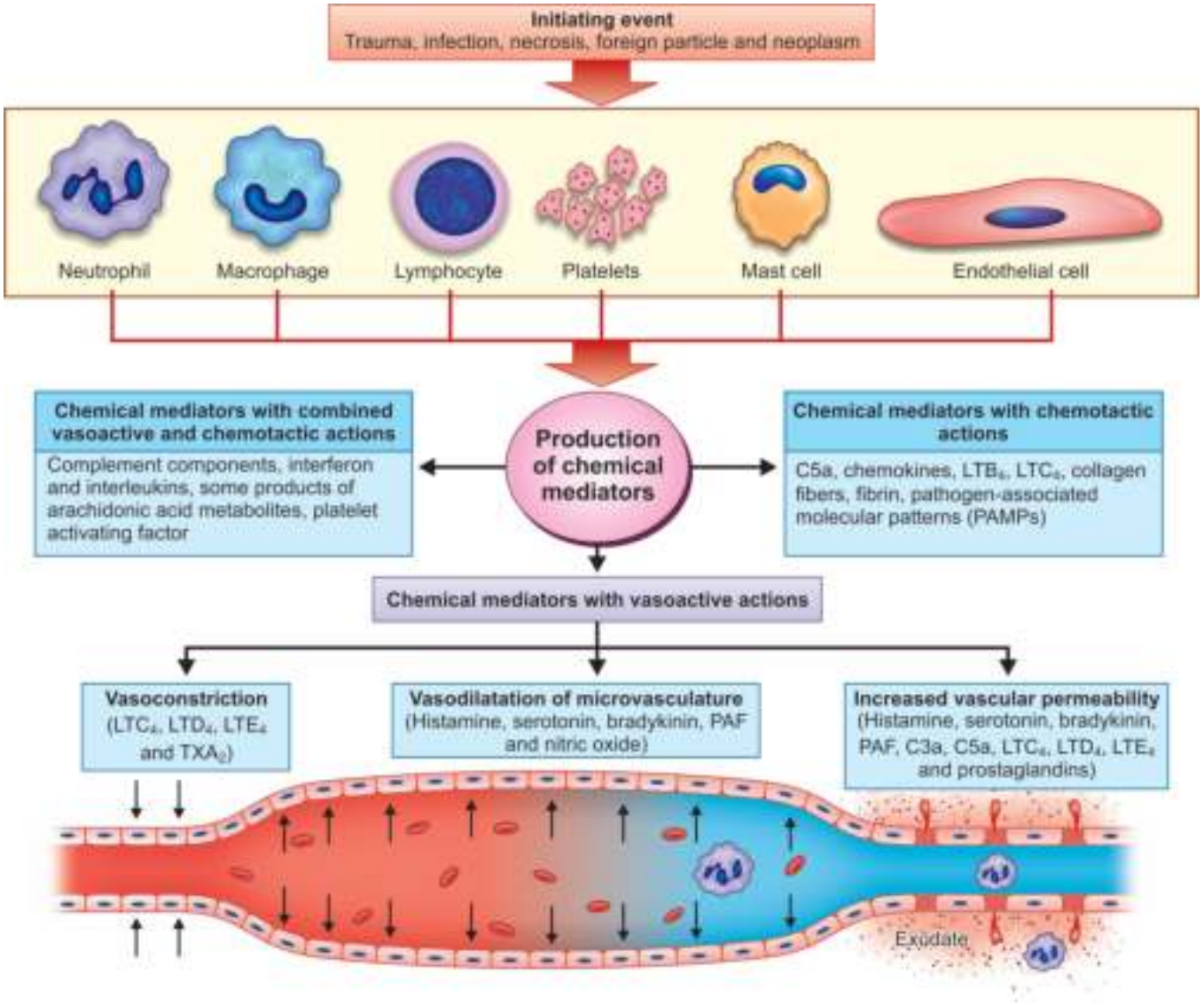


Fig. 2.22: Functional categories of chemical mediators and their inflammatory responses. Functional categories of chemical mediators include chemotactic, vasoactive and combined chemotactic and vasoactive functions.

Table 2.15 Functional categories of chemical mediators

Inflammatory Response	Chemical Mediators	Source
Chemical mediators with vasoactive actions		
Vasodilation of arterioles and capillaries	▪ Histamine ▪ Serotonin ▪ Bradykinin ▪ Platelet-activating factor (PAF) ▪ Nitric oxide ▪ Neuropeptide (substance P)	▪ Mast cells, basophils, platelets ▪ Platelets ▪ Liver (plasma-derived chemical mediators) ▪ Mast cells, basophils ▪ Macrophages, endothelium ▪ Neurons
Vasoconstriction	▪ Leukotrienes (LTC_4, LTD_4, LTE_4) ▪ Thromboxane A_2	▪ Leukocytes, mast cells ▪ Macrophages, mast cells, platelets, endothelium
Increased vascular permeability of venules	▪ Histamine ▪ Serotonin ▪ Bradykinin	▪ Mast cells, basophils, platelets ▪ Platelets ▪ Liver (plasma derived)

Contd...

Table 2.15 Functional categories of chemical mediators (*Contd...*)

Inflammatory Response	Chemical Mediators	Source
	▪ Platelet-activating factor (PAF) ▪ Anaphylatoxins (C3a, C5a) ▪ Prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$) ▪ Leukotrienes (LTC_4, LTD_4, LTE_4)	▪ Mast cells, basophils ▪ Liver (plasma derived) ▪ Macrophages, mast cells, platelets, endothelium ▪ Leukocytes, mast cells
Chemical mediators with chemotactic actions		
Chemotaxis of leukocytes	▪ C5a ▪ Chemokines ▪ Leukotrienes (LTB_4, LTC_4) ▪ Collagen fibers ▪ Fibrin (plasma derived) ▪ Pathogen-associated molecular patterns	▪ Liver (plasma derived) ▪ Leukocytes, endothelial cells ▪ Leukocytes, mast cells ▪ Tissues ▪ Liver (plasma derived) ▪ Bacterial peptides
Chemical mediators with combined vasoactive and chemotactic actions		
Vasoactive and chemotaxis of leukocytes	▪ Complement components (C3a, C5a) ▪ Interleukins ▪ Chemokines ▪ LTC_4, LTD_4, LTE_4 ▪ Platelet-activating factor	▪ Liver (plasma derived) ▪ Macrophages, dendritic cells, fibroblasts, hepatocytes ▪ Leukocytes, endothelial cells ▪ Macrophages, mast cells, endothelial cells ▪ Mast cells, basophils

PLASMA PROTEIN-DERIVED CHEMICAL MEDIATORS

Plasma protein-derived chemical mediators mediate inflammatory response. Hageman factor XII synthesized by liver is the key source of vasoactive chemical mediators.

- Hageman factor XII is activated by negatively charged surfaces (collagen, basement membrane), activated platelets and high molecular weight kininogen).
- Hageman factor activates kallikrein (kinin) system resulting in activation of fibrinolytic system, coagulation system (intrinsic pathway) and complement system at the site of injury.
- Kallikrein (kinin) system converts kininogen to bradykinin, which mediates arteriolar dilation, vascular permeability, and pain in acute inflammation.
- Kallikrein (kinin) system is activated by Hageman factor, plasmin and leukocyte protease.
- Kallikrein (kinin) is degraded by kininase and angiotensin converting enzyme (ACE) in the lungs.
- Kallikrein (kinin) system also activates complement system to generate C3, C5a, C5–9, which participate in chemotaxis of leukocytes, opsonization and phagocytosis of microbes. Plasma protein-derived chemical mediators in acute inflammation are shown in Fig. 2.23.

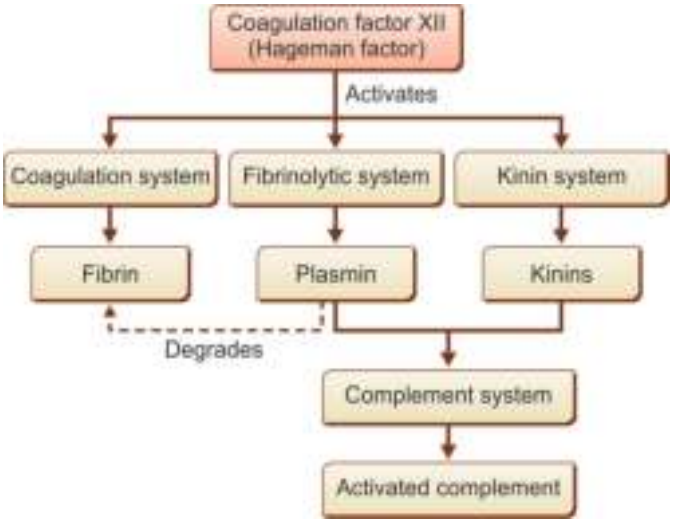


Fig. 2.23: Plasma protein derived chemical mediators in acute inflammation. These chemical mediators include complement system, coagulation cascade and kinin generation. Hageman factor (clotting factor XII) converts plasminogen to plasmin, prekallikrein to kallikrein and activation of the alternative complement pathway and coagulation system.

Fibrinolytic System

Plasminogen activator synthesized by endothelium and leukocytes activates fibrinolytic system, which converts plasminogen to plasmin, which degrades fibrin into fibrin peptides. Fibrinolytic system participates in vascular phase of inflammation is discussed as follows:

- Plasmin activates Hageman factor XII to synthesize plasma proteins related bradykinin (kinin).
- Bradykinin mediates vascular permeability, arteriolar dilation, and pain.
- Fibrinolytic system cleaves C3 to C3a. Plasmin, fibrin peptides, C3a and bradykinin increase vascular permeability.

Coagulation System

Coagulation system and inflammation are tightly linked. During final stage of coagulation, thrombin and

insoluble fibrin are formed. Thrombin and insoluble fibrin bind to seven transmembrane G protein-coupled receptors expressed on platelets, endothelial cells, smooth muscle cells and other cells. Thrombin and insoluble fibrin participate in vascular phase of acute inflammation.

- Thrombin and insoluble fibrin increase expression of adhesion molecule (P-selectin) by mobilization on vascular endothelium. These also induce integrin synthesis, resulting to leukocytic adhesion to vascular endothelium.
- Factor Xa and fibrin peptides increase vascular permeability.
- Factor Xa participates in emigration of leukocytes. Thrombin and fibrin peptides participate in recruitment of leukocytes to the site of injury. Thrombin and fibrin peptides also enhance the synthesis of chemical mediators such as chemokines, prostaglandins, platelet-activating factor, nitric oxide. Coagulation system products and their actions are given in Table 2.16.

Complement System

Complement system consists of a group of soluble plasma proteins synthesized by liver present in its inactive form that interact with one another in three distinct enzymatic-activation cascades, which are numbered C1 to C9 (C3 most abundant). Complement system cascade and its actions are shown in Fig. 2.24.

- Complement system plays important role in acute inflammation. It is a part of the innate and adaptative immune system by contributing to mediation of vascular permeability and vasodilation, leukocyte adhesion and chemotaxis, phagocytosis and cell lysis. C5a, the complement cleavage product is a powerful chemoattractant. C3b and Ic3b act as opsonins to coat bacteria. Many regulatory proteins expressed by mammals prevent inappropriate activation of complement system.
- Three pathways of complement have different triggers at starting points, but all converge at the same place, C3 convertase. This C3 convertase enzyme begins the formation of tiny openings in the cell membrane and the destruction of target pathogen.
- All these three pathways of complement system converge to form C3. C3 convertase enzyme converts C3 molecule into an activator C3b. C3 convertase enzyme and C3b are the central features of complement system activation. Activation of complement produces C3a, C5a and C5–9.

Three Pathways of Complement System

Three pathways of complement system are described as under:

- **Classical pathway of complement system:** Classical pathway of complement system is also known as immunologic pathway, which involves fixation of antibodies (IgG and IgM) and complement (C1). This pathway is rapid and nonspecific. C1q, C1, C1s are converted to form C4, C2, C3.
- **Alternate pathway of complement system:** Alternate pathway of complement system is antibody independent pathway. It is activated by bacteria, fungi, viruses and parasites and venom. Factor B and factor D are converted to C3. Deficiency of C3 affects alternate pathway of complement activation resulting to **recurrent infections** with fatal outcome, until treated.
- **Mannose-binding lectin pathway of complement system:** Mannose-binding lectin pathway of complement system is also antibody independent pathway. It binds mannose (carbohydrate) on pathogen surfaces. It is nonspecific for bacteria and viruses. MBL, MASP-1, MASP-2 are converted to form C4, C2, C3.

Mechanism of Action of Complement System

Mechanism of action of complement system is described as under:

- **C3 convertase enzyme:** It activates complement system and splits C3 into two functionally distinct C3a and C3b. C3a is released, while C3b binds to C5 convertase.

Table 2.16 Coagulation system products and their actions

Coagulation System	Actions
Thrombin and insoluble fibrin	▪ Mobilization of adhesion molecule (P-selectin) and its expression on vascular endothelium ▪ Induction of integrin synthesis, resulting to leukocytic adhesion to vascular endothelium
Factor Xa and fibrin peptides	Increased vascular permeability
Factor Xa	Emigration of leukocytes
Thrombin and fibrin peptides	▪ Recruitment of leukocytes to the site of injury ▪ Enhancing synthesis of chemical mediators such as chemokines, prostaglandins, platelet-activating factor, nitric oxide

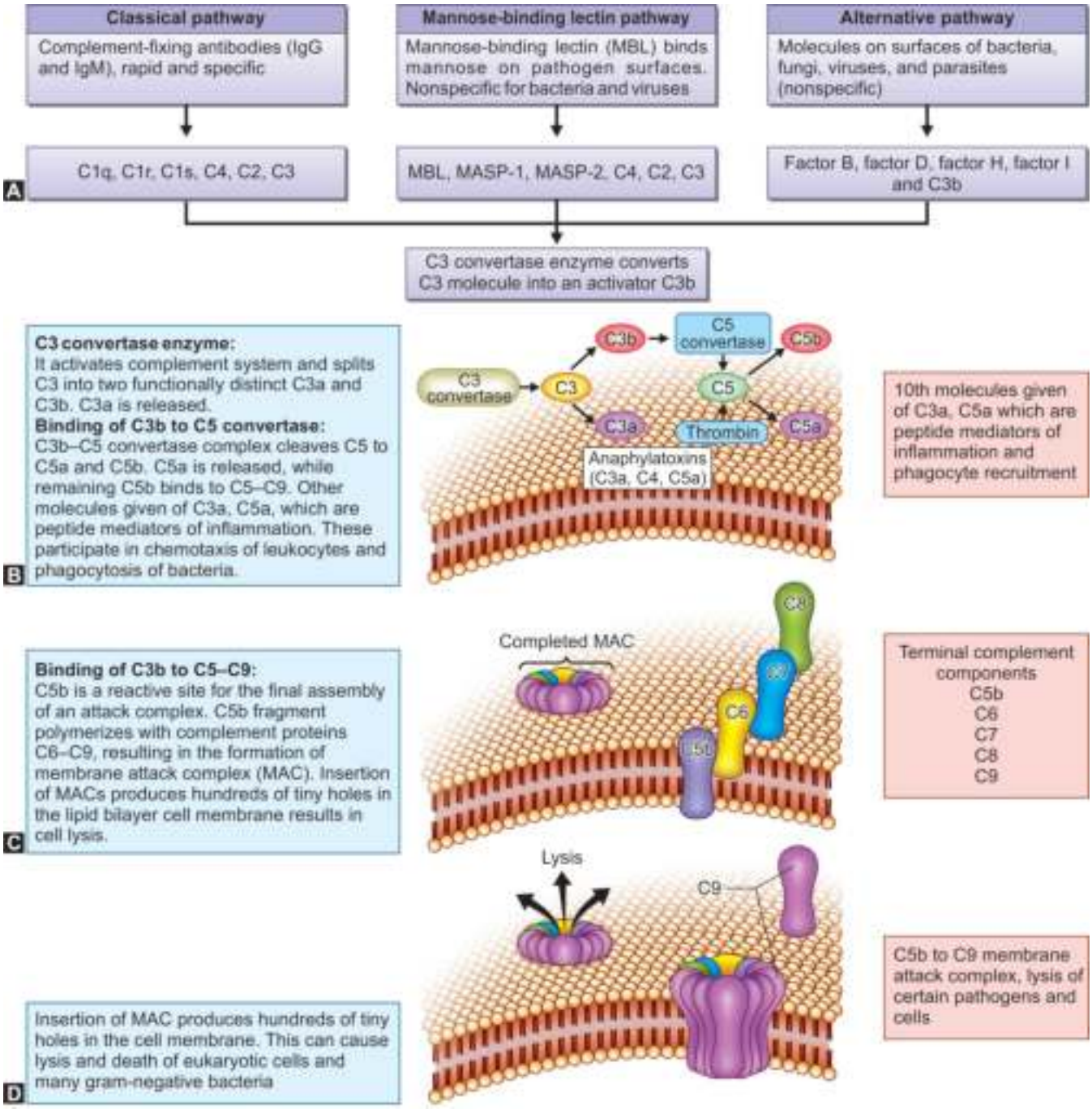


Fig. 2.24: Complement system cascade and its actions. Complement system consists of wide variety of proteins, which perform many functions: (A) defense against pyogenic bacterial infection by opsonization, chemotaxis, activation of leukocytes and degradation of microbes, (B) bridging innate and adaptive immunity, (C) disposal of immune and inflammatory products by clearance of immune complexes from the tissues and removal of apoptotic cells, and (D) MAC produces holes in the cell membrane of bacteria and kill them. The proteins involved in activating the complement system are themselves activated by three convergent pathways termed classical, mannose-binding lectin (MBL) and alternative.

- **Binding of C3b to C5 convertase:** Binding of C3b to C5 convertase forms C3b–C5 convertase complex, which cleaves C5 to C5a and C5b. C5a is released, while remaining C5b binds to C5–C9. C3a, C5a, are peptide mediators of inflammation. These participate in chemotaxis of leukocytes and phagocytosis of microorganisms in acute inflammation.
- **Binding of C3b to C5–C9:** C5b is a reactive site for the final assembly of an attack complex. C5b fragment polymerizes with complement proteins C6, C7, C8, and C9, resulting in the formation of membrane attack complex (MAC). Insertion of MACs produces hundreds of tiny holes in the lipid bilayer cell membrane results in cell lysis.

Pathology Pearls: Complement System Actions

Complement system cascade and its actions are given in Table 2.17. Breakdown products of complement system mediates acute inflammation by following mechanisms:

Opsonization and Phagocytosis

C3b acts as opsonins, which coat the bacteria leading to phagocytosis by neutrophils and macrophages.

Increased Vascular Permeability

C3a and C5a cause increased vascular permeability.

Leukocytic Adhesion, Transmigration and Chemotaxis

- C5a fragment is a chemotactic agent, which mediates the release of histamine from platelet-dense granules.
- C5a induces the expression of leukocyte adhesion molecules.
- C5a participates in transmigration and chemotaxis of leukocytes.

Anaphylactic Shock

- C3a, C5a and C4 are called anaphylatoxins, which stimulate degranulation of mast cells and basophils liberating histamine.
- Histamine causes increased vascular permeability, vasodilatation and smooth muscle contraction. Patient develops '**anaphylactic shock**'.

Synthesis of Arachidonic Acid Metabolites

C5a fragment activates lipoxygenase enzyme, which acts on membrane of neutrophils and monocytes resulting to synthesis of arachidonic metabolites (lipoxygenase pathway).

Membrane Attack Complex (MAC) Formation

- C5b-C9 is a lytic agent for bacteria and target cells, which stimulates arachidonic acid metabolism and produces reactive oxygen metabolites.
- Defective formation of membrane attack complex (MAC) leads to increased susceptibility to Neisseria organisms.

Clinical Pearls: Complement System Disorders

Patients develop increased susceptibility to infections due to defects in complement proteins, pathologic activation and deficiency of regulatory proteins. Disorders of complement system cascade are given in Table 2.18.

Recurrent Infections

- Patient presents with **increased susceptibility to infection** due to deficiency of complement components such as C2, C3, and C5.
- **Defective formation of membrane attack complex (MAC)** leads to increased susceptibility to Neisseria organisms.

Hereditary Angioneurotic Edema

- **Physiologic state:** Normally, classical pathway involves fixation antibodies (IgG and IgM) and complement (C1). C1 is inhibited by plasma protein C1 inhibitor (C1 INH).
- **Pathologic state:** Hereditary deficiency of C1 inhibitor (C1 INH) causes improper activation of C1 by immune complex resulting to excessive breakdown of C4 and C2. Complement C2 molecule generates vasoactive peptide (bradykinin), which produces painless nonpitting edema of soft tissues especially laryngeal edema, which may be life-threatening.

Systemic Lupus Erythematosus

Patient with C2 and C4 deficiencies may develop systemic lupus erythematosus (SLE) due to failure to clear immune complexes.

Paroxysmal Nocturnal Hemoglobinuria

- **Physiologic state:** Glycosylphosphatidylinositol (GPI) anchor regulatory proteins such as CD55, CD59, and C8, which are required for the protection of red blood cells, granulocytes, and platelets from complement-mediated lysis. CD55 known as DAF (decay accelerating factor) cleaves C3b. CD59 known as MIRL (membrane inhibitor reactive lysis) participates in cleaving C5–9.
- **Pathologic state:** PIG-A gene mutation encoding phosphatidylinositol linked membrane proteins leads to uncontrolled activation of complement resulting to recurrent bouts of intravascular complement mediated hemolysis.
  - **Clinical manifestations:** Recurrent bouts of intravascular complement-mediated hemolysis is often marked by the passage of hemoglobin-containing urine on awakening. During hemolytic episodes, patients develop normocytic or macrocytic anemia, accompanied by an appropriate reticulocytosis response. PNH may develop as a primary disorder or evolve from preexisting cases of aplastic anemia.
  - **Laboratory diagnosis:** Flow cytometry demonstrates diminished CD55 and CD59 expression on red blood cells, leukocytes and platelets. Traditional diagnostic test is based on hemolysis in sucrose (sucrose hemolysis test) or acidified serum (Ham test) *in vitro*.

CELL-DERIVED CHEMICAL MEDIATORS

Cell-derived chemical mediators are synthesized by monocytes, macrophages, lymphocytes, fibroblasts, mast cells, platelets, and endothelial cells of blood vessels. These participate in acute inflammation by increasing vascular permeability resulting in extravasation of plasma proteins rich fluid along with inflammatory cells in interstitial tissue '**exudate**'. Cell-derived preformed and newly synthesized chemical mediators are given in Table 2.19.

Vasoactive Amines

Vasoactive amines act directly or indirectly on the vascular system, which are stored as preformed molecules in mast cells or early inflammatory cells. Vasoactive amines include histamine, serotonin and bradykinin. Vasoactive amines chemical mediators are given in Table 2.20.

Table 2.17 Complement system cascade and its actions

Complement System Cascade	Actions
C3b	▪ Opsonization of microbes ▪ Phagocytosis of microbes
C3a and C5a	Increased vascular permeability
C5a	▪ Adhesion of leukocytes ▪ Chemotaxis of leukocytes ▪ Transmigration of leukocytes
C5a	Synthesis of arachidonic acid metabolites (lipoxygenase pathway)
C5b–C9 membrane attack complex (MAC)	Degradation of microbes and enhancing arachidonic acid metabolism and producing reactive oxygen metabolites

Defective formation of membrane attack complex (MAC) leads to increased susceptibility to Neisseria organisms. C3a, C5a and C4 cause anaphylactic shock due to excessive histamine release by mast cells.

Table 2.18 Hereditary and acquired deficiencies of complement linked to diseases in human beings

Complement Deficiency	Associated Disorder
Deficiencies of early complement components, C1, C4, C3	Systemic lupus erythematosus (SLE), glomerulonephritis, polymyositis
C1q	SLE-like syndrome; decreased secondary to agammaglobulinemia
C1r	SLE-like syndrome; dermatomyositis, vasculitis, recurrent infections and chronic glomerulonephritis, necrotizing skin lesions, arthritis
C1s	SLE-like syndrome
C1 inhibitor (C1 INH)	Hereditary angioedema, laryngeal edema. lupus nephritis
C2	Recurrent pyogenic infections, SLE-like syndrome, discoid lupus, membranoproliferative glomerulonephritis, dermatomyositis, synovitis, purpura, Henoch-Schonlein purpura, hypertension, Hodgkin disease, chronic lymphocytic leukemia, dermatitis, herpetiformis, polymyositis
C3 and factor B	Severe recurrent pyogenic bacterial infections, SLE-like syndrome, arthralgias, skin rash
C3b inactivator	Neisseria infections
C3 inactivator	Recurrent pyogenic infections, urticaria
C4	SLE-like syndrome, dermatomyositis-like syndrome, vasculitis
C5	Neisseria infections, SLE
C5 dysfunction	Leiner's disease, gram-negative skin and bowel infections
C6	Neisseria infections, SLE, Raynaud's phenomenon, scleroderma-like syndrome, vasculitis
C7	Neisseria infections, SLE, Raynaud's phenomenon, scleroderma-like syndrome, vasculitis
C8	Neisseria infections, xeroderma pigmentosum, SLE-like syndrome

(1) C2, C3, and C5 deficiency cause recurrent infections. (2) C5b–C9 defective formation of membrane attack complex (MAC) causes Neisseria infections. (3) C2 and C4 deficiency cause improper clearance of immune complex in systemic lupus erythematosus. (4) PIG-A gene mutation encoding phosphatidylinositol linked membrane proteins leading to uncontrolled activation of complement resulting to recurrent bouts of intravascular complement-mediated hemolysis in paroxysmal nocturnal hemoglobinuria. Flow cytometry demonstrating diminished CD55 and CD59 expression on red blood cells, leukocytes and platelets.

- **Histamine:** Histamine is stored in the secretory granules of mast cells, basophils and platelets. Histamine release is triggered by immune complexes, C3a, C5a, IL-1, IL-8, neuropeptide into the extracellular tissues. Histamine also increases secretion of salivary, lacrimal, respiratory and gastric glands. Histamine action on smooth muscle varies with location, which increases intestinal motility. Histamine is responsible for the wheal and flare reaction in skin, pruritus and headache. Histamine mediates acute inflammation, bronchial asthma and anaphylactic shock. Histamine is inactivated by histaminase enzyme.

Table 2.19 Cell-derived preformed and newly synthesized chemical mediators

Source	Chemical Mediators	
Preformed cell-derived chemical mediators		
Stored in the secretory granules of mast cells, basophils and platelets	Histamine	
Stored in dense bodies of platelets	Serotonin	
Neutrophils and macrophages	Lysosomal enzymes	
Newly synthesized cell-derived chemical mediators		
Cyclooxygenase pathway (arachidonic acid metabolites)	▪ Prostaglandins ▪ Prostacyclin (PGI_2)	▪ Thromboxane A_2 (TXA_2)
Lipoxygenase pathway (arachidonic acid metabolites)	▪ Leukotrienes	▪ Lipoxins
Cytokines	▪ Interleukins ▪ Tumor necrosis factor-α (TNF-α) ▪ Chemokines	▪ Interferons (IFN-α, IFN-β, IFN-γ) ▪ Growth factors
Other chemical mediators	▪ O_2-derived free radicals ▪ Nitric oxide (NO) ▪ Platelet-activating factor	▪ Neuropeptides (substance P) ▪ Hypoxia-inducible factor-α (HIF-α)

Table 2.20 Vasoactive amines chemical mediators

Chemical Mediators	Source	Actions
Histamine	Mast cell, basophils, platelets, enterochromaffin cells	▪ Increased vascular permeability (exudate) ▪ Bronchoconstriction (bronchial asthma) ▪ Anaphylactic shock (circulatory collapse)
Serotonin	Platelets	▪ Increased permeability of venules (exudate) ▪ Vasodilation ▪ Increased collagen synthesis
Bradykinin	Plasma proteins (kinin)	▪ Increased vascular permeability (exudate) ▪ Vasodilation ▪ Induction of pain

- **Acute inflammation:** Histamine binds to specific H1 receptors in the vascular wall, inducing endothelial cell contraction gap formation, increasing vascular permeability and edema formation (exudate) in acute inflammation. It occurs due to phosphorylation of contractile myosin and cytoskeleton proteins. Histamine causes smooth muscle relaxation of arterioles resulting in vasodilation.
- **Bronchial asthma:** Histamine is potent fast acting chemical mediator in allergic disorders, which causes smooth muscle contraction of bronchioles. Histamine increases mucus secretion in bronchioles and increases synthesis of 'eotaxin', which attract eosinophils.
- **Anaphylactic shock:** Massive release of histamine may cause circulatory collapse (anaphylactic shock). Patient develops hypotension, tachycardia, circulatory failure, and shock.

- **Serotonin:** Serotonin is stored in dense bodies of platelets during platelet aggregation. Serotonin release is triggered by immune complexes, C3a, C5a, IL-1, IL-8, neuropeptide into the extracellular tissues. Serotonin increases permeability of microvasculature and vasodilatation. It also causes smooth muscle contraction and inhibits gastric secretion.
- **Bradykinin:** Bradykinin is related to plasma proteins known as kinins. Kinin system converts kininogen to bradykinin, which causes vasodilation of peripheral arterioles, increased capillary permeability and induction of pain. In allergy, bradykinin causes prolonged smooth muscle contraction of bronchioles and increased mucus secretion. Bradykinin activates complement system to generate C3, C5a, C5–9, which participate in chemotaxis of leukocytes, opsonization and phagocytosis of microbes.

Neutrophil and Macrophage Lysosomal Enzymes

Macrophages and neutrophils contain lysosomal enzymes in their granules. During inflammation, lysosomal enzymes (preformed chemical mediators) are released at the site of tissue injury resulting in degradation of injurious agents. Liberation of lysosomal enzymes in the interstitial tissue may cause tissue damage.

- **Neutrophils:** Neutrophils contain azurophilic and specific granules. Specific granules are released more easily in extracellular compartment even in low concentration, in comparison to azurophilic granules. Myeloperoxidase, lactoferrin, lysozyme and major basic proteins degrade bacteria and debris within phagolysosomes. Neutral elastases degrade virulence factors of bacteria and thus combat bacterial infection. During acute inflammation, neutrophilic collagenase, hydrolase, protease and elastase degrade extracellular matrix (ECM) composed of collagen fibers, basement membrane, elastin and cartilage. Contents of polymorphonuclear cell granules are given in Table 2.21.
- **Monocytes/macrophages:** Monocytes/macrophages regulate acute and chronic inflammatory response, coagulation/fibrinolytic pathway and immune response. Primary inflammatory chemical mediators of monocytes/macrophages include enzymes (e.g. hydrolases collagenases, elastases, phospholipases), plasminogen activator, complement proteins, chemokines, cytokines, reactive oxygen species, antioxidants, coagulation factors and bioactive lipids, which participate in chronic inflammation.

Arachidonic Acid Metabolites Synthesis by Enzymatic Pathways

Arachidonic acid (AA) is 20-carbon polyunsaturated fatty acid (4 double bonds) obtained from dietary linoleic acid (essential fatty acid). It is present in its esterified form as a component of cell membrane phospholipids of tissue macrophages, mast cells, endothelial cells and recruited leukocytes.

- Tissue injury leads to influx of cytosolic calcium in tissue macrophages, mast cells, endothelial cells and recruited leukocytes.
- Increased cytosolic calcium activates membrane phospholipase A_2 enzyme resulting to synthesis of arachidonic acid known as 'eicosanoids. PGH2 serves as a substrate for cyclooxygenase and lipoxygenase enzymatic pathways. These enzymatic pathways synthesize various arachidonic acid metabolites.
- Generation of arachidonic acid metabolites and their roles in inflammation is shown in Fig. 2.25.
- Arachidonic acid metabolites, their source and actions are given in Table 2.22. The spectrum of reactions to inflammatory cytokines released by mast cells is given in Fig. 2.26.

Cyclooxygenase Pathway

- Cyclooxygenase pathway participate in synthesis of prostaglandins, prostacyclin and thromboxane A_2. Cyclooxygenase pathway is inhibited by anti-inflammatory drugs (NSAIDs and corticosteroids). COX2 inhibitor drugs produce less toxicity than COX1.
- Prolonged administration of nonsteroidal anti-inflammatory drugs may increase risk of arterial thrombosis in people possibly due to reduced

Table 2.21 Contents of polymorphonuclear cell granules

Primary (Azurophilic) Granules	Specific (Heterophilic) Granules	Tertiary Granules
▪ Myeloperoxidase ▪ Acid hydrolases ▪ Cathepsins G, B, D ▪ Defensins ▪ Bactericidal permeability increasing protein ▪ Cationic proteins ▪ Lysozyme ▪ Elastase ▪ Glucuronidase ▪ Mannosidase ▪ Phospholipase A_2	▪ Lysozyme ▪ Lactoferrin ▪ Alkaline phosphatase ▪ Nicotinamide adenine dinucleotide phosphate oxidase ▪ Collagenase ▪ Histaminase ▪ Phospholipase A_2 ▪ Complement activator ▪ CD11b/CD18 ▪ CD11c/CD18	▪ Glucuronidase ▪ Mannosidase ▪ Gelatinase ▪ Plasminogen activator ▪ Cathepsins

Neutrophil functions: Central role in acute inflammation, reactive oxygen species, phagocytosis of microorganisms and tissue debris, mediates tissue injury.

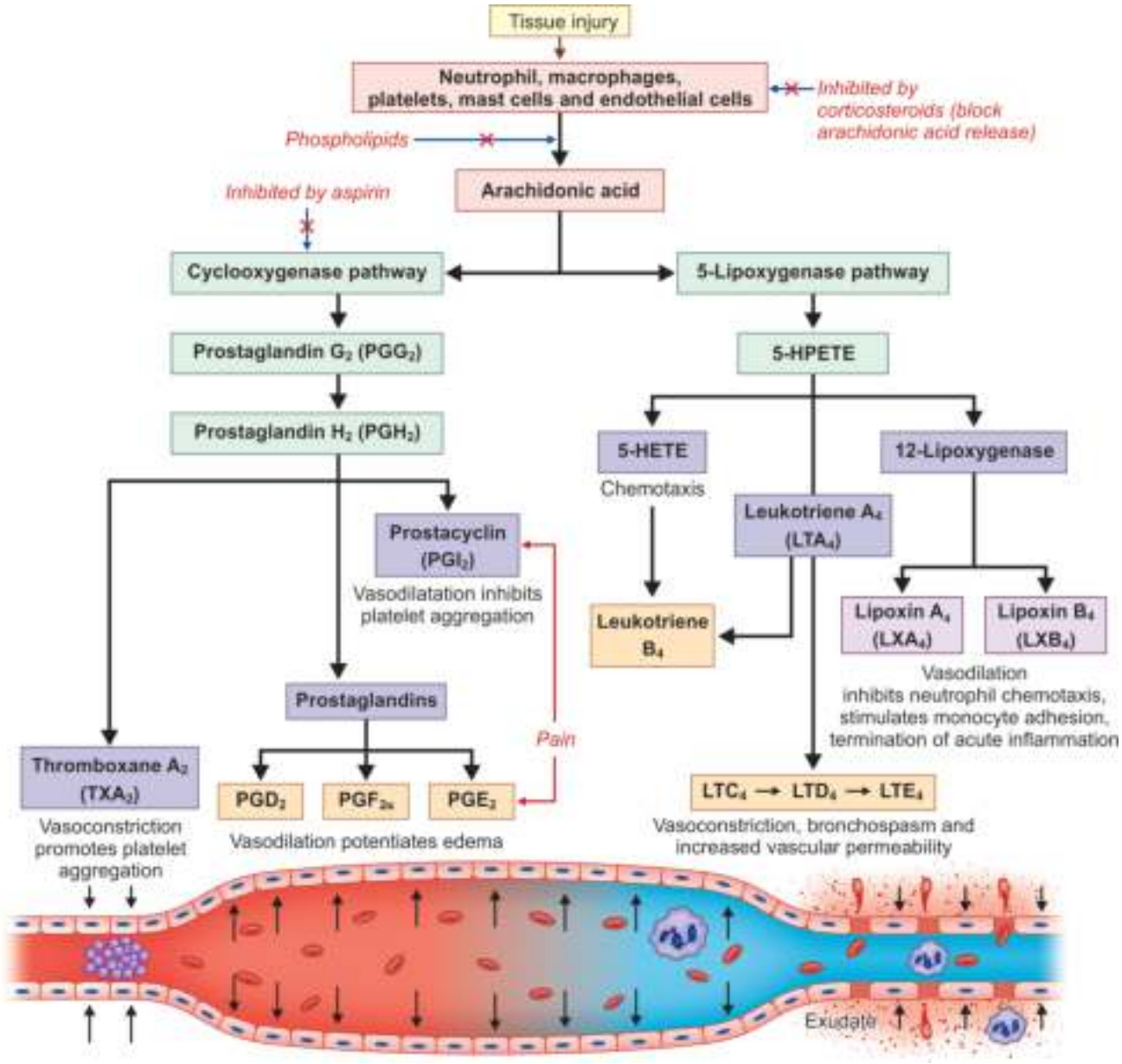


Fig. 2.25: Generation of arachidonic acid metabolites and their roles in inflammation. Arachidonic acid is produced by the actions of phospholipase A_2 on membrane phospholipids (linoleic acid). The arachidonic acid metabolites include prostaglandins, prostacyclin, thromboxane, leukotrienes and lipoxins, which play major role in the pathophysiology of inflammation.

Table 2.22 Arachidonic acid metabolites, their source and actions

Source	Chemical Mediators	Actions
Cyclooxygenase pathway		
Macrophages, endothelial cells, mast cell, platelets	▪ PGD_2 ▪ PGE_2 ▪ PGD_2, PGE_2	▪ Promotes platelets aggregation ▪ Vasodilation ▪ Increased vascular permeability ▪ Pain, fever, edema ▪ Modulation of phagocytic activity of leukocytes
Vascular endothelium (prostacyclin synthase enzyme)	PGI_2 (prostacyclin)	▪ Activating adenylyl cyclase and increases intracellular levels of cAMP ▪ Inhibiting platelets aggregation

Contd...

Table 2.22 Arachidonic acid metabolites, their source and actions (*Contd...*)

Source	Chemical Mediators	Actions
PGI_2 is converted to TXA_2 by thromboxane synthase in platelets	Thromboxane A_2 (TXA_2)	▪ Platelet aggregation (due to activation of guanylyl cyclase increasing intracellular levels of cGMP) ▪ Increased vascular permeability (serotonin released due to interaction of platelets, thrombin and collagen fibers) ▪ Vasoconstriction ▪ Smooth muscle contraction
Lipoxygenase pathway (5) in neutrophils		
Leukocytes and mast cells	▪ LTB_4 (leukotriene) ▪ LTC_4, LTD_4, LTE_4 (leukotrienes)	Adhesion, chemotaxis and activation of neutrophils ▪ Vasoconstriction ▪ Increased permeability of venules ▪ Bronchoconstriction (monteleukast therapeutic agent used to block leukotrienes receptors)
Lipoxygenase pathway (12) in platelets		
Platelets	Lipoxins (LXA_4, LXB_4)	▪ Termination of acute inflammation by inhibiting adhesion, chemotaxis of leukocytes ▪ Vasodilatation

prostacyclin in endothelial cells. Anti-inflammatory agents inhibiting cyclooxygenase pathway are given in Table 2.23.

- **Prostaglandins:** Prostaglandins are synthesized by macrophages, endothelial cells, mast cell and platelets. PGD_2 causes vasodilation, increased vascular permeability and aggregation of platelets. PGE_2 is responsible for pain, fever and edema. PGD_2 and PGE_2 modulate phagocytic activity of leukocytes.
- **Prostacyclin (PGI_2):** Prostacyclin synthase enzyme in vascular endothelium synthesizes prostacyclin. Prostacyclin activates adenylyl cyclase and increases intracellular levels of cAMP. Prostacyclin inhibits platelets aggregation. Prostacyclin participates in vasodilatation, increased vascular permeability and chemotaxis. Prostacyclin enhances the action of other chemical mediators leading to increased vascular permeability and chemotaxis.
- **Thromboxane A_2 (TXA_2):** Thromboxane A_2 is synthesized by platelets with the help of thromboxane synthase enzyme. Thromboxane A_2 activates guanylyl cyclase and increases intracellular levels of cGMP. TXA_2 promotes platelets aggregation and causes vasoconstriction. Thromboxane A_2 mediates smooth muscle contraction. Platelets come in contact with thrombin and fibrillar collagen fibers liberate serotonin resulting in increased vascular permeability.

Lipoxygenase Pathway

Lipoxygenase pathway participates in synthesis of leukotrienes (LTB_4, LTC_4, LTD_4, and LTE_4) and lipoxins (LXA_4, LXB_4). Arachidonic acid metabolites such as leukotrienes and lipoxins are synthesized by cell–cell interactions (neutrophils–platelets) by following mechanisms:

- **5-Lipoxygenase pathway in neutrophils:** Arachidonic acid metabolites of this pathway include leukotrienes and 5-HETE.
  - Leukotrienes (LTC_4, LTD_4, and LTE_4) are synthesized by leukocytes and mast cells. Injurious stimulus activates 5-lipoxygenase enzymatic pathway in neutrophils resulting in synthesis of LTA_4. Simultaneously LTA_4 is converted to leukotrienes, e.g. LTB_4 (chemotactic), LTC_4, LTD_4, and LTE_4.
  - Leukotrienes and histamine have similar actions. Histamine is less potent with rapid action. While leukotrienes are more potent chemical mediator of inflammation, but slow reacting substance of anaphylaxis (SRS-A).
  - Leukotrienes cause vasodilation and increased vascular permeability. Leukotrienes activate PMNs leukocytes leading to adhesion on venular endothelium, chemotaxis, and generation of oxygen-derived free radicals and release of lysosomal enzymes. Mechanism of actions of leukotrienes and therapeutic correlation are given in Table 2.24.
- **12-Lipoxygenase pathway in platelets:** This pathway coverts arachidonic acid to lipoxins (LXA_4 and LXB_4) in platelets. After an inflammatory response, neutrophils 'switch off' chemical mediators of inflammation such as prostaglandins and leukotrienes, and 'switch on' actions of anti-inflammatory **lipoxins**.
  - Lipoxins (LXA_4, LXB_4) terminate acute inflammation in the first few hours of injurious stimulus

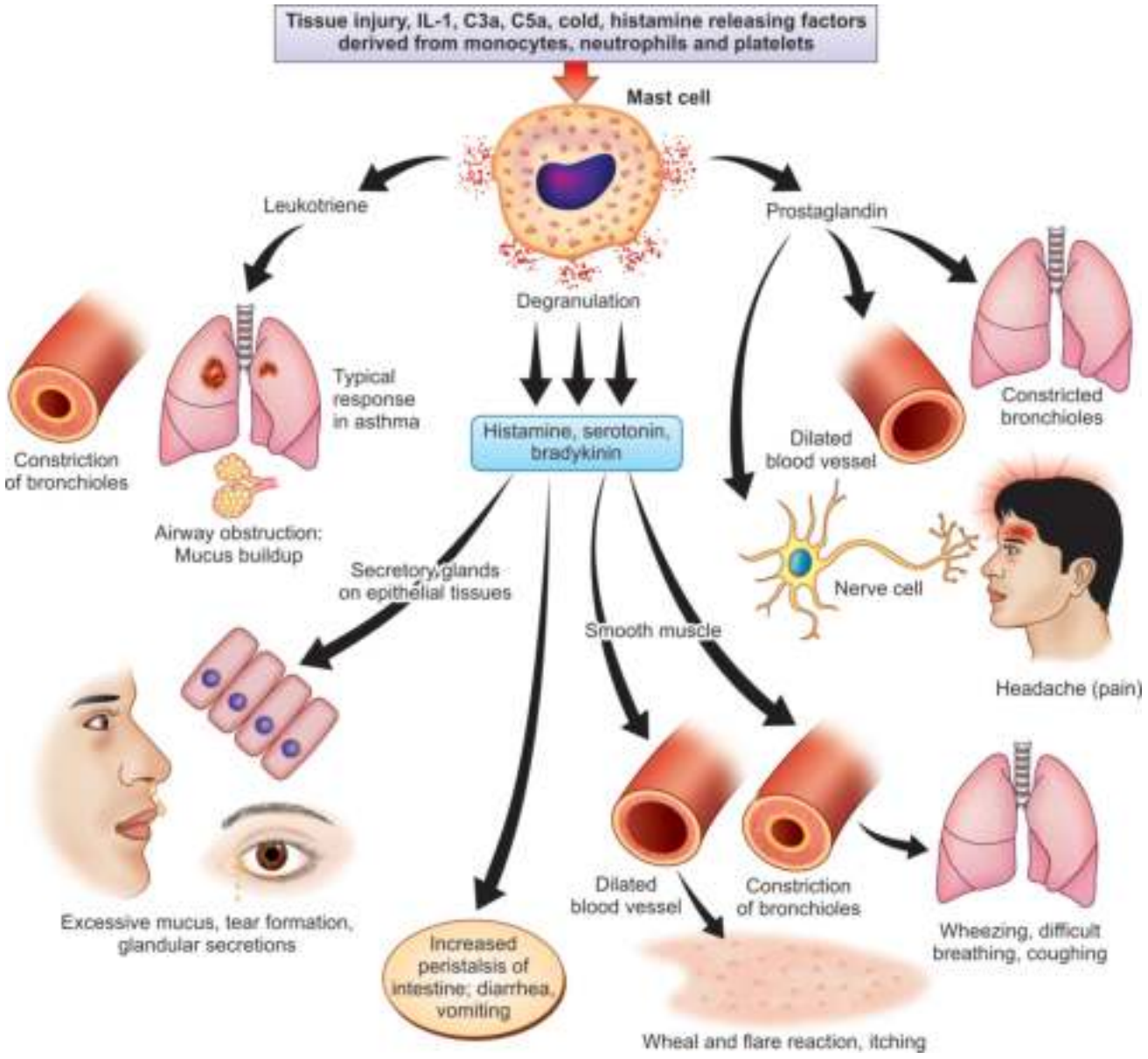


Fig. 2.26: The spectrum of reactions to inflammatory cytokines released by mast cells. These cytokines elicit overlapping extensive effects in target cells. In addition to inflammatory mediators such as histamine, prostaglandins and leukotrienes, mast cells can also synthesize wide variety of cytokines including tumor necrosis factor-α and Th2-associated cytokine such as IL-4, IL-5, IL-6, IL-10 and IL-13, which are important players in the pathogenesis of allergic reactions.

Table 2.23 Anti-inflammatory agents inhibiting cyclooxygenase pathway

Therapeutic Agents	Actions
Aspirin (NSAIDs)	Inhibiting the action of both COX1 and COX2
Meloxicam and carprofen (NSAIDs)	Inhibiting the action of COX2 without toxic effects
Glucocorticoids	Downregulating expression of gene encoding COX2 without toxic effects
Omega-3 fatty acid consumption	Serving as poor substrates for synthesizing prostaglandins

Prolonged administration of nonsteroidal anti-inflammatory drugs may increase risk of arterial thrombosis in people possibly due to reduced prostacyclin in endothelial cells.

Table 2.24 Mechanism of actions of leukotrienes and therapeutic correlation

Leukotrienes Actions	Therapeutic Agent Correlation
LTC_4, LTD_4 and LTE_4 bind to $CystLT_1$ and $CystLT_2$ receptors on bronchial smooth muscles and cause prolonged bronchospasm, increased vascular permeability, and mucus secretion of the asthmatic persons	▪ Monteleukast is used to treat bronchial asthma that blocks cysteinyl leukotrienes receptors ($CystLT_1$ and $CystLT_2$) expressed in bronchial smooth muscles ▪ Zileuton inhibits 5-lipoxygenase pathway that decreases synthesis of LTC_4, LTD_4 and LTE_4 ▪ Dietary fish rich in omega-3 fatty acid serves as poor substrates for synthesis of leukotrienes by lipoxygenase pathway ▪ Nonsteroidal anti-inflammatory drugs are not effective in inhibiting the synthesis of metabolites by lipoxygenase pathway
LTB_4 increases PMNs adhesion to endothelium and recruitment to site of tissue injury	Therapeutic correlation not applicable

Leukotrienes also participate in generation of oxygen-derived free radicals and release of lysosomal enzymes.

by inhibiting adhesion of leukocytes to endothelium as well as chemotaxis in the first few hours. Lipoxins also cause vasodilatation.

- Lipoxins transmit signal to macrophages, which phagocytose apoptotic bodies of neutrophils (programmed death).
- Lipoxins are potent anti-inflammatory chemical mediators that may have therapeutic significance.

Cytokines Synthesis

Cytokines are synthesized by activated macrophages, mast cells and endothelial cells. Cytokines usually act at short range in inflammation, which have multiple effects, such as activation of inflammatory cells resulting in degradation of injurious agents.Cytokines include interleukins, tumor necrosis factor-α, interferons (IFN-α, IFN-β, and IFN-γ), chemokines and growth factors. Cytokines chemical mediators and their actions are given in Table 2.25.

Interleukins Synthesis

Interleukins are soluble proteins synthesized by lymphocytes, monocytes–macrophages, NK cells, fibroblasts, hepatocytes and epithelial cells. Macrophages synthesize IL-1, IL-8, and IL-12. T cells synthesize IL-2, IL-3, IL-4,

Table 2.25 Cytokines chemical mediators and their actions

Source	Specific Cytokine	Actions
Interleukins		
Monocytes–macrophages, lymphocytes, dendritic cells, fibroblasts, hepatocytes and epithelial cells	▪ IL-1 ▪ IL-6 ▪ IL-8	▪ Adhesion, chemotaxis of leukocytes and phagocytosis and synthesis of acute-phase reactants by liver ▪ Synthesis of acute-phase reactants by liver ▪ Chemotaxis of leukocytes
Tumor necrosis factors		
TNF-α synthesis by macrophages, T cells, and NK cells	TNF-α	▪ Adhesion of leukocytes ▪ Synthesis of acute-phase reactants
TNF-β synthesis by T cells	TNF-β	▪ T cell proliferation ▪ Cytotoxic to some tumor cells
Interferons		
Macrophages, T cells, and NK cells	IFN-α	Antiviral activity
Fibroblasts	IFN-β	Antiviral activity
Natural killer cells (NK cells)	IFN-γ	▪ Antiviral activity ▪ Activation of macrophages and T cells during chronic inflammation ▪ Increasing cytotoxicity of natural killer cells

Contd...

Table 2.25 Cytokines chemical mediators and their actions (*Contd...*)

Source	Specific Cytokine	Actions
Chemokines: C-X-C (α), C-C (β), XC (γ) and CX3C		
Leukocytes, endothelial cells	▪ C-X-C (α) ▪ C-C (β-chemokine) ▪ XC (γ-chemokine) ▪ CX3C	▪ Chemotaxis of neutrophils ▪ Chemotaxis of monocytes ▪ Chemotaxis of lymphocytes ▪ Chemotaxis of monocytes and T cells
Growth factors: G-CSF, GM-CSF, IL-7: and IL-13		
Macrophages, neutrophils and T cells. fibroblasts and vascular endothelium	G-CSF	Activation and differentiation of neutrophils
Fibroblasts and vascular endothelium	▪ G-CSF ▪ GM-CSF	▪ Hematopoiesis ▪ Promoting the growth and development of macrophages from undifferentiated precursor stem cells ▪ Activation of natural killer cells and dendritic cells
Stromal cells in bone marrow, dendritic cells, hepatocytes, keratinocytes, neurons and epithelial cells except normal lymphocytes	IL-7	Acting as a growth factor for bone marrow stem cells
T cells	IL-13	Acting as a growth factor for bone marrow stem cells

IL-5, IL-9, IL-10, IL-13, and IL-16. Stromal cells synthesize IL-7. Interleukins mediate communications between leukocytes such as B cells, T cells, NK cells, monocytes, macrophages, hematopoietic cells and many other cell types. IL-2 and IL-4 favor lymphocytes growth. IL-10 and TGF-β disfavor lymphocytes growth. Interleukins, source and their actions are given in Table 2.26.

Table 2.26 Interleukins, source and their actions

Source	Interleukins	Actions
Chemotactic activity		
Monocytes/macrophages	▪ IL-1 ▪ IL-8	▪ Master cytokine stimulating T cells proliferation and IL-2 synthesis ▪ Adhesion of neutrophils ▪ Chemotaxis of neutrophils ▪ Phagocytosis of injurious agent by neutrophils ▪ Induces fever ▪ Chemotaxis of neutrophils
CD4+ T cells	IL-9	Chemotaxis of neutrophils and fever
T-suppressor cells and eosinophils	IL-16	Chemotaxis of T-helper cells
Synthesis of acute-phase reactants		
T cells, B cells, macrophages and fibroblasts	IL-6	Stimulating liver to synthesize acute-phase reactants
Bone marrow stromal cells	IL-11	Stimulating liver to synthesize acute-phase reactants
Synthesis of other cytokines		
T-helper cells	IL-17	Synthesis of IL-6, IL-8 G-CSF and PGE_2
Regulation of lymphocytes		
T cells, NK cells and macrophages	IL-2	▪ Stimulates proliferation of T cells, B cells (immunoglobulin synthesis), and NK cells; activates monocytes ▪ Activated natural killer cells kill of cancer cells, fungi and virally infected cells by antibody-dependent cell-mediated cytotoxicity (ADCC)
T cells	IL-4, IL-13 (Both have similar functions)	▪ IL-4 and IL-13 promoting growth of B and T cells ▪ IL-4 and IL-13 switching B cells to synthesize IgE antibody in type 1 hypersensitivity reactions ▪ IL-4 also enhancing expression of HLA class II antigens

Contd...

Table 2.26 Interleukins, source and their actions (*Contd...*)

Source	Interleukins	Actions
Specific immune response		
T cells	IL-5	▪ IL-5 promotes end-stage maturation of B cells into plasma cells ▪ Priming of basophils to release histamine and leukotrienes and chemotaxis of eosinophils in type 1 hypersensitivity reaction
T cells, B cells, macrophages and fibroblasts	IL-6	▪ Promoting maturation of B and T cells ▪ Inhibiting growth of fibroblasts ▪ Inducing synthesis of acute-phase reactants by liver ▪ Promoting mucus secretion
T cells	IL-9	Proliferation of T cells
T cells	IL-12	Activating T cells and natural killer cells
Monocytes/macrophages	IL-15	Proliferation of T cells and B cells
T cells	IL-10	Inhibiting macrophages, B cells and synthesis of interferons
Growth factors activity		
T cells	IL-3	Acting as a growth factor for tissue mast cells and hematopoietic stem cells
T cells	IL-4	Promoting growth of B and T cells; enhances expression of HLA class II antigens
Stromal cells in bone marrow, dendritic cells, hepatocytes, keratinocytes, neurons and epithelial cells	IL-7	Acting as a growth factor for bone marrow stem cells

Tumor Necrosis Factors Synthesis

Tumor necrosis factor-α (TNF-α, cachectin) is synthesized by macrophages, T cells, and NK cells, which participates in adhesion of leukocytes and synthesis of acute phase reactants in acute inflammation. Tumor necrosis factor-β (TNF-β) is synthesized by T cells, which stimulates T cell proliferation and IL-2 production. It is cytotoxic to some tumor cells. Tumor necrosis factors and their actions are given in Table 2.27.

Interferons Synthesis

Interferons (IFN-α, IFN-β, and IFN-γ) initiate an antiviral state in cells by blocking viral protein synthesis and inhibiting cell growth. When a cell is infected, its nucleus is triggered to transcribe and translate the interferon (IFN). The interferon diffuses out of the infected cells into nearby (uninfected) cells, where it enters the nucleus. IFN activates a gene for synthesizing a peptide that blocks viral replication. It is worth mentioning that IFN does not protect the original cells and that IFN prevents viruses from invading protected cells.

- Macrophages and T cells synthesize interferon-α (IFN-α). Fibroblasts synthesize interferon-β (IFN-β). Interferon-γ (IFN-γ) is synthesized by activated T cells and NK cells. Interferon-α and interferon-β activate natural killer cells. Interferon induces synthesis of antiviral molecules.
- Interferon-γ (IFN-γ) activates macrophages and T cells during chronic inflammation and enhances expression of HLA class II antigens. IFN-γ participates in differentiation of T and B cells, increases the cytotoxicity of natural killer cells, activates neutrophils and stimulates diapedesis. Human interferons (IFNs) are given in Table 2.28. Antiviral activity of interferon is shown in Fig. 2.27.

Chemokines Synthesis

- Chemokines are synthesized by macrophages and endothelial cells. Chemokines are classified into four groups according to the arrangement of the conserved cysteine (CC) residues in the mature

Table 2.27 Tumor necrosis factors and their actions

Characteristics	TNF-α	TNF-β
Principal source	▪ Macrophages ▪ T cells ▪ NK cells	T cells
Actions	▪ Adhesion of leukocytes ▪ Synthesis of acute-phase reactants	▪ T cell proliferation ▪ Cytotoxic to some tumor cells

Table 2.28 Human interferons (IFNs)

Characteristics	IFN-α	IFN-β	IFN-γ
Principal source	All cells	All cells	T lymphocytes (NK cells)
Inducing agent	Viral infection	Viral infection	Antigen
Chromosomal location of gene encoding IFN	22	1	1
Antiviral property	+++	+++	+
Macrophage action	Absent	Absent	++
MHC-I upregulation	+	+	+
MHC-II upregulation	Absent	Absent	+

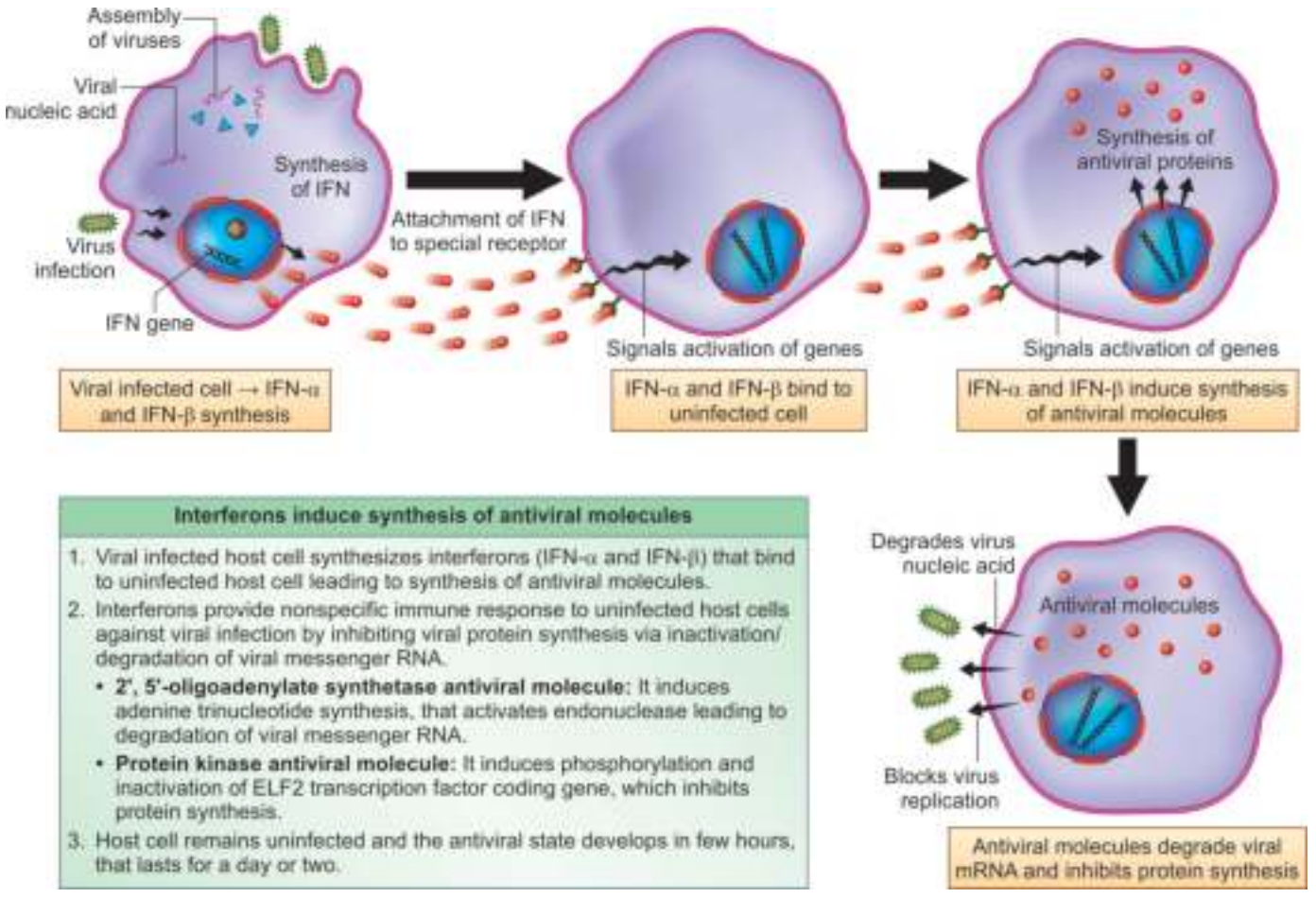


Fig. 2.27: Antiviral activity of interferon. When a cell is infected by a virus, its nucleus is triggered to transcribe interferon (IFN) gene. Interferon diffuses out of the cell and then binds to IFN receptors on nearby uninfected cells, where it induces production of proteins that eliminate viral genes and block viral replication. Note that the original cell is not protected by IFN and that does not prevent viruses from invading the protected cells.

proteins: (a) C-X-C chemokine (α-chemokine), (b) C-C chemokine (β-chemokine), (c) C chemokine (γ-chemokine) and (d) CX3C chemokine.

- Chemokines participate in recruitment of inflammatory cells (neutrophils, monocytes) to the injured site. These also increase the adhesion integrin affinity of leukocytes to ligand on vascular endothelium.
- Chemokines bind to G protein-coupled receptors and exert biological effects. HIV enters into lymphocytes via G protein-coupled receptors (CXCR4, CCR5). Other chemokines include C5a, IL-8 and platelet-activating factor. Chemokines and their actions are given in Table 2.29.

Growth Factors Synthesis

- **Interleukin-4 (IL-4):** It is synthesized by T cells, which promotes growth of B and T cells and enhances expression of HLA class II antigens.

Table 2.29 Chemokines and their actions

Specific Chemokine	Actions
C-X-C chemokine	
α-Chemokine	Activation and chemotaxis of neutrophils
***C-C chemokine (β-chemokine) examples**	
MCP-1 (monocyte chemotactic protein 1)	Chemotaxis of monocytes
RANTES (regulated and named T cell expressed and secreted)	Chemotaxis of lymphocytes
Eotaxin	Chemotaxis of eosinophils or basophils
C chemokine (γ-chemokine)	
Lymphotoxin	Chemotaxis of lymphocytes
CX3C chemokine	
Fractalkine	▪ Chemotaxis of monocytes and T cells ▪ Promoting strong adhesion of monocytes and T cells

**C-C chemokine (β-chemokine) molecule does not attract neutrophils.*

- **Interleukin-7 (IL-7):** It is synthesized by stromal cells in bone marrow, dendritic cells, hepatocytes, keratinocytes, neurons and epithelial cells except normal lymphocytes, which acts as a hematopoietic growth factor for bone marrow stem cells.
- **Interleukin-13 (IL-13):** It is synthesized by T cells, which acts as a growth factor for bone marrow hematopoietic stem cells.
- **Granulocyte colony-stimulating factor (G-CSF):** It is synthesized by macrophages, neutrophils and T cells, which participates in activation and differentiation of neutrophils.
- **Granulocyte-macrophage colony-stimulating factor (GM-CSF):** It is synthesized by fibroblasts and vascular endothelium, which participates in hematopoiesis. GM-CSF promotes the growth and development of macrophages from undifferentiated precursor stem cells. GM-CSF also activates natural killer cells and dendritic cells. Growth factors, source and their actions are given in Table 2.30.

Oxygen-derived Free Radicals Synthesis

Oxygen-derived free radicals (i.e. reactive oxygen species) play key role in microbial killing and tissue injury. Organisms killed by reactive oxygen species are given in Table 2.31.

- Injurious stimulus activates neutrophils and macrophages, which synthesize oxygen-derived free radicals via NADPH oxidase pathway (i.e. superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl

Table 2.30 Growth factors, source and their actions

Source	Growth Factors	Actions
T cells	IL-3	Acting as a growth factor for tissue mast cells and hematopoietic stem cells
T cells	IL-4	IL-4 promoting growth of B and T cells; enhances expression of HLA class II antigens
Stromal cells in bone marrow, dendritic cells, hepatocytes, keratinocytes, neurons and epithelial cells	IL-7	IL-7 acting as a growth factor for bone marrow stem cells
Monocytes/macrophages, neutrophils, T cells, fibroblasts and vascular endothelium	G-CSF	Activation and differentiation of neutrophils
Fibroblasts and vascular endothelium	GM-CSF	▪ Hematopoiesis ▪ Promoting the growth and development of macrophages from undifferentiated precursor stem cells ▪ Activation of natural killer cells and dendritic cells
Stromal cells in bone marrow, dendritic cells, hepatocytes, keratinocytes, neurons and epithelial cells except normal lymphocytes	IL-7	Acting as a growth factor for bone marrow stem cells
T cells	IL-13	Acting as a growth factor for bone marrow stem cells

Table 2.31 Organisms killed by reactive oxygen species

Bacteria	Fungi	Protozoa
▪ *Staphylococcus aureus* ▪ *Escherichia coli* ▪ *Serratia marcescens*	▪ *Candida albicans* ▪ Aspergillus	▪ Plasmodium ▪ *Leishmania donovani* (killed by nitric oxide)

ions (OH). Oxygen-derived free radicals combine with nitric oxide to form nitrogen intermediates.

- Oxygen-derived free radicals play an important role in microbial killing in phagolysosome and tissue injury. These cause damage to vascular endothelium resulting in increased vascular permeability. These also increase synthesis of cytokines and cell adhesion molecules, thus amplify the cascade of inflammatory mediators in acute inflammation.
- Serum, tissue fluids and host cells possess antioxidant mechanisms that protect against these potentially harmful oxygen-derived free radicals. These antioxidants include: (a) ceruloplasmin—copper binding serum protein, (b) transferrin—iron-free fraction of serum, (c) superoxide dismutase enzyme, (d) catalase—H_2O_2 detoxifier and (e) glutathione peroxidase—another powerful H_2O_2 detoxifier.

Nitric Oxide Synthesis

Nitric oxide (NO) is a free radical soluble gas synthesized and released by macrophages and vascular endothelium during conversion of arginine to citrulline by NO synthase. Actions of NO are shown in Fig. 2.28. Mechanism of action of nitric oxide on target cells is given in Table 2.32.

- **Physiologic state:** Nitric oxide, which was previously known as endothelium-derived relaxing factor. It is a free radical soluble gas synthesized in low concentration, which has half-life of seconds (short lived) *in vivo*. Nitric oxide synthase enzyme converts L-arginine to citrulline resulting in release of nitric oxide in vascular endothelium (endothelial inducible nitric oxide—eNO), macrophages (macrophage-inducible iNO) and neurons (nuron inducible nNO).
- **Pathologic state:** During acute inflammation, inflammatory cytokines (IL-1, TNF-α and IFN-γ) and bacterial endotoxin participate in synthesis of nitric oxide.
  - Calcium influx in vascular endothelium and neurons stimulates nitric oxide synthase enzyme resulting in synthesis of nitric oxide. TNF-α and IFN-γ stimulate macrophage-inducible synthase enzyme to synthesize nitric oxide.

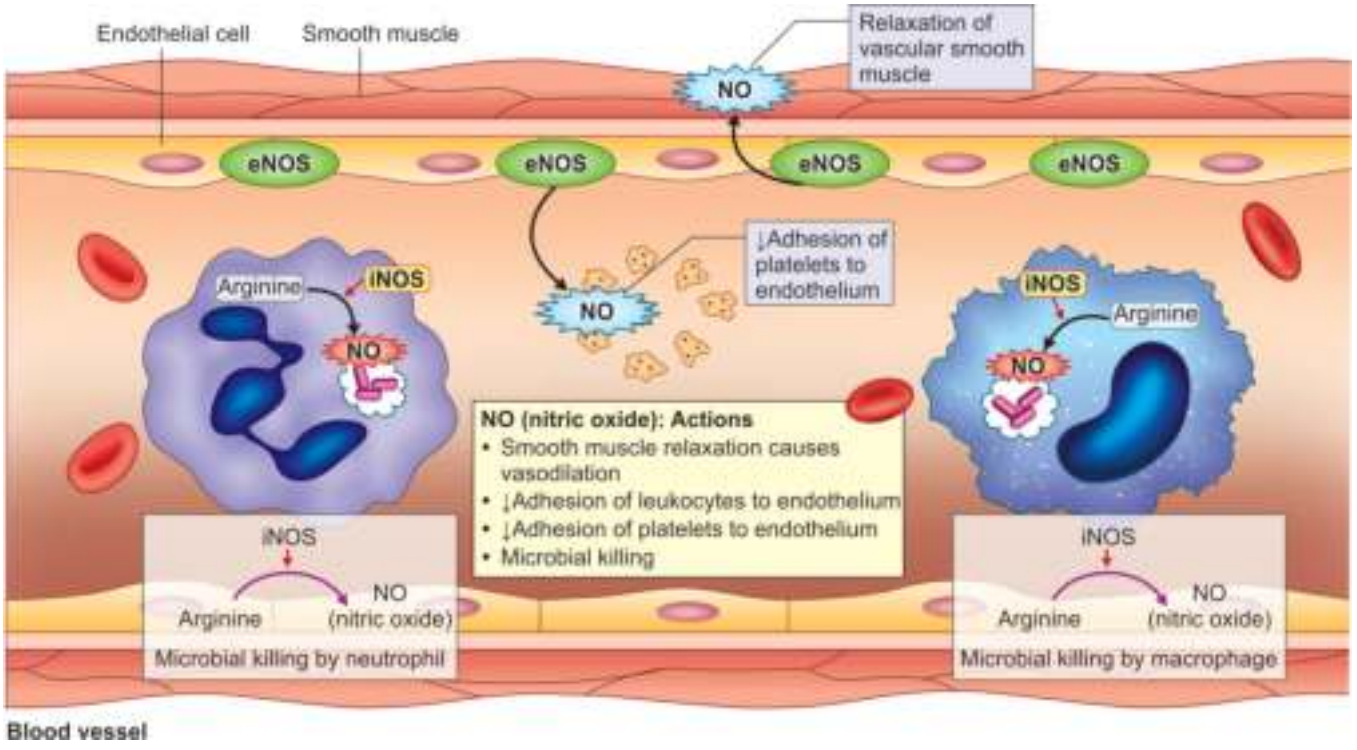


Fig. 2.28: Actions of nitric oxide. Nitric oxide is produced by vascular endothelium and macrophages. Nitric oxide causes vasodilatation and degradation of microbes, inhibits platelets aggregation and thus prevents thrombus formation. Sustained synthesis of nitric oxide in patient with septicemia results in hypotensive shock.

Table 2.32 Mechanism of action of nitric oxide on target cells

Target Organs	Action
Blood vessels	▪ Vasodilatation by relaxation of smooth muscle cells ▪ Hypotensive shock in septic patients
Leukocytes	Decreasing adhesion of leukocytes to vascular endothelium
Platelets	▪ Inhibits platelets aggregation and degranulation ▪ Preventing thrombus formation
Brain	Regulation of neurotransmitter by increasing blood flow to brain
Microbes	Killing of microbes (nitric oxide derived reactive oxygen metabolites toxic to microbes)
Tumor cells	Killing of tumor cells by macrophages

- Nitric oxide acts in paracrine manner on target cells through induction of cyclic guanosine monophosphate (GMP), which initiates intracellular events. Nitric oxide and platelet-activating factor (PAF) have opposite action.

Platelet-activating Factor Synthesis

Platelet-activating factor (PAF) is cell membrane phospholipids synthesized by platelets, mast cells/basophils, neutrophils, macrophages, monocytes, endothelial cells, and vascular endothelium.

- Platelet-activating factor acts via G protein-coupled receptor and can elicit most of the cardinal features of inflammation. Platelet-activating factor and nitric oxide have opposite action.
- Platelet-activating factor increases vascular permeability, smooth muscle contraction of bronchioles (bronchoconstriction), pulmonary edema, hypotension, and wheal and flare in the skin. PAF kills bacteria and tumor cells. PAF enhances leukocyte adhesion, chemotaxis, leukocyte degranulation and the oxidative burst. PAF also stimulates the synthesis of other chemical mediators, particularly eicosanoids.
- Physiologic actions of platelet-activating factor and histamine are similar. Platelet-activating factors and their functions are given in Table 2.33. Differences between nitric oxide and platelet-activating factor are given in Table 2.34.

Neuropeptides Synthesis

Neuropeptides are small proteins synthesized by nerve fibers mainly in lungs and gastrointestinal tract. Neuropeptides are related to kinin family.

Table 2.33 Platelet-activating factors and their functions

Platelet-activating Factor	Functions
Mast cells/basophils derived PAF	▪ Increases vascular permeability leading to edema ▪ Vasoconstriction
Endothelial derived PAF	Priming and chemotaxis of leukocytes
Neutrophils derived PAF	▪ Increasing leukocyte adhesion to endothelium by integrin mediated mechanism ▪ Chemotaxis, degranulation and oxidative burst of leukocytes ▪ Enhances synthesis of eicosanoids by leukocytes
Monocytes/macrophages derived PAF	Adhesion of leukocytes of vascular endothelium
Platelets derived PAF	▪ Platelets aggregation ▪ Thrombus formation

Platelet-activating factor is more potent mediator than histamine.

Table 2.34 Differences between nitric oxide and platelet-activating factor

Characteristics	Nitric Oxide	Platelet-activating Factor
Blood vessels	Vasodilatation	Vasoconstriction and increased vascular permeability
Leukocytes	Inhibits leukocyte recruitment	Enhances chemotaxis, degranulation and oxidative burst
Platelets	Inhibits platelet aggregation	Enhances platelet aggregation
Killing of microbes/tumor cells	Absent	Present
Respiratory bronchi/ bronchioles	No effect	Bronchospasm

- Neuropeptides and bradykinin have similar functions. Neuropeptide synthesis is activated by capsaicin (chiles), which transmits pain signals, regulates blood pressure, modulates vascular permeability; and stimulates secretion of endocrine cells.
- Neuropeptides also synthesize other proinflammatory molecules, which are thought to link the sensing of dangerous stimuli to development of protective host responses. Neuropeptides and their actions are given in Table 2.35.

Hypoxia-inducible Factor Synthesis

Hypoxia-inducible factor-α (HIF-α), a protein released from injured cells, mediates cell injury and inflammatory response. HIF-α activates many genes involved in inflammation, including VEGF, which increases vascular permeability. Local and systemic effects of chemical mediators are given in Table 2.36.

MORPHOLOGIC PATTERNS OF ACUTE INFLAMMATION

Vascular and cellular responses in acute inflammation produce distinctive morphologic patterns in various tissues. Location, cause, and duration of inflammation determine the morphology of an inflammatory reaction. Reactive gliosis is a normal response of the brain to injury and infection but is not visible on the cut surface of the brain at autopsy. Morphologic patterns of acute inflammation are described below.

SEROUS INFLAMMATION

Serous inflammation is a type of acute inflammation characterized by the copious effusion of non-viscous serous fluid, commonly produced by cells of serous membranes but may be derived from blood plasma.

Table 2.35 Neuropeptides and their actions on nerves in lungs and gastrointestinal tract

Transmission of pain signals
Regulation of blood pressure
Modulation vascular permeability
Stimulation of secretion of endocrine cells
Synthesis of other proinflammatory molecules, which are thought to link the sensing of dangerous stimuli to that development of protective host responses

- **Serous inflammation of coelomic cavities:** Serous inflammation occurs in coelomic cavities (e.g. pleural, pericardial and peritoneal cavities) and skin. Serous fluid is yellow and straw colored with scant cells, which is either derived from plasma proteins or secretion of mesothelial cells of serous cavities. Tissue looks dull engorged with blood vessels.
- **Skin blisters:** Skin blisters are caused by burns and viral infection. Serous fluid is accumulated either within or immediately beneath the epidermis of the skin.

FIBRINOUS INFLAMMATION

Deposition of fibrin-rich exudates in meninges and coelomic surfaces (pleura, pericardium and peritoneum) is known as fibrinous inflammation, which occurs as a result of increased vascular permeability due to acute inflammation and malignancy leading to extravasation of fibrinogen over coelomic surfaces. Surface of the heart is covered by shaggy fibrinous exudates giving 'bread and butter' appearance. Fibrinous exudate may be removed by fibrinolysis. Cellular debris is cleared by macrophages. When fibrin is not removed, it may stimulate the growth of fibroblasts and capillaries and thus leading to organization (fibrous strands) and obliteration of the pericardial sac.

Table 2.36 Local and systemic effects of chemical mediators

Local and Systemic Effects	Chemical Mediators
Increased vascular permeability and edema	Histamine, anaphylatoxins (C3a, C5a), bradykinin, leukotrienes (LTC_4, LTD_4, and LTE_4), nitric oxide, platelet-activating factor and neuropeptide (substance P)
Vasodilatation	Histamine, serotonin, bradykinin, platelet-activating factor, nitric oxide
Vasoconstriction	LTC_4, LTD_4, LTE_4, TXA_2
Adhesion of neutrophils to endothelium	C5a, LTB_4, IL-1, TNF-α, chemokines and platelet-activating factor
Chemotaxis of leukocytes and their activation	C5a, LTB_4, IL-1, TNF-α, chemokines and bacterial products
Pain	Prostaglandins, bradykinin, and neuropeptides (substance P)
Fever	Prostaglandins, IL-1 acting on hypothalamic thermoregulatory centers
Bronchoconstriction	Histamine and leukotrienes
Tissue damage	Lysosomal enzymes, oxygen-derived free radicals, hypochlorous acid, prostaglandins, leukotrienes, and nitric oxide

PURULENT (SUPPURATIVE) INFLAMMATION

Pyogenic bacteria such as staphylococci cause liquefactive necrosis of tissue leading to formation of opaque pus due to excessive production of exudate. Pus contains cellular debris derived from damaged tissue and polymorphonuclear cells. The destroyed tissue is restored and eventually replaced by fibrosis. Patient presents with pain induced by bradykinin and edema fluid. Complications of purulent (suppurative) inflammation include thrombophlebitis, massive bacteremia, and septic shock, embolization to various solid organs producing septic infarcts, lymphangitis and lymphadenitis.

- **Abscess in organs:** Abscesses in various organs include skin abscess, brain abscess, pyogenic meningitis, lung abscess, lobar pneumonia, bronchopneumonia, splenic abscess, breast abscess, empyema gall bladder and acute appendicitis. There is danger of perforation of suppurative acute appendicitis; hence early appendectomy is the treatment of choice. Multiple splenic abscess is shown in Fig. 2.29. Histology of acute appendicitis is shown in Figs 2.30 and 2.31. Histology of acute lymphadenitis is shown in Fig. 2.32. Histology of lobar pneumonia is shown in Fig. 2.33.

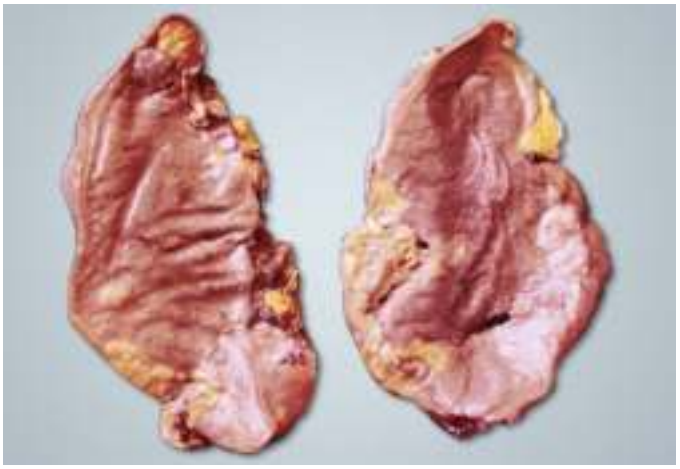


Fig. 2.29: Splenic abscesses. Splenic abscesses are most commonly seen as complications of infective endocarditis caused by streptococci and staphylococci, which occur in 5% of patients. Splenic abscesses can have a fatal outcome, healing of abscesses or chronic clinical course. (*Courtesy:* Department of Pathology, Sapthagiri Institute of Medical Sciences, Bengaluru, Karnataka).

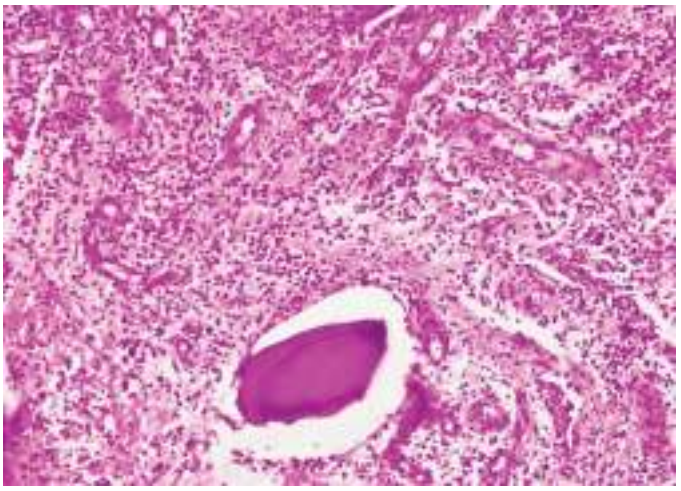


Fig. 2.30: Histology of acute appendicitis. Pathognomonic feature of acute appendicitis on histopathology is predominantly neutrophilic infiltration in muscular coat or all layers of appendix (400X).

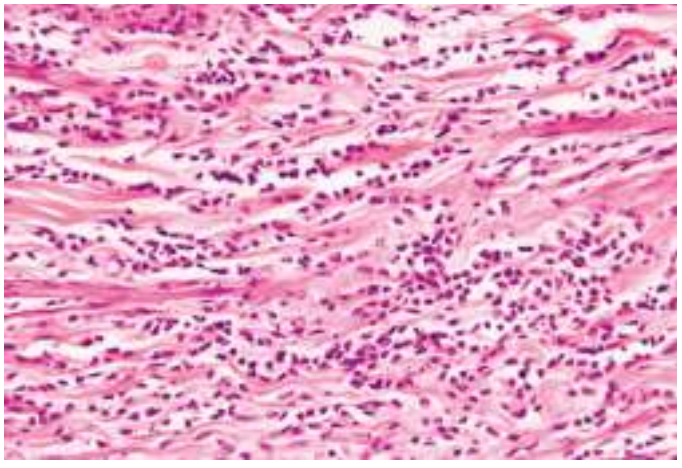


Fig. 2.31: Histology of acute appendicitis. Pathognomonic feature of acute appendicitis on histopathology is predominantly neutrophilic infiltration in muscular coat or all layers of appendix. Pathogenesis includes obstruction of appendiceal orifice and subsequent bacterial infection. Most common symptom is periumbilical pain radiating to the right lower quadrant. On clinical examination, there may be rebound tenderness and percussion pain over McBurney point and guarding (400X).

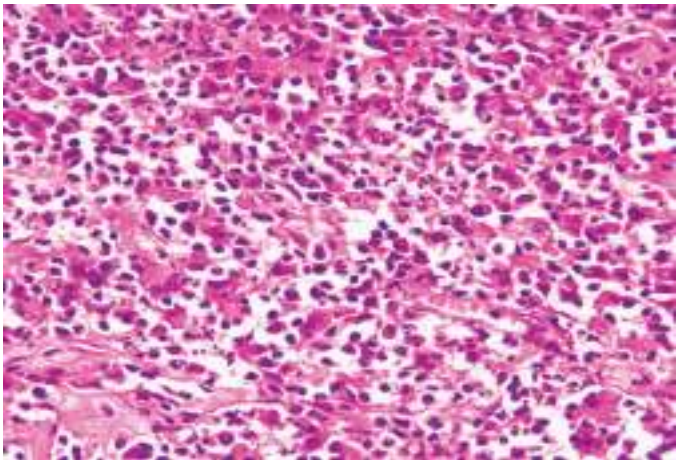


Fig. 2.32: Histology of acute nonspecific lymphadenitis. Acute nonspecific lymphadenitis most commonly affects children in cervical region caused by bacterial infection. Children present with enlarged, painful and tender lymph nodes, redness on overlying skin, low-grade fever and malaise. Microscopic examination of lymph node, is performed will show sinus dilatation followed by accumulation of polymorphonuclear cells, vascular dilatation and edema of the capsule (400X).

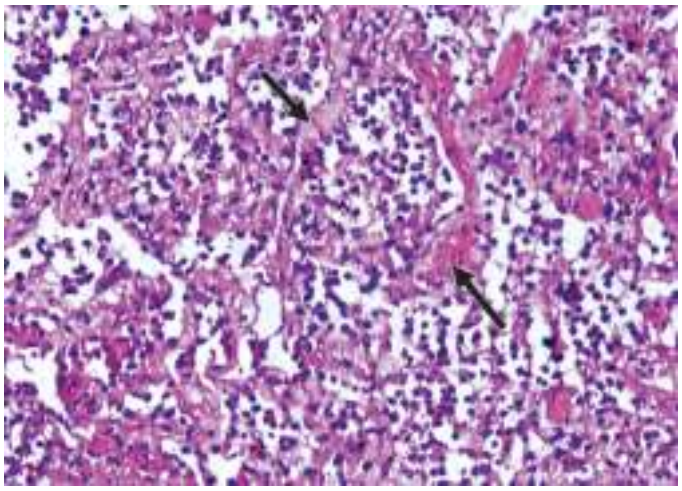


Fig. 2.33: Histology of lobar pneumonia in congestion stage. Lobar pneumonia has four stages: congestion, red hepatization, grey hepatization and resolution. Congestion stage occurs in the first 24 hours, that is characterized histologically by vascular congestion, intra-alveolar fluid, small number of neutrophils, often numerus bacteria. Gross examination reveals heavy and hyperemic lung (arrows) (400X).

- **Cellulitis:** Cellulitis refers to acute diffuse suppurative inflammation caused by streptococci, which synthesize hyaluronidase and streptokinase enzymes that dissolve the ground substances and facilitate the spread of infection in areolar tissue of orbit, pelvis, and subcutaneous tissue.
- **Carbuncle:** Carbuncle is an extensive form of abscess in the back of the neck and scalp due to collection of pus in multiple loci. Patient presents with multiple discharging sinuses at the surface.
- **Furuncle or boil:** Furuncle or boil is small abscess related to hair follicles or sebaceous glands.

PSEUDOMEMBRANOUS INFLAMMATION

Pseudomembrane formation is the term for an acute inflammatory process, in which there is overlay of fibrin and debris on mucous membrane. Deposition of yellow-colored exudate over mucosal surfaces produces a shaggy membrane composed of necrotic tissue. *Corynebacterium diphtheriae* liberates toxin, which forms pseudomembrane in the pharynx and trachea of children leading to severe respiratory distress. *Clostridium difficile* toxin in adults produces pseudomembranous colitis.

HEMORRHAGIC INFLAMMATION

Hemorrhagic inflammation is characterized by bleeding within or around the pancreas. It is usually a complication of acute pancreatitis. Intrapancreatic activation of pancreatic enzymes causes acute inflammation and necrosis of pancreatic parenchyma, pancreatic fat and blood vessel. Patient presents with upper abdominal pain radiating to back, tenderness, fever, nausea, vomiting and rapid pulse.

GANGRENOUS INFLAMMATION

Gangrenous inflammation is seen in ischemic necrosis with superadded bacterial infection in appendix and intestine, which results in partial liquefaction of the tissues by lytic activity of bacteria and inflammatory cells in the affected tissue. Gangrenous inflammation needs to be treated immediately, because it spreads quickly and can be fatal.

CATARRHAL INFLAMMATION

Catarrhal inflammation involves mucosal surfaces (e.g. catarrhal rhinitis, catarrhal conjunctivitis) containing mixture of serous and mucous secretions. Catarrhal inflammation is characterized by increased blood flow to the mucosal blood vessels, edema of the interstitial tissue, and enlargement of the secretory epithelial cells and profuse discharge of mucus and epithelial debris.

OUTCOME OF ACUTE INFLAMMATION

Acute inflammation may have one of the following outcomes (complete resolution, pus formation, fibrosis, and progression to chronic inflammation). Outcome of acute inflammation is shown in Fig. 2.34.

COMPLETE RESOLUTION

Resolution of normal structure and function of tissue occurs without scarring, if the injurious agent is eliminated. Lymphatics and macrophages play central role in complete resolution of acute inflammation (e.g. restoration of structure and function of tissue without scarring).

- Complete resolution occurs with mild injury (bee sting or first-degree burns) to those cells, which are capable to enter the cell cycle (e.g. labile and stable cells). Neutrophil cells start disappearing as a result of apoptosis from injury site. Leukotrienes undergo decay after neutralizing the injurious agent. Normalization of vascular permeability occurs.
- Macrophages phagocytose necrotic cellular debris and apoptotic neutrophils leading to removal from injured site. Edema fluid and proteins are finally drained into the lymphatics. Injured cells with intact supporting stroma undergo regeneration (e.g. labile and stable cells) with restoration of normal structure and function.

TISSUE DESTRUCTION

Oxygen-derived free radicals released by mononuclear cells are injurious to the own tissues as well as invading agents.

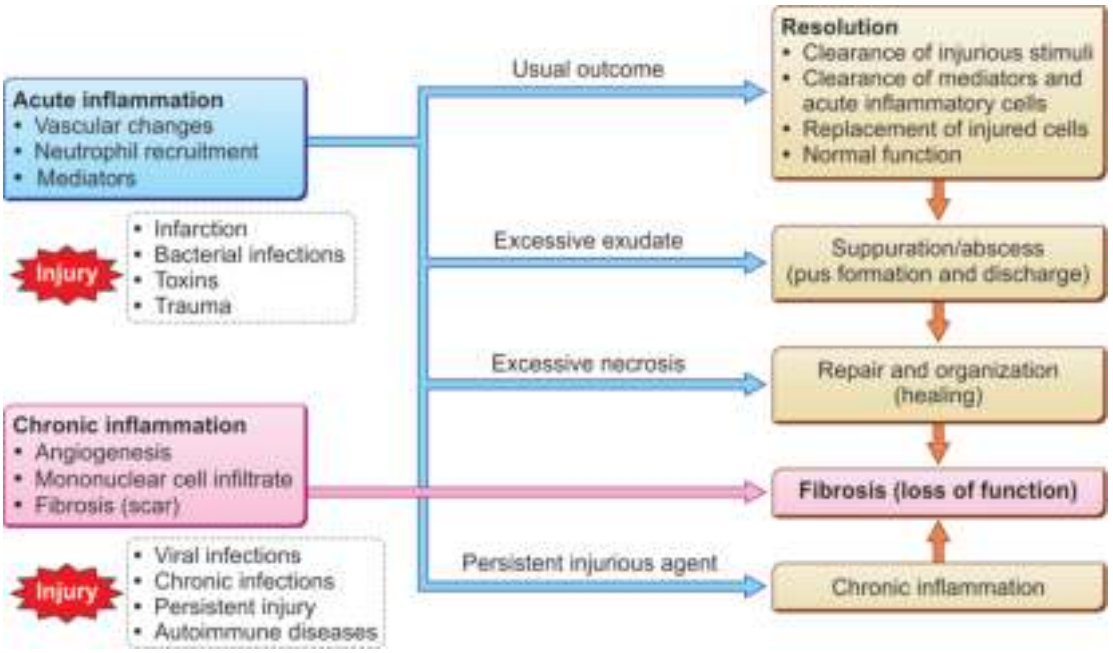


Fig. 2.34: Outcome of acute inflammation. The outcome depends on the type of tissue involved and the amount of tissue destruction has occurred, which are in turn related to the cause of the injury. Outcome of inflammation includes complete resolution by clearance of injurious agent, chemical mediators and replacement of injured cells, suppuration, healing by fibrosis and scar formation, loss of function and chronic inflammation.

- **Skin ulcer:** Loss of surface epithelium or skin results in ulcer formation, which can be caused by acute inflammation of epithelial surfaces (e.g. ulcer of the skin and peptic ulcer).
- **Abscess:** Production of large amounts of thick yellow pus or purulent exudate (PMN cells) by pyogenic bacteria such as *Staphylococcus aureus* is accompanied by significant liquefactive necrosis. The destroyed tissue is restored and eventually replaced by fibrosis.
- **Suppurative fistula:** Suppurative fistula is an abnormal communication between two organs or between an organ and a surface due to suppurative infection.
- **Gastric and duodenal ulcers:** Histologic examination of gastric shows focal destruction of the mucosa and full-thickness replacement of the muscularis with collagen-rich connective tissue.
  - Gastric ulcers are usually single and less than 2 cm in diameter and deep, with its floor covered by debris and neutrophils. Beneath to the ulcer is present in the granulation tissue and dense fibrosis replacing muscle coat. Ulcers on the lesser curvature are commonly associated with chronic gastritis, whereas those on the greater curvature are often related to nonsteroidal anti-inflammatory drugs (NSAIDs).
  - Grossly, chronic peptic ulcers may closely resemble gastric carcinoma. Peptic ulcer may heal by regeneration of mucosa close to its edge. Peptic ulcer may perforate involving full thickness of the wall. Chronic peptic ulcer may persist for years.
  - Therefore, the endoscopist must take multiple biopsies from the edges and bed of any gastric ulcer. Although contraction and scarring of gastric ulcers may occur leading to pyloric stenosis.

PROGRESSION TO CHRONIC INFLAMMATION

Persistent or recurrent acute infection greater than 48 hours progress to chronic inflammation, which occurs when host response fails to degrade the injurious agent (e.g. pathogen, tissue debris and foreign bodies) that incites gradual onset of inflammatory reaction. Chronic inflammation usually occurs as *de novo*, without a preceding acute inflammation.

- Chronic inflammation is characterized by disappearance of neutrophils from the injury site; followed by influx of mononuclear cells (e.g. macrophages, lymphocytes and plasma cells), angiogenesis and fibrosis in the injured tissue. Plasma cells produce immunoglobulins. Some of the macrophages may become multinucleated giant cells.
- Fibroblasts migrate into the injured area and lay down collagen fibers. Consequently, chronic inflammation is almost always accompanied by tissue destruction.

HEALING BY FIBROSIS

Healing by fibrosis is the final result of tissue destruction, with resultant distortion of structure and, in some cases, altered function. Growth factors synthesized by macrophages initiate the subsequent process of repair forming scar composed of primarily of collagen fibers. The scar does not contain parenchymal cells, hence functional impairment may occur.

- **Third degree burns:** Third degree burns occur with extensive injury or damage to permanent cells, which are incapable to regenerate. Fibrous scarring occurs in the areas of damage, forming a scar composed primarily of collagen, which leads to functional impairment of tissue.
- **Fibrinous exudate organization:** Organization occurs in fibrinous inflammation seen in coelomic cavities (pericardium, pleura and peritoneum), in which excessive fibrinous exudates cannot be cleared. Coelomic cavities are organized by growing of capillary loops into the exudates accompanied by fibroblasts and capillaries forming adhesions. Mass of fibrous tissue is called organization.

ANTI-INFLAMMATORY THERAPEUTIC AGENTS

The acute inflammatory response can be treated with anti-inflammatory drugs, which can prevent production of key chemical mediators of inflammation. Phospholipase A2 activity is inhibited by corticosteroids, limiting the production of arachidonic acid and, therefore, the formation of arachidonic acid metabolites. Anti-inflammatory therapeutic agents can be directed at many targets along the eicosanoid biosynthetic cyclooxygenase and lipoxygenase pathways. Anti-inflammatory agents inhibiting cyclooxygenase pathway are given in Table 2.37.

- **Cyclooxygenase pathway:** Various anti-inflammatory therapeutic agents are used to inhibit the action of COX-1 and COX-2 (cyclooxygenase pathway). COX-2 inhibitor drugs produce less toxicity than COX-1 inhibitors.
  - **Nonselective therapeutic agents:** NSAIDs such as aspirin inhibit the action of both COX-1 and COX-2 responsible for blocking production of prostaglandins.
  - **Selective anti-inflammatory therapeutic agents:** Meloxicam and carprofen inhibit the action of COX-2 without toxic effects. Prolonged administration may increase risk of arterial thrombosis in people possibly due to reduced prostacyclin synthesis in endothelial cells, while sparing of COX-1 mediated production of TXA_2 in platelets.
- **Lipoxygenase pathway:** 5-Lipoxygenase pathway is not affected by nonsteroidal anti-inflammatory drugs (NSAIDs). Pharmacological agents such as monteleukast are used to inhibit leukotrienes synthesis or block leukotrienes receptors (e.g. $CystLT_1$ and $CystL_2$), which are useful in the treatment of bronchial asthma. Zileuton inhibits 5-lipooxygenase and thus decreasing synthesis of LTC_4, LTD_4 and LTE_4.
  - **Glucocorticoids:** Glucocorticoids are broad spectrum powerful anti-inflammatory agents, which downregulate expression of genes encoding phospholipase A2, COX-2, proinflammatory cytokines (IL-1 and TNF-α) and nitric oxide synthase. Glucocorticoids also upregulate genes that encode potent anti-inflammatory protein such as lipocortin 1.
  - **Omega-3 fatty acid consumption:** Fish oil is a source of omega-3 fatty acid. Consumption of fish oil (omega-3 fatty acid) may modify inflammatory response. Omega-3 fatty acid serves as poor substrates for synthesizing prostaglandins (cyclooxygenase pathway) and leukotrienes (lipoxygenase pathway). In contrast, omega-3 fatty acid serves as excellent substrate for the synthesis of anti-inflammatory products (e.g. resolvins and protectins).

Table 2.37 Anti-inflammatory agents inhibiting cyclooxygenase pathway

Therapeutic Agents	Actions
Aspirin (NSAID)	Inhibiting the action of both COX-1 and COX-2
Meloxicam and carprofen (NSAIDs)	Inhibiting the action of COX-2 without toxic effects
Glucocorticoids	Down regulating expression of gene encoding COX-2 without toxic effects
Omega-3 fatty acid consumption	Serving as poor substrates for synthesizing prostaglandins

Prolonged administration of nonsteroidal anti-inflammatory drugs may increase risk of arterial thrombosis in people possibly due to reduced prostacyclin in endothelial cells.

CHRONIC INFLAMMATION

Chronic inflammation is an immune reaction to persistent and recurrent injury greater than 48 hours to weeks or months or years. It occurs when host response fails to degrade the injurious agent (e.g. pathogen, tissue debris and foreign bodies) that incites gradual-onset of inflammatory reaction, tissue destruction and attempts tissue repair.

- Chronic inflammation usually occurs as *de novo*, without a preceding acute inflammation. Mononuclear cells (macrophages, lymphocytes, plasma cells) predominate in chronic inflammation. Macrophages accumulate at the site of chronic inflammation as a result of continued recruitment and local proliferation.
- Chronic inflammation occurs in two major histologic patterns: chronic nonspecific inflammation and granulomatous inflammation.
- Granuloma is an aggregation of epithelioid histiocytes surrounded by rim of lymphocytes, plasma cells and fibroblasts. There are two types of granulomas: foreign body granuloma and immune granuloma.
- A nonabsorbable suture left in the body over a long time produces foreign body granuloma. Immune granuloma with central necrosis is seen in tuberculosis.
- Granulomatous inflammation is a pattern of chronic inflammation defined by the presence of granulomas.

COMMON CAUSES OF CHRONIC INFLAMMATION

Chronic inflammation occurs due to persistent infectious agents, prolonged exposure to potentially toxic agents and autoimmune disorders.

- **Progression of acute inflammation:** Persistent acute inflammation period can progress to chronic inflammation accompanied by tissue destruction. Pneumonic focus can induce extensive destruction and cavity formation resulting in extensive fibrosis. Peptic ulcer disease is manifested by acute and chronic inflammatory reactions.
- **Persistent infection:** Chronic inflammation occurs due to persistent infection for longer period by microorganisms with low-virulence resisting elimination by neutrophils resulting in delayed hypersensitivity reaction. Causative organisms include Mycobacterium tubercle bacilli, *Mycobacterium leprae* bacilli, *Treponema pallidum*, cat-scratch disease, fungi, brucellosis, and viruses. All these pathogens cause granulomatous inflammation except brucellosis and viral infections. Later two organisms resist phagocytosis and intracellular killing, but granulomas are absent.
- **Prolonged exposure to potentially toxic agents:** Prolonged exposure to exogenous and endogenous toxic substances may cause chronic inflammation. Exogenous toxic agents inducing chronic inflammation include suture materials, implanted prosthesis, asbestos fibers, silicosis or berylliosis. Necrotic adipose tissue and bone, and uric acid crystals (gouty tophus) are endogenous toxic materials that cause chronic inflammation.
- **Autoimmune disorders:** Autoimmunity is altered long-standing immune response to self-antigens that can result in chronic tissue damage in various organs. Autoimmunity can occur in the settings of Hashimoto's thyroiditis, pernicious anemia, rheumatoid arthritis, contact hypersensitivity reaction (exposure to nickel), ulcerative colitis, Crohn's disease and sarcoidosis.

ESSENTIAL MORPHOLOGIC FEATURES OF CHRONIC INFLAMMATION

Essential morphologic features in chronic inflammation include: (a) disappearance of neutrophils and appearance of mononuclear cells (e.g. macrophages, T cells, B cells, and plasma cells) causing tissue destruction, (b) formation of granulation tissue (proliferation of fibroblasts and angiogenesis), and (c) proliferation of fibroblasts with collagen fibers production leading to tissue fibrosis. Macrophage is the key cell that directs the various cells involved in chronic inflammation. Macrophages play an important role in removal of dead polymorphonuclear cells, presentation of antigenic material and granuloma formation.

MONONUCLEAR CELLS INFILTRATE

Chronic inflammation is characterized by a predominantly mononuclear inflammatory infiltrate composed of macrophages, lymphocytes and plasma cells along with few neutrophils. Mononuclear cells interact dynamically over the course of chronic inflammation. Macrophages are present in higher numbers replacing neutrophils. CD4+ helper T cells predominate in the chronic inflammatory infiltrate. CD8+ cytotoxic T cells are diminished in number.

TISSUE DESTRUCTION

Tissue destruction occurs due to persistent infection that leads to loss of organ function, and healing by fibrosis. Inappropriate activation of macrophages causes tissue destruction in chronic inflammation. Some of the macrophages may become multinucleated giant cells. Fibroblasts migrate into the injured area and lay down collagen.

- Necrotic tissue also stimulates leukocytes to release cell derived chemical mediators. Necrotic cells also activate plasma derived chemical mediators (kinin system, coagulation system, complement system and fibrinolytic system).
- Necrosed cells liberate uric acid at persisting injurious agent. In cellular immune reactions, T cells may directly kill cells. This ongoing tissue destruction can activate the inflammatory cascade by diverse mechanisms, so that features of both acute and chronic inflammation may coexist in certain circumstances.

TISSUE HEALING BY FIBROSIS

Tissue healing occurs by connective tissue replacement of damaged tissue by formation of highly vascular granulation tissue composed of newly formed blood vessels and myofibroblasts (activated fibroblasts) and some inflammatory cells that replace a fibrin clot. Granulomas are not present in granulation tissue. Fibronectin is required for granulation tissue formation. Fibronectin is a cell adhesion glycoprotein which binds collagen, fibrin, and cell surface receptors (e.g. integrins).

- **Angiogenesis:** Angiogenesis is a process of new blood vessels from existing blood vessels by pro-angiogenic factor (VEGF, FGF). It is essential in tissue development, reproduction, and wound healing. Angiogenesis occurs by mobilization of endothelial precursor cells (EPCs) from bone marrow and preexisting blood vessels at the site of injury. Chemotactic factors recruit endothelial cells from preexisting blood vessels to form new blood vessels (angiogenesis).
- **Collagen fibers synthesis:** Growth factors (PDGF, EGF, TGF-α, FGF-β, IGF-1, IL-1 and TGF-β) synthesized by macrophages participate in migration of fibroblasts, their proliferation, and synthesis of collagen fibers in early wound healing (3–5 days). Collagen fibers are the most abundant proteins in the body, which form the major structural component of many organs. Collagen fibers provide tensile strength of healing wounds. Fibrogenic cytokines synthesized by macrophages serve to eliminate injurious agents, and enhance process of tissue repair in chronic inflammation.

CELLS INVOLVED IN CHRONIC INFLAMMATION

Chronic inflammation is characterized by a predominantly mononuclear cell infiltrate (includes monocytes/macrophages, lymphocytes, plasma cells) with a few neutrophils present. Other cells include dendritic cells and eosinophils, tissue fibroblasts and endothelial cells. Neutrophils and macrophages originate from the bone marrow and serve as antigen presenting cells, which are professional phagocytes involved in the innate immunity. Macrophages detect and phagocytose pathogens enhance inflammation and promote tissue repair. Some important cells involved in chronic inflammation are given in Table 2.38.

MONOCYTES/MACROPHAGES

Macrophages are present in acute and chronic inflammation. Monocytes/macrophages are the dominant player of chronic inflammation. Monocytes begin to emigrate into extravascular tissues quite early in acute inflammation. In chronic inflammation, macrophage accumulation persists as a result of continuous recruitment from the circulation and their local proliferation at the site of inflammation. Macrophages regulate lymphocyte responses to antigens and secrete a variety of chemical mediators that modulate the proliferation and function of fibroblasts and endothelial cells.

Macrophage Origin and Distribution

Mononuclear phagocytes (monocytes/macrophages) are derived from a common precursors in the bone marrow, which give rise to monocytes. Blood monocytes migrate and differentiate into tissue macrophages known as reticuloendothelial cells diffusely distributed in the connective tissues in various organs. Macrophages have different names in different tissues, e.g. macrophages in connective tissue, alveolar macrophages in lung, mesangial cells in kidneys, sinus histiocytes in lymph node, Kupffer cells in liver, littoral cells in spleen, microglial cells in brain, osteoclasts in bone, lipophages in adipose tissue. Reticuloendothelial cells distribution in various tissues are depicted in Fig. 2.35 and Table 2.39.

Macrophage Receptors

Some macrophage receptors such as mannose and glucan and scavenger receptors bind cell-wall carbohydrates of bacteria, fungi and yeast. **Toll-like receptors** (TLRs) are present on macrophages, dendritic cells and other immune cells, which recognize different microbial components.

- TLR1, TLR2 and TLR6 recognize lipid and carbohydrates of gram-positive bacteria. TLR3, TLR7, TLR8

Table 2.38 Some important cells involved in chronic inflammation

Immune Cell	Functions	
Reticuloendothelial cell		
Monocyte/macrophage	▪ Antigen-presenting cell (APC) ▪ Phagocytosis and elimination of microorganisms ▪ IL-1 synthesis that activates T cells	▪ Monocyte life span 1–3 days in peripheral blood ▪ Macrophage life span months to years in tissues
T lymphocytes		
T helper cell (Th1)	▪ IL-2 and interferon-γ (IFN-γ) synthesis	▪ Activate macrophages and cytotoxic T cells
T helper cell (Th2)	▪ IL-4 and IL-6 synthesis	▪ B cell activation
T regulatory cell (Treg)	▪ T regulatory cell (Treg) suppresses immune response by inhibiting T cell proliferation and cytokine production	▪ T regulatory cell (Treg) maintains homeostasis and self-tolerance ▪ T regulatory cell (Treg) prevents autoimmunity
Memory T cell	▪ Memory T cell protects against previously encountered pathogen	▪ On re-encounter to specific invading pathogen, memory T cells are quickly converted into a large number of effector T cells to eliminate it
B lymphocytes		
B cell	▪ B cell is precursor of plasma cell that synthesizes immunoglobulins	▪ B cell acts as an antigen-presenting cell to T helper cell (Th2)
Plasma cell	▪ B cell differentiates to form plasma cell	▪ Plasma cell synthesizes IgM and then IgG and IgA immunoglobulins
Natural killer cells (granular lymphoid-like cells)	Unlike lymphocytes lack antigen specific receptors	
Circulating granulocytes		
Neutrophil	▪ Acute inflammatory cell involved in bacterial phagocytosis and killing, neutrophilic granules involved in increased vascular permeability, chemotaxis, killing and digesting extracellular matrix	▪ Neutrophil has 6 hours life span in peripheral blood ▪ Neutrophil has 4 days life span in tissues
Eosinophil	Acute inflammatory cell involved in allergic and parasitic conditions. Eosinophilic granules include major basic proteins	
Basophil	Basophil is circulating cell that gives rise to mast cell. Basophil granules contain histamine	

and TLR9 recognize viral nucleic acid derivatives (viral DNA). TLR9 also recognizes bacterial DNA containing unmethylated CpG motifs.

- TLR4 recognizes lipopolysaccharides of gram-negative bacteria. TLR5 recognizes bacterial flagellin. TLR10 recognizes unknown ligand. TLR11 is present on macrophages and neutrophils, that recognizes bacterial profilin. Pathogen recognition toll-like receptors are given in Table 2.40.

Monocyte/Macrophage Half-life Span

Half-life of blood monocytes is one day, while life span of tissue macrophage is several weeks to two years. If the injurious agent is eliminated, macrophages eventually disappear either by dying off or making their way into the lymphatic channels and lymph nodes.

Macrophage Activation

Activated macrophages have many salient features, which include: increase in cell size, lysosomal enzymes level, metabolic activity, phagocytic activity and secretion of large variety of biologically active products. Activated macrophages synthesize TNF-α, IL-1, chemokines that promote leukocyte recruitment and participate in chronic inflammation, tissue injury and tissue repair.

- Activation of macrophages occurs by immune and nonimmune mechanisms. T cells synthesize interferon-γ (IFN-γ), which activate macrophages by immune mechanism.
- Bacterial endotoxins, necrotic cells, fibronectin-matrix proteins and chemical mediators activate macrophages by nonimmune mechanism.

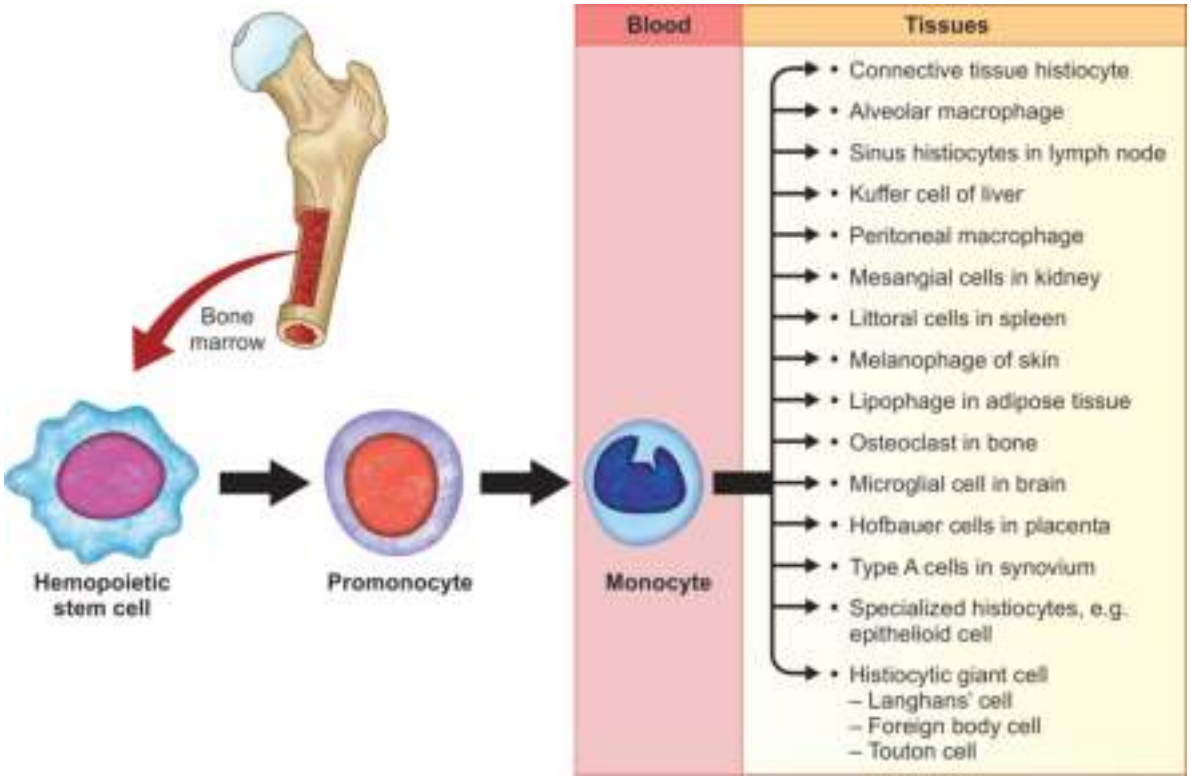


Fig. 2.35: Reticuloendothelial cells distribution in various tissues. The reticuloendothelial system consists of macrophages in tissue descending from the blood monocytes, which are able to perform phagocytosis of foreign materials and particulate material. The tissue component is concentrated in liver (90%), spleen, lymph nodes, bone marrow, lungs and other tissues. Reticuloendothelial system removes immune complexes from the circulation in healthy persons. Reticuloendothelial system has affinity for encapsulated bacteria, such as *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*.

Table 2.39 Reticuloendothelial cells distribution in various tissues

Tissue	Reticuloendothelial Cells
Blood	Monocytes
Connective tissue	Macrophages
Lung	Alveolar macrophages
Peritoneum	Peritoneal macrophages
Kidney	Mesangial cells
Liver	Kupffer's cells
Lymph nodes	Sinus histiocytes
Spleen	Littoral cells
Placenta	Hofbauer cells
Skin	Melanophages
Brain	Microglial cells
Synovium	Synovial macrophages (type A synoviocytes)
Bone	Osteoclasts
Adipose tissue	Lipophage
Specialized histiocytes	▪ Epithelioid cells ▪ Histiocytic giant cells ▪ Langhans' giant cells ▪ Foreign body giant cells ▪ Touton giant cells

Monocytes/Macrophage Recruitment

Chemotaxis plays key role in migration of circulating monocytes similar to polymorphonuclear cells to injured site by chemokines, where these are called tissue macrophages. Chemokines are derived from macrophages, T cells, bacterial products, products derived from injured tissue (e.g. break down products of collagen, fibronectin), growth factors (PDGF, TGF-α), fibrinopeptides, and complement cascade product (C5a). Sources of chemokines and their role in chemotaxis of macrophages are given in Table 2.41.

Macrophage Proliferation and Immobilization in Tissues

Growth factors derived from necrotic tissue and T cells play important role in proliferation of macrophages leading to increased number of macrophages at persistent injury site. Macrophages engulf particulate material, process and present to CD4+ helper T cells. Activated T cells synthesize IFN-γ, which activates macrophages and transform into epithelioid cells and multinucleated giant cells inflammation. As macrophages lead to formation of fibrous scar tissue; it has been hypothesized that immobilization of macrophages allows resolution of chronic inflammation.

Table 2.40 Pathogen recognition toll-like receptors

Toll-like Receptors (TLRs)	TLRs Expression on Cell	TLRs Recognize Pathogen Component
TLR1	Macrophages and neutrophils	Lipid and carbohydrates from gram-positive bacteria
TLR2	Macrophages, neutrophils and basophils	Lipid and carbohydrates from gram-positive bacteria and fungi
TLR3	Macrophages	Nucleic acid derivatives virus (viral DNA)
TLR4	Macrophages, neutrophils and basophils	Lipopolysaccharides from gram-negative bacteria
TLR5	Macrophages, neutrophils and basophils	Bacterial flagellin
TLR6	Macrophages and neutrophils	Lipid and carbohydrates from gram-positive bacteria
TLR7	Macrophages and neutrophils	Nucleic acid derivatives (viral DNA)
TLR8	Macrophages and neutrophils	Nucleic acid derivatives (viral DNA)
TLR9	Macrophages and neutrophils	Nucleic acid derivatives (viral DNA) and bacterial DNA containing unmethylated CpG motifs
TLR10	Macrophages and neutrophils	Ligand unknown
TLR11	Macrophages and neutrophils	Bacterial profilin

Table 2.41 Sources of chemokines and their role in chemotaxis of macrophages

Source of Chemokines	Examples of Chemokines
Blood cells	Macrophage chemotactic protein 1 (MCP-1)
Bacteria	Bacterial products
Injured tissue	Breakdown products of collagen fibers and fibronectin
Growth factors	PDGF, TGF-α
Fibrinopeptides	Fibrin degradation products (FDPs)
Complement	C5a

Activated Macrophages and their Products

Macrophages possess receptors for IgG and C3b, which process antigen, enhance immune response, and participate in phagocytosis of injurious agent. Activated macrophages synthesize many products, which participate in elimination of injurious agent and initiation of tissue repair by fibrosis and tissue destruction in chronic inflammation. This ongoing tissue destruction can activate the inflammatory cascade by diverse mechanisms, so that features of both acute and chronic inflammation may coexist in certain circumstances.

- Activated macrophages synthesize variety of cytokines such as IL-1, TNF-α, IL-6, IL-8 and monocyte chemoattractant protein 1 (MCP-1). These cytokines attract inflammatory cells at the site of tissue injury. Macrophages synthesize reactive oxygen and nitrogen oxygen species, which are toxic to microbes as well as host cells. Macrophages synthesize neutral proteases, which may cause degradation of extracellular matrix.
- Matrix metalloproteinases (MMPs) are synthesized by polymorphonuclear cells, macrophages, fibroblasts, synovial cells and some epithelial cells. MMPs cause breakdown of type 3 collagen fibers. Deposition of type 1 collagen fibers increase tensile strength by 80%.
- Macrophages synthesize variety of growth factors (PDGF, FGF, and TGF-β), which are involved in proliferation of fibroblasts, deposition of collagen fibers and new blood vessels formation (angiogenesis).
- Macrophages synthesize arachidonic acid metabolites such a prostaglandins and leukotrienes involved in chronic inflammatory process. Macrophage products and their actions are given in Table 2.42. Role of activated macrophages in chronic inflammation is shown in Fig. 2.36.

LYMPHOCYTES

Chronic inflammation is a response to prolonged exposure to injurious stimuli that harm and destroy tissues and promote lymphocytic infiltration into inflamed sites. Lymphocytes have life span of 2 weeks to 2 years. Lymphocytes mediate innate and adaptive immunity.

- There are three main types of lymphocytes: CD8+ cytotoxic T cells, B cells and natural killer cells. B cells produce antibodies that can destroy invading pathogens. CD8+ cytotoxic T cells attack foreign cells, cancer cells and cells infected with virus.
- Natural killer cells represent the major source of interferon-γ, a proinflammatory cytokine acting as a master regulator of different immune cell response.

Table 2.42 Macrophage products and their actions

Inflammatory Mediators	Actions
Cytokines	
TNF-α	Endothelial cell-leukocyte adhesion; can trigger or inhibit apoptosis
IL-1, TNF-α	Chemotaxis of inflammatory cells
IL-1, IL-6	Synthesis of acute phase reactant by liver
IL-2	Autocrine action
IL-8	Leukocyte recruitment
Chemokines	
IL-8 and MCP-1	Chemotaxis of inflammatory cells and paracrine action
Lysosomal enzymes	
Neutral hydrolases	Toxic to extracellular matrix
Acid hydrolase and serine protease	Hydrolyzing the muramic acid-N-acetylglucosamine bond of bacterial glycopeptides leading to killing of microbes
Metalloproteinases (MMPs)	▪ Degradation of type 3 collagen fibers ▪ Deposition of type 1 collagen fibers leading to increased tensile strength
Cell derived arachidonic metabolites	
Prostaglandins	▪ Platelet aggregation ▪ Vasodilatation ▪ Increasing vascular permeability with formation of inflammatory exudate ▪ Modulation of phagocytic activity of leukocytes ▪ Pain ▪ Fever
Leukotrienes	▪ Vasoconstriction ▪ Increased vascular permeability ▪ Bronchospasm
Plasminogen activator	
Plasminogen activator	Cleaves plasminogen to form plasmin, that degrades fibrin strands resulting in dissolution of blood clot
Procoagulant activity	
Procoagulant activity	Activation of coagulation system
Reactive oxygen and nitrogen species	
Superoxide, highly reactive hydroxyl molecule and hypochlorous acid (bleach)	Toxic to microbes and host cells
Reactive oxygen and nitrogen species	
PDGF, FGF, and TGF-β	▪ Fibroblast proliferation ▪ Collagen deposition ▪ Angiogenesis

Metalloproteinases (MMPs) also known as collagenases synthesized by macrophages, neutrophils, fibroblasts, synovial cells and some epithelial cells.

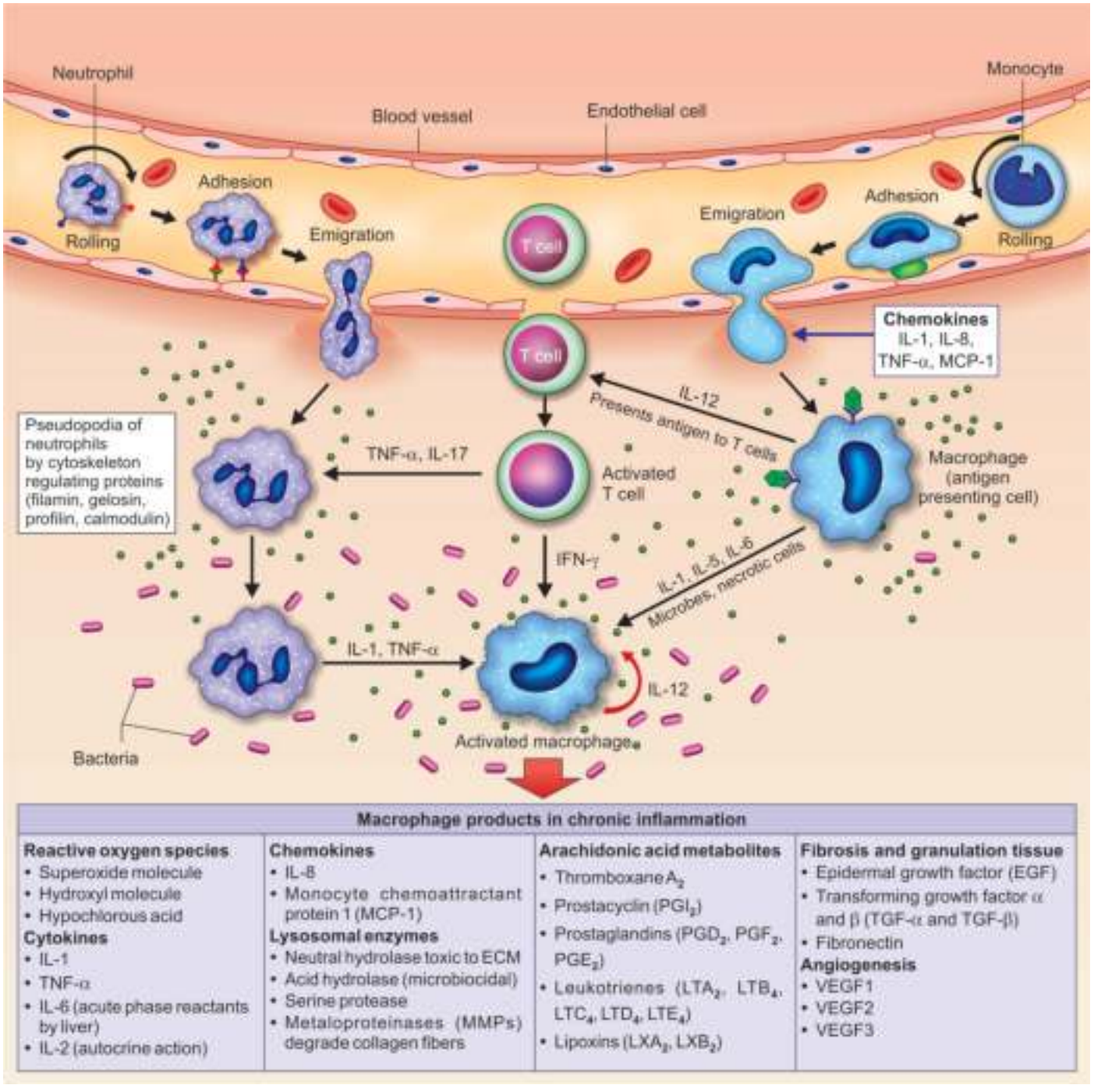
T Lymphocytes

T cells participate in cell-mediated immunity. Activated macrophages display antigens to CD4+ helper T cells. Macrophages synthesize IL-12 that stimulates T cell responses. Activated T cells recruit monocytes from the circulation interferon-γ (IFN-γ) is a powerful activator of macrophages.

- **T helper 1 cell:** T helper 1 (Th1) participates in synthesis of interferon-γ (IFN-γ) and IL-2, which activate macrophages and cytotoxic natural killer cells.
- **T helper 2 cell:** T helper 2 (Th2) synthesizes IL-4 and IL-6 that activates B cell.
- **CD4+ regulatory T cell:** CD4+ regulatory T cell (Treg) suppresses immune response by inhibiting T cell proliferation and cytokine production. Treg maintains homeostasis and self-tolerance. Treg prevents autoimmunity.
- **Memory T cell:** Memory T cell provides protection against previously encountered pathogen. On re-encounter to specific invading pathogen, memory T cells are quickly converted into a large number of effector T cells to eliminate it.

B Lymphocytes and Plasma Cells

B cell is precursor of plasma cell that synthesizes immunoglobulins. B cell acts as an antigen-presenting



Macrophage products in chronic inflammation

Reactive oxygen species	Chemokines	Arachidonic acid metabolites	Fibrosis and granulation tissue
• Superoxide molecule • Hydroxyl molecule • Hypochlorous acid **Cytokines** • IL-1 • TNF-α • IL-6 (acute phase reactants by liver) • IL-2 (autocrine action)	• IL-8 • Monocyte chemoattractant protein 1 (MCP-1) **Lysosomal enzymes** • Neutral hydrolase toxic to ECM • Acid hydrolase (microbiocidal) • Serine protease • Metaloproteinases (MMPs) degrade collagen fibers	• Thromboxane A_2 • Prostacyclin (PGI_2) • Prostaglandins (PGD_2, PGF_2, PGE_2) • Leukotrienes (LTA_2, LTB_4, LTC_4, LTD_4, LTE_4) • Lipoxins (LXA_2, LXB_2)	• Epidermal growth factor (EGF) • Transforming growth factor α and β (TGF-α and TGF-β) • Fibronectin **Angiogenesis** • VEGF1 • VEGF2 • VEGF3

Fig. 2.36: Role of activated macrophages in chronic inflammation. Macrophages are activated by immunological or nonimmunological mechanisms. In inflammation, macrophages perform three major functions: antigen presentation, phagocytosis and immunomodulation through production of various cytokines and growth factors. Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation. Various products synthesized by activated macrophages can induce tissue fibrosis.

cell to T helper cell (Th2). B cells, when stimulated with antigen, become plasma cells. Plasma cells participate in humoral immunity.

- Plasma cells are large cells with amphophilic to basophilic cytoplasm with eccentric nucleus with chromatin arranged in a characteristic cartwheel pattern.
- Plasma cell cytoplasm contains a pale perinuclear zone that on electron microscopy demonstrates an extensive Golgi appratus and centrioles.
- Plasma cells have abundant rough endoplasmic reticulum coupled with a well-develop Golgi appratus, which together are responsible for immunoglobulins synthesis (e.g. IgG, IgA, IgM, IgD and IgE).

Natural Killer Cells

Natural killer cells are large granular, lymphoid-like cells with important functions in innate immunity especially against intracellular viral infections or tumor cells. Unlike T and B lymphocytes, natural killer cells lack antigen-specific receptors. Natural killer cells controlling human viral infections are given in Table 2.43.

Pathology Pearls: Macrophages and Lymphocytes Interaction

- Macrophages can act as antigen-presenting cells to CD4+ helper T cells. Poorly digested antigen is presented by macrophages to CD4+ helper T cells. Interaction between macrophages and CD4+ helper T cells triggers an immune response. As a result, stimulated T cells can secrete interleukin-2 (IL-2), which mediates activation and proliferation of lymphocytes. Interaction between macrophages and helper T cells is shown in Fig. 2.37.
- Cellular activation can also result in secretion of varying effector molecules. For example, T helper cells (Th1) synthesize interferon-γ (IFN-γ), IL-2 and TNF-β, which activate macrophages and cytotoxic CD8+ T cells and also mediate the transformation of monocytes/macrophages to epithelioid cells and multinucleated giant cells. T helper cell (Th-2) secretes IL-4, IL-5, IL-6, IL-10 and IL-13, which activates B cell.
- Contact-mediated activation of macrophages by activated lymphocytes triggers production of reactive oxygen species (ROS), nitric oxide (NO), IL-1 and TNF-α.

Table 2.43 Natural killer cells (NKCs) controlling human viral infections

Human cytomegalovirus
Human herpesvirus 5
Vesicular stomatitis virus (VSV)
Herpes simplex virus (HSV)
Human papillomavirus (HPV)
Human immunodeficiency virus (HIV)
Epstein-Barr virus (EBV)

Natural killer cells are rapid but nonspecific means of controlling viral and other intracellular infections.

OTHER CELLS INVOLVED IN CHRONIC INFLAMMATION

Other cells involved in chronic inflammation include eosinophils, mast cells, neutrophils, platelets and fibroblasts.

Eosinophils

The eosinophil is a specialized cell of the immune system. Eosinophils are particularly prominent in allergic and parasitic infection mediated by IgE, which are recruited by eotaxin chemokine.

- Eosinophilic granules contain major basic protein, a highly cationic protein that is toxic to parasites but also causes lysis of host epithelial cells. Eosinophilia is highly suggestive of response to invasive helminths, arthropods, allergic rhinitis and bronchial asthma.

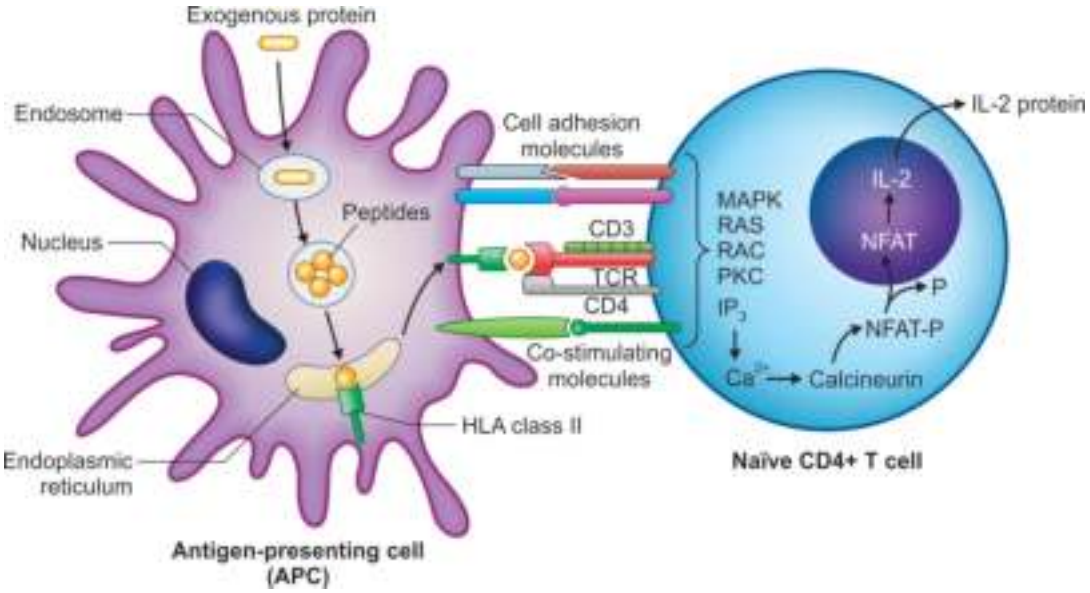


Fig. 2.37: Interaction between macrophages and CD4+ helper T cells. Macrophages interact with CD4+ helper T cells in order to bring about T cell activation in target cells. CD4+ helper T cells play important role in adaptive immunity by activating B cells form plasma cells to secrete immunoglobulins, and activating cytotoxic T cells to kill infected target cells. Macrophages stimulated by T cells need to produce a tumor necrosis factor α (TNF-α) cytokine, which acts on macrophages to produce nitric oxide (NO).

- Eosinophilic specific granules contain preformed chemical mediator, i.e. major basic protein (MBP), which kills only invasive helminths by type 2 hypersensitivity reaction. It has no effect on pinworms and adult worms (*Ascaris lumbricoides*), which are not invasive.
- Eosinophils modulate hypersensitivity reactions with the help of histaminase and arylsulfatase. Histaminase neutralizes histamine, whereas arylsulfatase neutralizes leukotrienes. Eosinophilic red granules contain crystalline material in cytoplasm, which become 'Charcot-Leyden crystals' in the sputum of asthmatic patients. Primary inflammatory mediators of eosinophils are given in Table 2.44.

Mast Cells

Mast cells are widely distributed in connective tissue, which participate in acute and chronic inflammation. Mast cells express surface receptors that bind the Fc portion of IgE antibody. Mast cell contains electron-dense granules, which release histamine, leukotrienes (LTC_4, LTD_4, LTE_4), prostaglandins, and platelet-activating factor (PAF) during allergic reactions to foods, insect venom, or drugs, sometimes mast cells have catastrophic results (e.g. anaphylactic shock). Mast cells may produce cytokines (TNF-α and IL-4), that contribute to fibrosis in chronic inflammation. Mast cells degranulation and their actions are shown in Fig. 2.38. Primary inflammatory mediators of mast cells are given in Table 2.45.

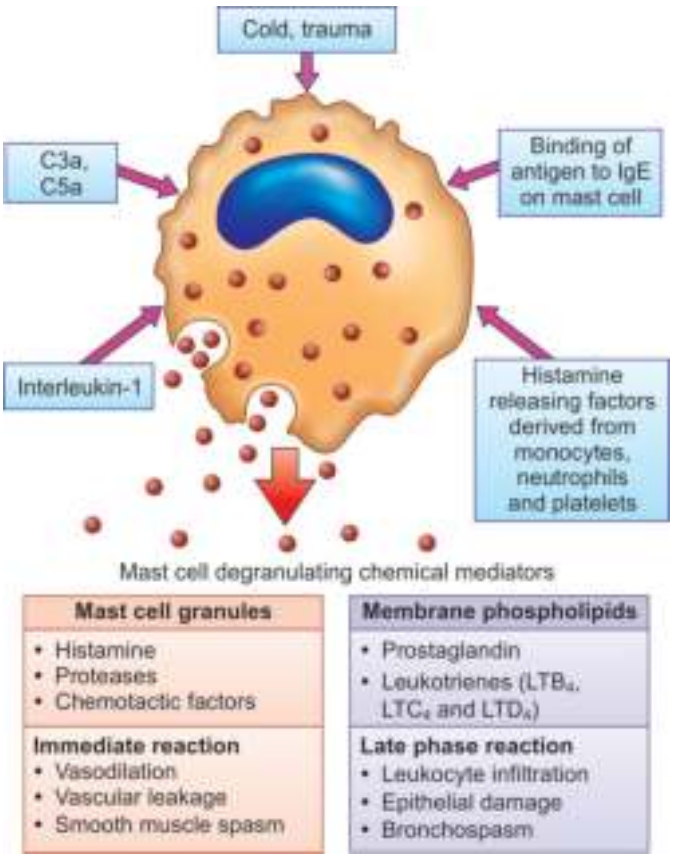


Fig. 2.38: Mast cells degranulation and their actions. Mast cell degranulation is a central event in the development of lesions in urticaria, and histamine levels are elevated in biopsies skin. Upon stimulation by an allergen, mast cells degranulate and release contents into surrounding tissues.

Table 2.44 Primary inflammatory mediators of eosinophils

Specific Mediator	Actions
Reactive oxygen species (ROS)	
Superoxide, highly reactive hydroxyl molecule and hypochlorous acid	These participate in inflammatory reactions
Lysosomal enzymes granules (also known as primary crystalloid granules)	
Major basic proteins (highly cationic protein)	▪ Modulation of hypersensitivity reactions ▪ Killing only invasive helminths by type 2 hypersensitivity reaction ▪ No effect on noninvasive parasites such as pinworms and *Ascaris lumbricoides* ▪ Lysis of host epithelial cells
Histaminase	▪ Modulation of hypersensitivity reactions ▪ Neutralization of histamine
Aryl sulphatase B	Neutralization of leukotrienes
Eosinophil cationic protein	Damaging schistosomula of *Schistosoma mansoni*
Eosinophil peroxidase	Functions unknown
Acid hydrolase	Functions unknown
β-Glucuronidase	Functions unknown
Other inflammatory mediators of eosinophils	
Phospholipase D	Specific functions unknown
Prostaglandins of the E series	Specific functions unknown
Cytokines	Specific functions unknown

Table 2.45 Primary inflammatory mediators of mast cells

Histamine
Leukotrienes (LTC_4, LTD_4, LTE_4)
Platelet-activating factor
Eosinophil chemotactic factors
Cytokines (TNF-α and IL-4)

Neutrophils

Neutrophils characteristically participate in acute inflammation and sometimes present in during chronic inflammation due to ongoing persistent infection for many months in osteomyelitis and tissue damage as a result of chemical mediators produced by activated macrophages and T cells.

Platelets

Platelets regulate vascular permeability and proliferation of mesenchymal cells in chronic inflammation. Platelets also participate in thrombosis and clot formation. Platelets contain primary inflammatory mediators in dense granules and α-granules. Dense granules of platelets contain serotonin, calcium and ADP. Platelets' α-granules contain cationic proteins, fibrinogen and coagulation proteins, platelet-derived growth factors, acid hydrolases and thromboxane A_2.

Fibroblasts

Fibroblasts mediate chronic inflammation and wound healing. These cells are derived from mesoderm or neural crest and differentiate into other connective tissue cells (e.g. adipocytes, smooth muscle cells, chondrocytes and osteocytes).

- Fibroblasts respond to immune signals, which produce extracellular matrix proteins. Fibroblasts interact with lymphocytes, via surface molecules and receptors on fibroblasts and lymphocytes. For example, when CD40 on fibroblasts binds with its ligand on lymphocytes, both cells are activated.
- Fibroblasts synthesize variety of inflammatory mediators that include IL-6, IL-8, cyclooxygenase 2, hyaluronan, PGE_2, CD40 expression, extracellular proteins and matricellular proteins.

CHRONIC NONSPECIFIC INFLAMMATION

Tissue injury and wound healing proceed simultaneously in chronic inflammation. Neutrophils have disappeared from the injury site. There is influx of macrophages, lymphocytes, plasma cells in chronic nonspecific inflammation. Synthesis of cytokines is associated with granulation tissue formation (proliferation of fibroblasts and capillaries) resulting in tissue scarring and architecture distortion.

- Chronic nonspecific inflammation is mediated by the interaction of monocytes–macrophages with lymphocytes. Cytokines synthesized by macrophages and lymphocytes activate each other.
- Macrophages display antigens to B cells resulting in synthesis of antibody-producing plasma cells. Other inflammatory cells such as eosinophils, mast cells, platelets and neutrophils also participate in chronic inflammation.

GRANULOMATOUS INFLAMMATION

Granulomatous inflammation is a distinctive form of chronic inflammation, which develops when acute inflammatory cells are unable to digest the injurious agent (e.g. suture or talc). Fusion of macrophages within the persistent inflammatory lesion results in the formation of granulomas admixed with mononuclear infiltration (macrophages, lymphocytes and plasma cells) and multinucleated giant cells.

- **Granuloma composition:** Granuloma consists of three components: (a) epithelioid cells (activated macrophages) contain eosinophilic cytoplasm and oval or elongate nuclei, which may show folding of nuclear membrane, (b) collar of lymphocytes plasma cells and reactive fibroblasts, and (c) multinucleated giant cells (40–50 microns in size with 20 or more nuclei arranged peripherally) formed by fusion of epithelioid cells. Epithelioid cells show increased endoplasmic reticulum and few phagolysosomes on electron microscopy. Demonstration of granulomas in biopsy specimen has diagnostic significance associated with the persistent inflammatory lesions.
- **Granulomas in granulomatous inflammation:** Granulomas are formed in various pathologic conditions due to bacteria, fungi, parasites, inorganic compounds (silicosis, berylliosis), foreign bodies (sutures, implants, accidents) and sarcoidosis (unknown etiology). Brucellosis and viral infections resist phagocytosis and intracellular killing, but granulomas are absent. Granulomas are seen in secondary or primary chronic granulomatous conditions. Some examples of chronic granulomatous inflammation are given in Table 2.46. Differences between granuloma and granulation tissue are given in Table 2.47.

TYPE OF GRANULOMAS

There are two types of granulomas in granulomatous inflammation: immunologic mediated granulomas (caseating and noncaseating granulomas) and

Table 2.46 Some examples of chronic granulomatous inflammation

Disorder	Characteristics
Persistence of infectious agents due to failure of phagocytosis	
Tuberculosis	Mycobacterium tubercle bacilli induced caseous necrosis, epithelioid granulomas and Langhans' giant cells. AFB may be demonstrated by Ziehl-Neelsen staining
Leprosy	*Mycobacterium leprae* bacilli induced noncaseating granulomas
Syphilis	*Treponema pallidum* induced with gumma (microscopic to grossly visible lesion), with central necrotic cells, surrounded by macrophages and plasma cell infiltrate
Cat-scratch disease	Gram-negative bacteria forms rounded or stellate granuloma containing central granular debris
Prolonged exposure to exogenous and endogenous substances	
Endogenous materials	▪ Necrotic adipose tissue and bone ▪ Uric acid crystals (gouty tophus)
Exogenous materials	▪ Suture materials ▪ Implanted prosthesis ▪ Asbestos fibers ▪ Silicosis ▪ Berylliosis
Autoimmune disorders	
Organ-specific diseases	▪ Hashimoto's thyroiditis ▪ Pernicious anemia ▪ Rheumatoid arthritis
Contact hypersensitivity reactions	Self-antigens altered by exposure to nickel
Diseases of unknown etiology	▪ Ulcerative colitis ▪ Crohn's disease ▪ Sarcoidosis

Table 2.47 Differences between granuloma and granulation tissue

Characteristics	Granuloma	Granulation Tissue
Features	Well circumscribed collection of modified macrophages (epithelioid cells) surrounded by lymphocytes and fibroblasts	Hallmark of healing process composed of proliferation of fibroblasts and new blood vessels formation (angiogenesis) embedded in loose edematous matrix along with inflammatory cells such as neutrophils, lymphocytes and plasma cells
Granulomas	Present	Absent
Multinucleated giant cells	Present	Absent
Conditions	Tuberculosis, tuberculoid leprosy, sarcoidosis, rheumatoid arthritis	Normal physiological response after an injury
Vascularization	Absent	Present due to angiogenesis
Fibroblasts	Minimal	Marked
Cytokines	IL-1, IL-12 and IFN-γ	Angiogenetic and fibrogenic growth factors involved, e.g. VEGF, PDGF, FGF, TNF
Consequence	Damage to host tissues	Physiological process

nonimmunologic mediated granulomas (foreign body granulomas).

- Immunologic granulomas are mediated by lymphocytes. Examples of immunologic mediated granulomas are tuberculosis, leprosy, cat scratch fever, sarcoidosis, histoplasmosis and Hodgkin's disease.
- Nonimmunologic mediated foreign body granulomas are formed by poorly digestible particles seen in silicosis, surgical sutures, implanted prosthesis, asbestosis, and beryllium exposure. Granuloma formation by immunologic and nonimmunologic mechanisms are given in Table 2.48.

Table 2.48 Granuloma formation by immunologic and non-immunologic mechanisms

Granuloma Formation Mediated by Immunologic Mechanism
Tuberculosis
Leprosy
Cat-scratch fever
Sarcoidosis
Histoplasmosis
Blastomycosis
Cryptococcosis
Coccidioidomycosis
Hodgkin's disease (mixed cellularity variant)
Granuloma Formation Mediated by Nonimmunologic Mechanism
Silicosis
Berylliosis
Asbestosis
Surgical sutures
Implanted prosthesis
Foreign body induced pneumonia

Granulomas Formation Mediated by Immunologic Mechanism

Exposure to antigen leads to activation of macrophages resulting in synthesis of IFN-γ, which activates CD4+ helper T cells leads to synthesis of IFN-γ.

- IFN-γ synthesized by activated macrophages and CD4+ helper T cells plays key role in formation of granuloma. IFN-γ activates macrophages to become epithelioid cells. IFN-γ also participates in proliferation and differentiation of T cells.
- Cytokines TNF-α and IFN-γ participate in recruitment of chronic inflammatory cells, granuloma formation and maintenance of tuberculous and systemic fungal granulomas.
- Inhibitors of TNF-α cause the breakdown of granulomas leading to dissemination of disease. Mechanism of caseating and noncaseating granulomas formation is shown in Fig. 2.39. Steps in granuloma formation are shown in Fig. 2.40.

Caseating Granulomas

Tuberculosis is caused by a bacterium called *Mycobacterium tuberculosis*, that often attacks lungs. The bacteria spread from person to person through tiny droplets released into the air via coughing and sneezing.

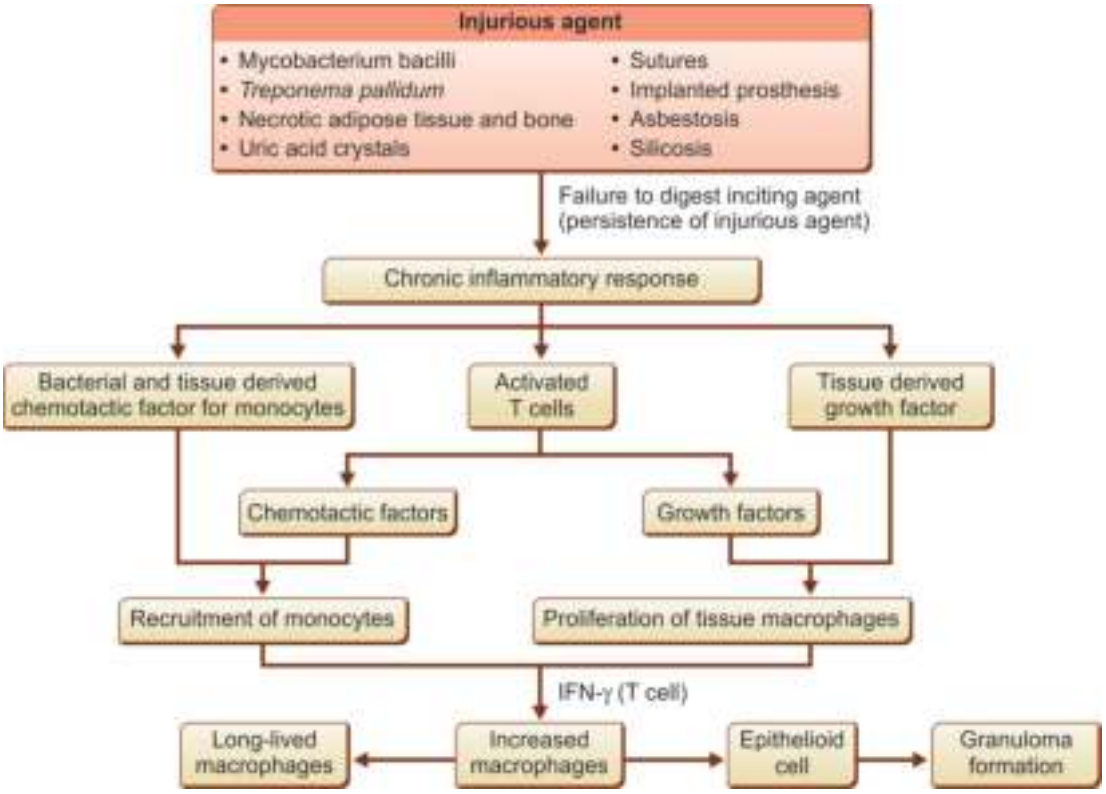


Fig. 2.39: Mechanism of caseating and noncaseating granulomas formation. Granulomas are collection of macrophages, often surrounded by helper-T cells and rim of fibroblasts. Most granulomas fall into one of two categories: caseating with necrotic center; or noncaseating without any necrosis caused by persistent infection. Granulomas are formed when the immune system responds to causative agent, eventually leading macrophage transformation to epithelioid cells, which may bind tightly together, forming granuloma.

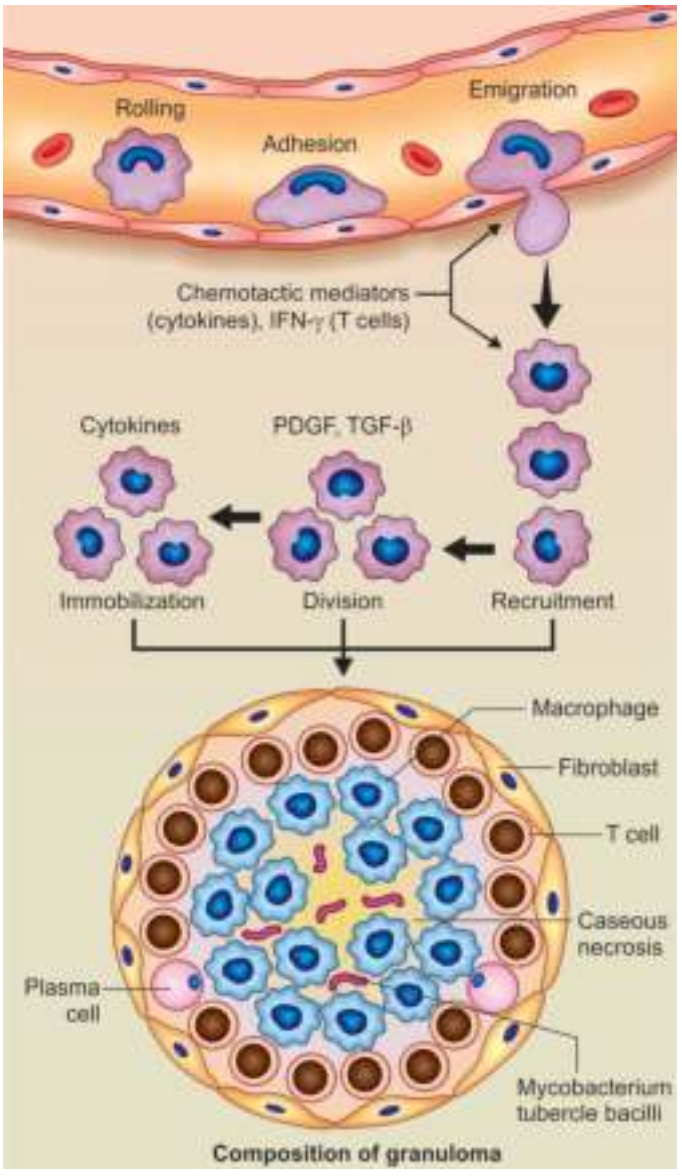


Fig. 2.40: Steps in granuloma formation. Macrophages migrate and accumulate involving cellular recruitment into the site of infection initiate granuloma formation, when the immune system responds to causative agent, eventually leading macrophage transformation to epithelioid cells, which may bind tightly together forming granuloma. A fully formed tuberculous granuloma consists of a central zone of caseating necrosis, surrounded by cellular infiltrate comprising activated macrophages surrounded by a rim of lymphocytes and plasma cells.

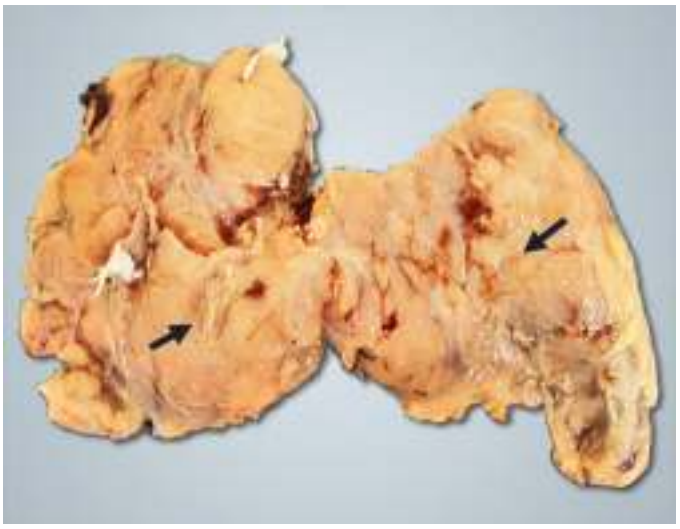


Fig. 2.41: Gross morphology of tubercular lymphadenitis. Cut surface tubercular lymphadenitis shows cheesy appearance on cut surface. (*Courtesy:* Department of Pathology, Dr DY Patil Medical College, Pune, Maharashtra.)

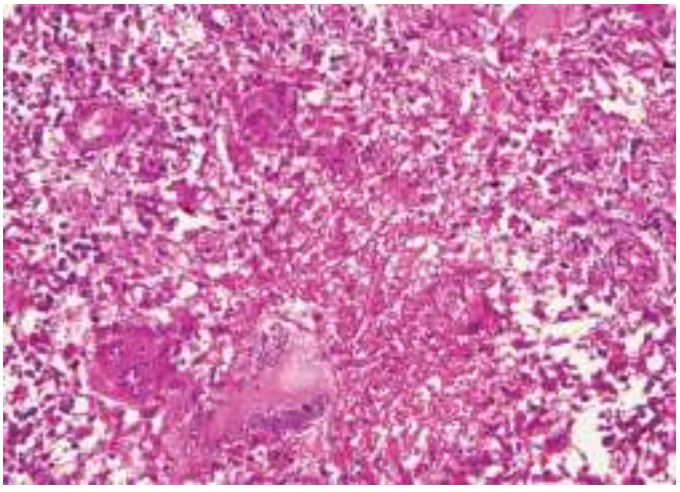


Fig. 2.42: Granulomatous inflammation with multiple Langhans' giant cells. Granulomatous inflammation is seen in bacterial, fungal, protozoal infections and other granulomatous diseases. A granuloma is a focus collection of granulomatous inflammation consisting of microscopic aggregation of macrophages surrounded by a rim of lymphocytes and plasma cells. Langhans' giant cell is formed by the fusion of epithelioid cells that shows an arc of nuclei toward the periphery of the outer membrane giving horseshoe/ring appearance (400X).

- Caseating granulomas are demonstrated in tuberculosis. The caseating granulomas are formed when tubercle bacilli are poorly degraded by macrophages.
- Mycobacterium tubercle bacilli cause caseous necrosis, epithelioid granulomas and Langhans' giant cells with nuclei arranged peripherally in horseshoe/ring form or clustered at two poles in tuberculosis. AFB is seen in macrophages. Mycobacterium tubercle bacillus is strict aerobe. It is acid-fast due to presence of mycolic acid in its cell wall and stained by Ziehl-Neelsen staining (decolorized by 20% H_2SO_4).
- Diagnostic techniques for tuberculosis include: demonstration of acid-fast bacilli (Ziehl-Neelsen stain), culture on Lowenstein-Jensen medium (LJ medium), serological polymerase chain reaction (PCR) technique.
- Gross morphology of tubercular lymphadenitis is shown in Fig. 2.41. Granulomatous inflammation with multiple Langhans' giant cells is shown in Fig. 2.42. Mycobacterium tubercle bacilli stained by Ziehl-Neelsen (ZN) stained in tubercular lymphadenitis are shown in Fig. 2.43.

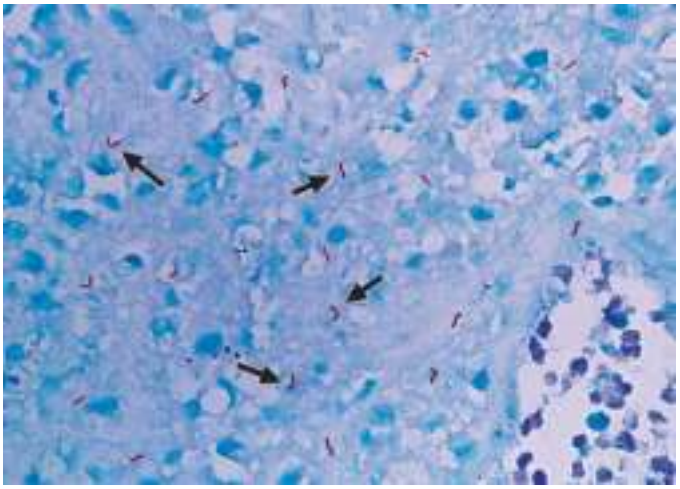


Fig. 2.43: Mycobacterium tubercle bacilli stained by Ziehl-Neelsen (ZN) stained in tubercular lymphadenitis. Ziehl-Neelsen (ZN) stain is a rapid method for detecting acid-fast bacilli (AFB) due to its rapidity, low cost and high predictive values of tuberculosis. Acid-fast bacilli have lipoid capsule that has a high molecular weight and is waxy at room temperature. This makes the AFB impermeable by aqueous-based staining solutions (arrows) (400X).

Noncaseating Granulomas

Persistence of infectious agents due to failure of phagocytosis leads to formation of noncaseating granulomas, which are composed of multinucleated giant cells without caseous necrosis. Noncaseating granulomas are formed due to fungi, cat scratch fever, syphilis, sarcoidosis, schistosomiasis and Hodgkin's disease.

- **Leprosy:** Leprosy is an infectious disease caused by a bacillus, *Mycobacterium leprae*, which multiplies slowly. *Mycobacterium leprae* are transmitted from human body by nasal discharge and digital impregnation of skin, as bacilli can be carried under nails and are inoculated under the skin by scratching. The incubation period is usually 3–5 years. Skin of extremities, mouth, eyes and peripheral nerves involving Schwann cells are commonly involved. The organisms may be demonstrated in bone marrow, liver, spleen and lymph nodes. Macrophages are laden with *Mycobacterium leprae* bacilli. Noncaseating granulomas are present in leprosy.
- **Syphilis:** Syphilis is caused by *Treponema pallidum* with formation of gumma (microscopic to grossly visible lesion), with central necrotic cells, surrounded by macrophages and plasma cell infiltrate. *Treponema pallidum* is the only organism that causes venereal disease transmitted through sexual contact, anogenital and orogenital contact. *Treponema pallidum* is a very tiny organism that is not visible on light microscopy. Thus, it can be identified by its distinct spiral movements on darkfield microscopy. The organism cannot survive outside the body.
- **Deep fungal infections:** Some fungi occur in both the yeast and mycelial forms, which are called dimorphic fungi, which cause pneumonia and disseminated infection. Noncaseating granulomas may be seen in fungal infections (e.g. histoplasmosis, blastomycosis, cryptococcosis, coccidioidomycosis). *Blastomyces dermatitidis* involves skin, bone and genitourinary system. *Coccidioides immitis* disseminates to central nervous system and bone. *Histoplasma capsulatum* involves lungs, liver, spleen and bone marrow. *Paracoccidioides brasiliensis* disseminates to lung, mucosa of mouth and nose; and central nervous system. *Histoplasma capsulatum* is shown in Fig. 2.44. *Cryptococcus neoformans* is shown in Fig. 2.45.
- **Parasitic infections:** Noncaseating granulomas are seen in schistosomiasis. Schistosomiasis, also called

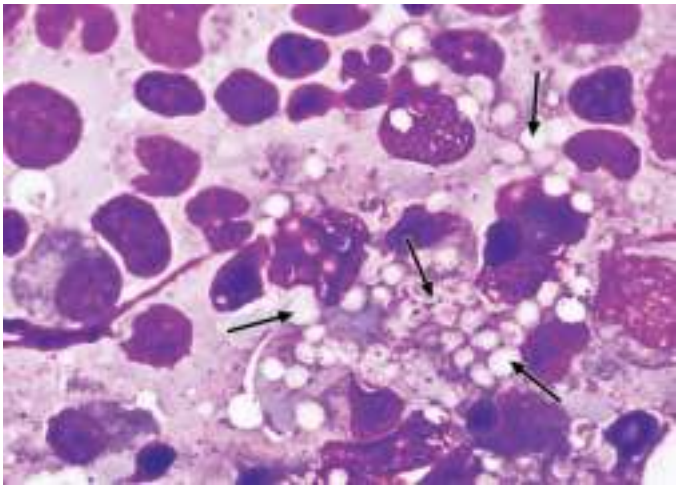


Fig. 2.44: *Histoplasma capsulatum.* Bone marrow demonstrates *Histoplasma capsulatum* as empty spaces in the reticuloendothelial cells in immunocompromised state (arrows) (1000X).

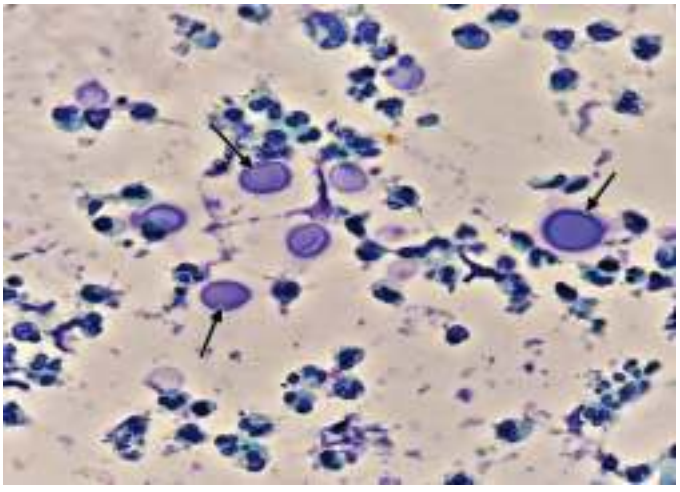


Fig. 2.45: *Cryptococcus neoformans.* Endobronchial ultrasound bronchoscopy (EBUS) demonstrates *Cryptococcus neoformans* (arrows) (400X). (*Courtesy:* Dr. Tushar Kalonia.)

bilharzia, or snail fever or Katayama fever, which is an acute and chronic disease caused by trematode parasitic flatworms of the genus Schistosoma. The parasite may infect intestine or urinary tract. Lack of proper hygiene, swimming or fishing in infested water make the persons vulnerable to infection.

- **Cat-scratch disease:** Cat-scratch disease is a gram-negative bacterial infection spread by infected cats due to cat lick into person's open wounds or bites a person by breaking the surface of the skin. Histologic examination of tissue shows rounded or stellate noncaseating granuloma containing central granular debris.

Granulomas Formation Mediated by Nonimmunologic Mechanism

Prolonged exposure to exogenous and endogenous toxic substances causes chronic inflammation. Granulomas are seen in vicinity of necrotic adipose tissue, necrotic and bone and gouty tophus (uric acid crystals).

Noncaseating Granulomas Formation in Autoimmune Disorders

Noncaseating granulomas are observed in the settings of autoimmune disorders such as Hashimoto's thyroiditis, pernicious anemia, rheumatoid arthritis, contact hypersensitivity reactions, ulcerative colitis, Crohn's disease and sarcoidosis.

- **Hashimoto's thyroiditis:** Hashimoto's thyroiditis is an autoimmune disease characterized by destruction of thyroid follicles by autoantibodies such as antithyroid peroxidase antibody, antithyroglobulin antibody and anti-TSH antibody. Histologic examination reveals noncaseating granulomas along with dense chronic inflammatory infiltrate and destruction of thyroid follicles. Patient presents with diffuse painless thyroid enlargement, transient hyperthyroidism resulting from excessive release of thyroid hormones from damaged thyroid follicles, but later hypothyroidism in 40–50% of cases.
- **Pernicious anemia:** Parietal cells in stomach synthesizes intrinsic factor required for absorption of vitamin B_{12}. Autoantibodies are formed against parietal cells in stomach cause pernicious anemia.
- **Rheumatoid arthritis:** In long-standing cases, rheumatoid nodules are formed in synovium and composed of macrophages, lymphocytes and plasma cells.
- **Contact hypersensitivity reactions:** Self-antigens are altered by exposure to nickel. Patient develops contact hypersensitivity reactions.
- **Ulcerative colitis:** Ulcerative colitis is characterized by ulcerations of mucosa and submucosa with formation of pseudopolyps in colon. Disease begins in the rectum as friable red mucosa and extends in continuity to involve segment or the entire colon, which does not form fistulous tract. Light microscopy shows crypt abscesses due to PMN cells infiltration and noncaseating granulomas. Patient may develop adenocarcinoma. Patient presents with abdominal cramping, diarrhea with blood and mucus, rectal bleeding, and tenesmus (ineffective and painful straining of stool).
- **Crohn's disease:** Crohn's disease is an autoimmune disorder characterized by discontinuous/skipped lesions involving all layers of small or large intestine. On light microscopy, intestinal wall shows noncaseating granulomas along with dense chronic inflammatory infiltrate. Patient presents with colicky pain in right lower quadrant due to intestinal obstruction, diarrhea and anal bleeding. It is located in the terminal ileum (30%), colon and small bowel (50%), colon alone (20%).
- **Sarcoidosis:** Sarcoidosis is an idiopathic disorder in which abnormal immune system leads to formation of noncaseating granulomas and collection of activated macrophages, which trigger an inflammatory response leading to extensive tissue damage and scarring. It is essential to exclude tuberculosis, fungal infection and berylliosis. Schaumann bodies calcium and protein inclusions in noncaseating granuloma in sarcoidosis are shown in Fig. 2.46. Relative frequency of organs involved in sarcoidosis is given in Table 2.49. Differences between sarcoidosis and tuberculosis are given in Table 2.50.

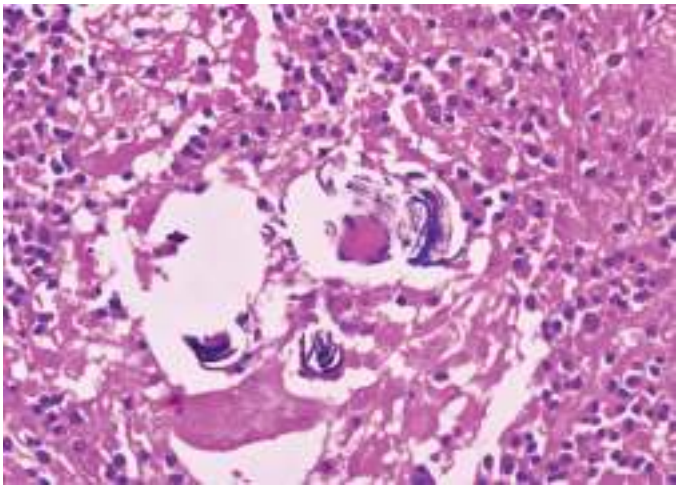


Fig. 2.46: Calcium and protein inclusions in noncaseating granuloma in sarcoidosis. The morphologic feature of sarcoidosis of unknown etiology is noncaseating epithelioid cell granulomas formed by epithelioid cells in any organs especially in intrathoracic lymph nodes and lungs in over 90% of patients. Diagnosis of sarcoidosis is made by exclusion of other systemic granulomatous conditions In pathology, **Schaumann bodies** are calcium and protein laminated inclusions inside of Langhans' giant cells as a part of granuloma in the settings of sarcoidosis, hypersensitivity pneumonitis, and berylliosis (400X).

Table 2.49 Relative frequency of organs involved in sarcoidosis

Organs	Frequency	Comments
Lymph nodes	80%	Hilar, mediastinal, cervical, epitrochlear, preauricular and postauricular lymph nodes
Lungs	80%	Diffuse consolidation without cavities
Liver	70%	Noncaseating granulomas in portal tracts
Skin	30%	Small erythematous nodules on back
Eyes	20%	Lacrimal gland involvement
Heart	20%	Light microscopy showing noncaseating granulomas in heart
Brain	15%	Noncaseating granulomas are present in brain
Spleen	15%	▪ Nodules visible in 15% cases on gross examination ▪ Light microscopy showing noncaseating granulomas in 75% cases
Bone	15%	Destruction of terminal phalanges
Nasal and pharyngeal mucosa and tonsils	10%	Light microscopy showing noncaseating granulomas in these regions
Salivary gland	1%	Bilateral parotid gland enlargement but may involve all salivary glands

Table 2.50 Differences between tuberculosis and sarcoidosis

Characteristics	Sarcoidosis	Tuberculosis
Etiology	Unknown etiology	Mycobacterium tubercle bacilli
Granuloma	Noncaseating granulomas	Caseating granuloma
Cytoplasmic inclusions in giant cells	Presence of Schaumann bodies, asteroid bodies and birefringent crystals	Absence of Schaumann bodies, asteroid bodies and birefringent crystals
Steroid therapy	Improvement	Worsening the disease
Diagnosis	Done by excluding causes of granulomatous lesions	Acid-fast bacilli demonstration

Foreign Body Granulomas

A foreign body granuloma is formed when the immune host system fails to digest the inert foreign body, resulting in the accumulation of macrophages in the tissues. As macrophages surround and isolate the foreign body, some of them will fuse to form multinucleated giant cells. T cells and fibroblasts also participate in this inflammatory response. Foreign bodies can usually be identified in the center of the granulomas, particularly if viewed with polarized light, in which it appears refractile. Foreign body granulomas are shown in Fig. 2.47.

- Substances that cause foreign body granulomas include such as surgical suture materials, implanted prosthesis, minerals and metallic particles (e.g. silica, aluminum, zinc, nickel, calcium, asbestos), carbon pigments in cosmetic tattoo, cosmetic fillers (e.g. collagen, silicon, paraffin and hyaluronic acid), ruptured cyst, hairs, and other biotic and abiotic materials, such as talc, cactus, spins, glass, retained sutures, splinters and natural and artificial hair.
- Natural history of foreign body granuloma varies depending on the etiology. Foreign body granulomas and abscesses due to bovine collagen injections often regress spontaneously within 1–2 years. Other types of foreign body granuloma may persist for decades.

Special Granuloma Formation

Special granuloma formation includes recurrent pyogenic granulomas with satellitosis and Durck's granuloma.

- **Recurrent pyogenic granulomas with satellitosis:** Development of recurrent pyogenic granulomas with multiple satellite lesions is rare and most often associated with trauma or carbon dioxide laser excision of a previous recurrent primary pyogenic granuloma. Pyogenic granuloma with satellitosis is tumor-like non-neoplastic growth of the oral mucosa or skin. Pyogenic granulomas with satellitosis can be demonstrated in cat-scratch fever and lymphogranuloma venereum. Pyogenic granulomas with satellitosis show central neutrophilic infiltrate.
- **Durck's granulomas:** Durck's granulomas are demonstrated in brain containing activated microglia/macrophages appearing at the site of a prior ring hemorrhage in cerebral malaria caused by *Plasmodium falciparum*. Malaria infestation of central nervous system can cause a severe neurological syndrome

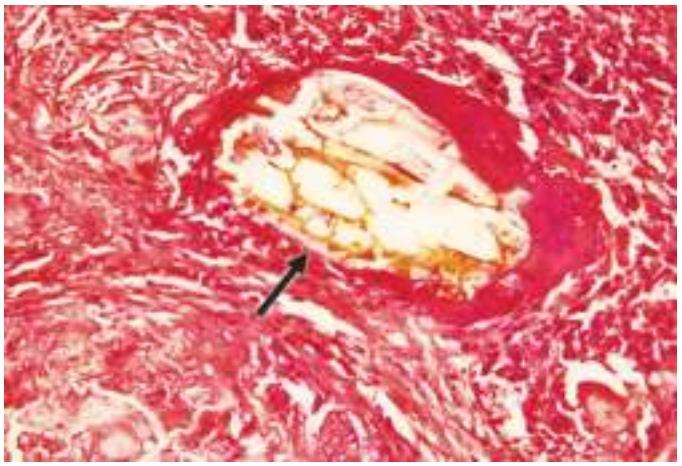


Fig. 2.47: Foreign body granulomas. Foreign body granulomas may develop, when immune system is unable to digest exogenous foreign body, resulting in the accumulation of macrophages. As macrophages surround and isolate foreign body, some of them will fuse to form multinucleated giant cells, T cells and fibroblasts also participate in this inflammatory response (arrow) (400X).

termed cerebral malaria. **Cerebral malaria** is characterized by preferential sequestration of parasitized red blood cells in the cerebral microvasculature. Immunohistochemistry and electron microscopy studies have revealed widespread cerebral endothelial cell activation, focal endothelial damage and necrosis in a patient of cerebral malaria.

MULTINUCLEATED GIANT CELLS

Normally, multinucleated giant cells are present in bone marrow (megakaryocytes), bone (osteoclasts) and placenta (syncytiotrophoblasts). Multinucleated giant cells are formed by fusion of activated macrophages (epithelioid cells) showing multiple nuclei.

- Epithelioid cells are recruited by IL-4 and IFN-γ, which measure 40–50 micron in diameter. Cytoplasm of giant cells is abundant and contains 15 or more small nuclei. Multinucleated giant cells include Langhans' giant cells, foreign body giant cells, Touton giant cells, Reed-Sternberg cells, Aschoff's giant cells, and tumor giant cells.
- Types of multinucleated giant cells are shown in Fig. 2.48A to G. Giant cells in various pathologic disorders are given in Table 2.51. Giant cells seen in bone tumors and tumor-like conditions are given in Table 2.52. Differences between Langhans' giant cell and tumor giant cell are given in Table 2.53.

Langhans' Giant Cells

Langhans' giant cells are large multinucleated cells found in granulomatous inflammatory conditions. These cells are formed by the fusion of epithelioid cells (modified macrophages) oriented around tuberculosis antigen with the multiple nuclei arranged in a horseshoe-shaped pattern in the cell periphery and an eosinophilic cytoplasm, representing the most successful type immune response. Langhans' giant cell in tissue section is shown in Fig. 2.49.

Foreign Body Giant Cells

A foreign body giant cell is a collection of fused macrophages which are generated in response to the presence of a large foreign body formed through signaling from

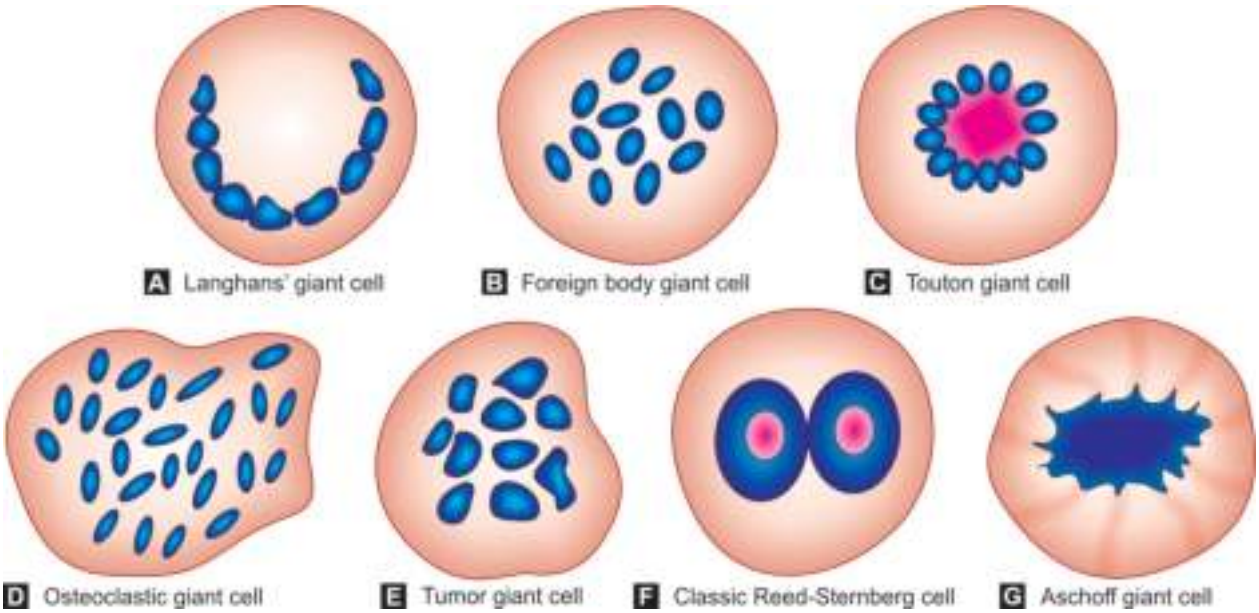


Fig. 2.48: Types of multinucleated giant cells. (A) Langhans' giant cell, (B) foreign body giant cell, (C) Touton giant cell, (D) osteoclastic giant cell, (E) tumor giant cell, (F) classic Reed-Sternberg cell, (G) Aschoff giant cell.

Table 2.51 Giant cells in various pathologic disorders

Pathological Disorder	Morphology and Condition
Langhans' giant cells	
Tuberculosis	Langhans' giant cells contain peripherally arranged nuclei in a horseshoe-shaped pattern
Foreign body giant cells	
Prolonged exposure to exogenous or endogenous substances	Foreign body giant cells show haphazardly scattered nuclei around
Foreign body granulomas present around foreign material	Surgical suture materials, implanted prosthesis, asbestos fibers, silica, beryllium, necrotic adipose tissue, necrotic and bone and gouty tophus (uric acid crystals)
Touton giant cells	
Juvenile xanthogranuloma, xanthoma disseminatum and dermatofibroma	Touton giant cells showing ring of nuclei separating outer foamy cytoplasm
Aschoff's giant cells	
Rheumatic heart disease	Aschoff's nodule composed of swollen eosinophilic collagen fibers, central fibrinoid necrosis surrounded by macrophages, lymphocytes, plasma cells and occasional multinucleated giant cells
Reed-Sternberg cells	
Hodgkin's disease	▪ Popcorn cells (lymphohistiocytic cells) in lymphocytic rich variant ▪ Lacunar cells in nodular sclerosis variant ▪ Classic Reed-Sternberg cells in mixed cellularity variant
Warthin-Finkeldey giant cells	
Measles	Giant cells
Tumor giant cells	
Tumors	Benign and malignant bone lesions
Giant cells in normal tissues	
Bone	Osteoclasts
Bone marrow	Megakaryocytes
Placenta	Syncytiotrophoblastic giant cells

Table 2.52 Giant cells seen in bone tumors and tumor-like conditions

Benign Tumors or Tumor-like Conditions
Osteoclastoma
Benign fibrous histiocytoma
Chondroblastoma
Chondromyxoid fibroma
Fibrous dysplasia
Metaphyseal fibrous defects (nonossifying fibroma)
Aneurysmal bone cyst
Malignant Tumors
Malignant fibrous histiocytoma
Fibrosarcoma
Giant cell variant of osteosarcoma

IL-3 and IL-4. Foreign body giant cell contains up to 200 nuclei within cytoplasm, which produce reactive products associated with phagocytosis and respiratory burst that can degrade the surface of the biomaterial leading to potential failure of the implanted biomaterial.

Touton Giant Cells

Touton giant cells are observed in lesions with high lipid content such as fat necrosis, xanthoma, xanthelasma, xanthogranuloma and dermatofibroma. Touton giant cells contain a ring of nuclei surrounding a central homogenous amphophilic and eosinophilic cytoplasm, while foamy cytoplasm near the periphery of the cell. Touton giant cells are formed by the fusion of macrophage-derived foam cells. It has been suggested that IL-3, IFN-γ and M-CSF may be involved in the production of Touton giant cells, which are positive for CD14, CD68, CD163, factor XIIIa and fascin, suggesting that Touton giant cells are dermal dendrocytes.

Aschoff's Giant Cells

Aschoff giant cells are associated with rheumatic heart disease. Normally, cardiac macrophages are present in small number. But, in rheumatic heart disease,

Table 2.53 Differences between Langhans' giant cell and tumor giant cell

Features	Langhans' Giant Cell	Tumor Giant Cell
Origin	Fusion of epithelioid cells in response to some chronic inflammation	Anaplastic giant cells, dividing nuclei of neoplastic cells
Number of nucleus/nuclei	Containing 20 or more small nuclei—either around the periphery in horseshoe/ring form or clustered at two poles	Prominent nuclei—either single huge polymorphic nucleus or may have two or more nuclei
Nucleus morphology	Normal appearing nucleus, same as macrophage and epithelioid cells	Nucleus hyperchromatic
Size of nucleus	Normal	Large in relation to cells
Associated features	Absent	Showing other features of malignancy

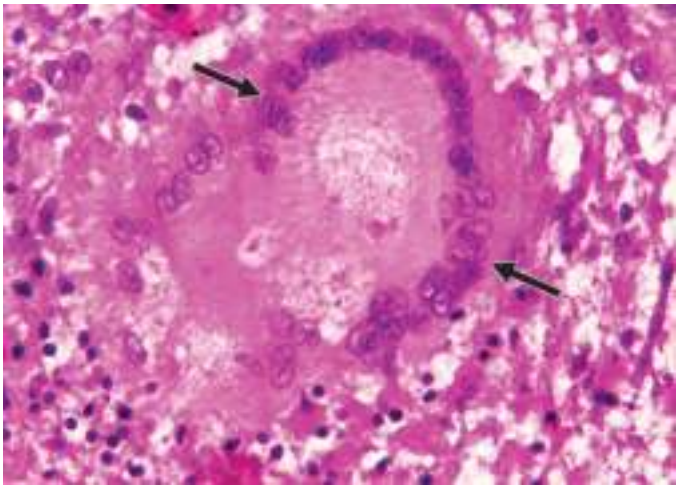


Fig. 2.49: Langhans' giant cell in tissue section. Langhans' giant cell is a large cell characterized by an arc of nuclei toward the periphery of the outer membrane giving horseshoe/ring appearance. The cell is formed by the fusion of epithelioid cells, which are derived from immune cells, called macrophages (arrows) (400X).

macrophage number is increased, which participate in the formation of Aschoff nodule surrounding centers of fibrinoid necrosis. Aschoff giant cells are known as Anitschkow cells, which contain basophilic cytoplasm, up to four nuclei with central band of clumped chromatin that gives caterpillar-like appearance in longitudinal sections also called caterpillar cells, which have owl-eyed appearance in cross sections.

Reed-Sternberg Cells

Diagnostic criteria of Hodgkin disease include demonstration of neoplastic Reed-Sternberg cells in the lymph nodes. Classic, mononuclear, pleomorphic and lacunar variants of Reed-Sternberg cells are demonstrated in various histologic types of Hodgkin disease. Classic Reed-Sternberg cells are seen in mixed cellularity variant of Hodgkin disease. Mononuclear Reed-Sternberg cells can be observed in any variant of Hodgkin disease. Pleomorphic Reed-Sternberg cells are seen in lymphocytic depletion variant of Hodgkin disease. Lacunar Reed-Sternberg cells are observed in nodular sclerosis variant of Hodgkin disease. Lymphohistiocytic Reed-Sternberg cells are sometimes called popcorn cells and seen in nodular lymphocytic predominant Hodgkin disease.

Warthin-Finkeldey Giant Cells

Warthin-Finkeldey giant cells are found in hyperplastic lymph nodes early in the course of measles, systemic lupus erythematosus and HIV-infected persons, as well as in Kimura disease, but rarely observed in lymphoma and non-neoplastic lymph node disorders. Warthin-Finkeldey giant cells have an appearance akin to 'grape-like clusters' or 'Mulberry'. Warthin-Finkeldey giant cells are large cells enclosing multiple, round, regular-sized nuclei having inconspicuous nucleoli.

SYSTEMIC EFFECTS OF INFLAMMATION

In some cases, tissue injury can result in prominent systemic inflammatory manifestations as a result of release of chemical mediators into the circulation.

ACUTE-PHASE REACTANTS SYNTHESIS

Within hours of onset of inflammation, acute phase response is triggered by infection, tissue injury, prostaglandin E, interferons associated viral infection, cytokines synthesis (IL-1, IL-6, and TNF-α), and synthesis of acute-phase reactants by liver such as C-reactive proteins, serum amyloid A, haptoglobin and fibrinogen. These acute-phase reactents reach to the site of inflammation to kill the pathogens by opsonization (CRP and SAA), clearing necrotic cells and activating complement system pathways.

- Acute-phase reactants are analyzed to monitor the progress of disease activity and assess response to therapy in inflammatory disorders (e.g. rheumatoid arthritis, juvenile chronic arthritis, ankylosing

spondylitis, Reiter's syndrome, psoriatic arthropathy, vasculitis, rheumatic fever).

- Measurement of acute-phase reactants is useful to detect complications of a known disease (e.g. immune complex deposition, postsurgical infection).
- Acute-phase proteins and their functions are given in Table 2.54. Measurement of acute-phase reactants in clinical practice is given in Table 2.55.

Immune-mediated Acute-phase Reactants

Immune-mediated acute-phase reactants include C-reactive protein (CRP), mannose-binding lectin (MBL) protein, complement proteins and α_1-acid glycoprotein.

- **C-reactive protein (IL-6):** C-reactive protein has opsonization property, which is a sensitive marker of cell necrosis associated with acute inflammation and disease activity. Its concentration is increased in

Table 2.54 Acute-phase proteins and their functions

Protein	Functions
Immune-related acute-phase reactants	
C-reactive protein	Opsonization
Mannose-binding lectin (MBL)	Opsonization, complement activation
Complement proteins	Inflammatory response to kill pathogens
α_1-Acid glycoprotein	Binding and transport of specific drugs
Antiproteases (antienzymes) acute-phase reactants	
α_1-Antitrypsin (α_1-AT)	Serine protease inhibitor
α_2-Macroglobulin (α_2-M)	Antiprotease
Antioxidants acute-phase reactants	
Ceruloplasmin	Antioxidant and binds copper
Coagulation factors acute-phase reactants	
Fibrinogen	Coagulation cascade
Factor VIII	Coagulation cascade
Other acute-phase reactants	
Serum amyloid A protein	Apolipoprotein
Haptoglobin	Haptoglobin binds hemoglobin
Plasma fibronectin	Tissue repair
Ferritin	Makes iron available for cellular processes and protects lipids, DNA and proteins from toxic effects of iron
Lipopolysaccharide-binding protein (LBP)	Binds to bacterial lipopolysaccharide and presents to cell surface recognition receptors called TLR4 and CD31 and elicits response
Cysteine proteinase inhibitor	Antiprotease

Decreased concentration of acute-phase reactants in acute inflammation include albumin, transferrin, transthyretin (TBPA) and retinol-binding protein (RBP).

Table 2.55 Measurement of acute-phase reactants in clinical practice

Acute-Phase Reactants (Proteins)	Normal Concentration Range	Concentration in Acute Inflammation	Response Time
C-reactive protein	0.0008–0.004 g/L	0.4 g/L	6–10 hours
α-Antichymotrypsin	0.3–0.6 g/L	3.0 g/L	10 hours
α-Antitrypsin	2.0–4.0 g/L	7.0 g/L	24 hours
Orsomucoid	0.5–1.4 g/L	3.0 g/L	24 hours
Haptoglobin	1.0–3.0 g/L	6.0 g/L	24 hours
Fibrinogen	2.0–4.5 g/L	10.0 g/L	24 hours
C3	0.55–1.2 g/L	3.0 g/L	48–72 hours
C4	0.2–0.5 g/L	1.0 g/L	48–72 hours
Ceruloplasmin	0.15–0.6 g/L	2.0 g/L	48–72 hours

disrupted atheromatous plaques in coronary arteries, which may predispose to thrombosis and myocardial infarction. On this basis; anti-inflammatory agents are being administered in these patients to reduce the risk of myocardial infarction. C-reactive protein is excellent marker to monitor of the disease activity in patient suffering from rheumatoid arthritis. C-reactive protein can be analyzed in the serum as a nonspecific marker of inflammation. Serial measurements of c-reactive proteins can be used to monitor progress of an inflammatory disease.

- **Mannose-binding lectin protein:** Mannose-binding lectin (MBL) protein is an acute-phase reactant produced in the liver in response to acute inflammation, which plays key role in complement activation and opsonization of pathogens. MBL recognizes and binds to sugars, such as mannose, fucose and glucose, that are found on the surface of bacteria, viruses and yeast and turning on complement system. Normal serum levels of MBL range from 800 to 1000 ng/ml in healthy Caucasians. MBL protein synthesis is initiated when pattern-recognition molecules bind to the pathogen-associated molecular patterns (PAMPs) on the surface of pathogens or to necrotic or apoptotic cell.
- **α_1-Acid glycoprotein:** α_1-Acid glycoprotein is an acute-phase protein. It is an important plasma protein involved in binding and transport of specific drugs such as diazepam, disopyramide and chlorpromazine. α_1-Acid glycoprotein concentration rises several folds during an acute inflammatory response.

Other Acute-phase Reactants Synthesis

Other acute-phase reactants include serum amyloid A (SAA) protein, haptoglobin, plasma fibronectin ferritin, lipopolysaccharide-binding protein (LBP) and cysteine proteinase inhibitor.

- **Serum amyloid A protein:** Serum amyloid associated protein belongs to a family of apolipoproteins associated with high density lipoprotein (HDL) in plasma produced in the liver. High density lipoproteins are used by macrophages as a source of energy. Prolonged synthesis of SAA results in secondary amyloidosis. Different isomers of serum amyloid associated protein are expressed constitutively at different levels in response to inflammatory stimuli (acute-phase response). SAA can be produced by macrophages in inflammatory tissue. SAA deposition in organs is found in patients with sarcoidosis, rheumatoid arthritis, inflammatory bowel disease (ulcerative colitis, Crohn's disease) and chronic persistent infections.
- **Haptoglobin:** Haptoglobin binds hemoglobin, which is an acute-phase reactant that scavenges hemoglobin in the event of independently of the degree of intravascular or extravascular hemolysis associated with tissue injury process. Haptoglobin prevents iron loss and renal damage. Haptoglobin also acts as antioxidant activity and plays a role in modulating many aspects of the acute phase response. High haptoglobin levels may be a sign of inflammatory disease.
- **Fibronectin and vitronectin:** Fibronectin and vitronectin are also acute phase reactant proteins. Fibronectin plays a role in tissue repair.
- **Ferritin:** Ferritin makes iron available for cellular processes and protects lipids, DNA and proteins from toxic effects of iron.
- **Lipopolysaccharide-binding protein:** Lipopolysaccharide-binding protein binds to bacterial lipopolysaccharide and presents to cell surface recognition receptors called TLR4 and CD31 and elicits response.

Antiproteases (Antienzymes) Acute-phase Reactants

Antiproteases acute-phase reactants include α_1-antitrypsin (ATT) and α_2-macroglobulin.

- **α_1-Antitrypsin:** α_1-Antitrypsin is a serine protease inhibitor produced by liver. It is one of the classical acute-phase response proteins with high serum ATT level indicates state of acute inflammation.
- **α_2-Macroglobulin:** α_2-Macroglobulin is an antiprotease, which functions as an inhibitor of coagulation by inhibiting thrombin. It binds foreign peptides and serves as host defense barrier against pathogens in the plasma and tissues. Its level may be increased in nephrotic syndrome.
- **Cysteine proteinase inhibitor:** Cysteine proteinase inhibitor has antiprotease activity. Human mucous membrane secretions contain a proteinase inhibitor, which is secreted locally inhibits trypsin, chymotrypsin, granulocyte elastase and cathepsin G as well as mast cell chymase and tryptase.

Antioxidants Acute-phase Reactants

Ceruloplasmin is an antioxidant that binds copper. It is an acute-phase plasma protein produced principally by liver and activated monocytes and macrophages. The plasma level of ceruloplasmin nearly doubles in response to inflammation, infection or trauma, which are mediated by cytokines. High ceruloplasmin levels indicate abnormally high levels of copper.

Coagulation Proteins as Acute-phase Reactants

Fibrinogen and factor VIII play key role in coagulation cascade.

- **Fibrinogen:** Fibrinogen plays key role in coagulation cascade, which is a marker of inflammation, which when elevated indicates the presence of inflammation and identifies persons with a high-risk for cardiovascular disorders. Increased synthesis of

fibrinogen promotes red blood cells (RBCs) rouleaux formation, which is the basis of increased erythrocyte sedimentation rate (ESR) in inflammation. In addition, fibrinogen acts as an acute-phase reactant.

- **Factor VIII:** Coagulation factor VIII is an acute-phase reactant protein in humans that plays key role in coagulation cascade. Factor VIII has been recently shown to be transcriptionally responsive to interleukin-6 (IL-6). Elevated levels of factor VIII and von Willebrand factor may indicate endothelial dysfunction and inflammation in different settings including chronic autoimmune diseases, that could serve as potential candidate biomarkers of chronic graft-versus-host disease.

CLINICAL MANIFESTATIONS IN INFLAMMATION

Systemic inflammation-associated clinical syndromes (sepsis and septic shock) have high mortality and remain as challenge in emergency medicine. Systemic inflammation is usually accompanied by changes in body temperature (fever or hypothermia), and lethargy.

Septic Shock

Septic shock is a life-threatening condition that occurs when a systemic infection leads to dangerously low-blood pressure (hypotension), generalized vasodilatation, platelet aggregation, low tissue perfusion and widespread organs dysfunction, particularly lungs, kidneys, liver, and heart. Septic shock can be a complication of infections due to gram-negative bacteria, fungi or viruses (flu or COVID-19).

- Septic shock occurs in concert with activation of monocytes/macrophages and synthesis of large quantity of cytokines IL-1 and TNF-α and activation of neutrophils that interact with the endothelium through pathogen recognition receptors and result in further involvement of cytokines, proteases, kinins, reactive oxygen species (ROS) and nitric oxide.
- Primary site response of these chemical mediators induces microvascular injury and also activates the coagulation and complement cascades which further exacerbate the vascular injury leading to capillary leakage. This cascade of events is responsible for the clinical manifestations of sepsis to septic shock.

Fever

Patient has above normal temperature (>1 to 4°C) due to bacterial products and cytokines, which act as pyrogen. Fever plays important role in providing a hostile environment for bacterial and viral reproduction. More oxygen is available for the oxygen-dependent myeloperoxidase (MPO) system. Mechanisms of fever and therapeutic agent are described as under.

- Exogenous pyrogens released by bacteria or injured cells stimulate macrophages to synthesize IL-1 and TNF-α (endogenous pyrogens). IL-1 stimulates prostaglandin E_2 (PGE_2) synthesis in the hypothalamic thermoregulatory centers, thereby resets the body temperature set point at higher level (fever).
- Profound chills with shivering, sweating and piloerection are associated with fever. Inhibitors of cyclooxygenase (e.g. aspirin) block the fever response by inhibiting PGE_2 synthesis in the hypothalamus.
- Nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin reduce fever by inhibiting cyclooxygenase and thus blocking prostaglandins synthesis.

Lethargy and other Manifestations

Lethargy, a common feature of the acute-phase response, results from the effects of IL-1 and TNF-α on the central nervous system. Patient presents with increased pulse rate, hypotension, rigors (shivering), chills (search for warmth), anorexia, somnolence, and malaise (probably because of the actions of cytokines on brain cells). There is decreased sweating due to redirection of blood flow from cutaneous to deep vascular beds leads to minimize heat loss.

SYSTEMIC INFLAMMATORY RESPONSE

Systemic inflammatory response is a clinical triad characterized by (a) disseminated intravascular coagulation, (b) cardiovascular failure and hypoglycemia.

- **Disseminated intravascular coagulation (DIC):** Patient develops DIC by the action of bacterial products (lipopolysaccharide) and TNF-α. These bacterial products and TNF-α stimulate endothelial cells to express tissue factor and promote platelets aggregation, which also inhibit anticoagulant mechanism by (a) decreasing synthesis of thrombomodulin by endothelial cells, and (b) decreasing the expression of tissue factor pathway inhibitor (TFPI). This condition is always fatal.
- **Cardiovascular failure:** Cytokines (IL-1 and TNF-α) activate cardiac myocytes and vascular smooth muscle cells to synthesize nitric oxide (NO), which causes heart failure and hemodynamic shock due to loss of perfusion pressure. Patient may develop acute respiratory distress syndrome (ARDS) is neutrophil-mediated endothelial injury characterized by escape of fluid from blood into the lung air spaces.

METABOLIC ALTERATIONS IN INFLAMMATION

Various metabolic alterations can occur in inflammation, which include negative nitrogen balance, skeletal muscle catabolism, glucose metabolism, lipid metabolism, mineral metabolism and acid–base balance.

- **Negative nitrogen balance:** Negative nitrogen balance occurs due to protein catabolism. Metabolic products increase the osmotic pressure in interstitial

space which attracts water and thus contribute to edema (swelling—tumor).

- **Skeletal muscle catabolism:** Skeletal muscle catabolism provides amino acids that can be used in the immune response and tissue repair.
- **Glucose metabolism:** Cytokines (IL-1 and TNF-α) cause liver injury and impair liver function, resulting in a failure to maintain normal blood glucose levels due to lack of glycolysis from stored glycogen. Anaerobe utilization of glucose is increased because of hypoxia with increased formation of lactic acid and pyruvic acid.
- **Lipid metabolism:** Lipid metabolism leads to increased formation of ketone bodies and fatty acids.
- **Mineral metabolism:** There is increased extracellular K^+ concentration in inflammation.
- **Acid–base balance:** Metabolic acidosis occurs as a result of formation of ketone bodies and lactic acid.

RELEASE OF CHEMICAL SUBSTANCES IN INFLAMMATION

Interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) synthesized by macrophages play a key role in release of norepinephrine, vasopressin and activation of renin-angiotensin-aldosterone system.

HEMATOLOGIC ALTERATIONS IN INFLAMMATION

Hematologic findings in acute inflammation include leukocytosis, leukemoid reactions, leukopenia, monocytosis, lymphopenia, lymphocytosis, eosinophilia and elevated erythrocyte sedimentation rate (ESR).

- **Leukocytosis:** Leukocytosis is defined as an absolute increase in the circulating white blood cell count. In acute inflammation (bacterial infection), cytokines (IL-1, TNF-α) released by leukocytes stimulate bone marrow leading to leukocytosis (TLC 15000–20,000/cu mm). In prolonged infection, colony stimulating factors (CSFs) induce proliferation of myeloid precursors in the bone marrow. There is shift to the left, which is defined as >10% band form neutrophils or the presence of metamyelocytes. Neutrophils contain toxic granules (e.g. prominence of azurophil granules in lysosomes).
- **Leukemoid reactions:** In some cases, bone marrow produces more immature leukocytes in increased number into the circulation (i.e. shift to the left) showing leukocytosis as high as 40,000–100,000/cu mm (leukemoid reactions). It is sometimes difficult to differentiate leukemoid reaction from leukemia.
- **Leukopenia:** Leukopenia is defined as an absolute decrease in the circulating WBC count. Leukopenia is occasionally encountered under conditions of chronic inflammation, especially in patients who are malnourished or who suffer from a chronic debilitating disease such as typhoid fever, viruses, rickettsiae, certain protozoa, disseminated cancer and rampant (difficult to treat) tuberculosis.
- **Monocytosis:** Peripheral blood shows absolute monocytosis in chronic debilitating disease (e.g. tuberculosis, rheumatoid arthritis). There is increase in serum IgG levels.
- **Lymphopenia:** Lymphopenia occurs due to sequestration of B and T cells in lymph nodes, which indicates apoptosis of lymphocytes.
- **Lymphocytosis:** Lymphocytosis is defined as an increase in the absolute peripheral blood lymphocyte count above the normal range (<4,000/μl in children and 9,000/μl in infants) in acute viral infections (infectious mononucleosis, mumps and German measles), whooping cough, tuberculosis, brucellosis and lymphoproliferative diseases.
- **Eosinophilia:** Eosinophilia is demonstrated in bronchial asthma, hay fever and parasitic infestations.
- **Elevated erythrocyte sedimentation rate (ESR):** Increased plasma levels of acute phase reactants accelerate erythrocyte sedimentation rate. ESR index estimated by Wintrobe's tube or Westergren's pipette expressed as mm/hour is important to monitor the activity of many inflammatory diseases such as tuberculosis and rheumatoid arthritis.

TISSUE REPAIR

Tissue repair is a natural process in which architecture and function of the tissue is restored by compensatory regeneration of active resident cells as well as maturation of stem cells, following loss of blood supply, mechanical trauma, surgical procedure, or chemical-induced injury.

- Tissue repair requires the highly coordinated interaction of vascular component, cellular component and chemical mediators.
- If the injured tissue is incapable of complete restoration to original state, tissue repair occurs by laying down of connective tissue and scar formation.
- Fibrosis develops in a tissue space by organization of the inflammatory exudate in chronic inflammation occupying the tissue space by extensive deposition of collagen in the parenchymal organ.

NORMAL CELL PROLIFERATION AND TISSUE GROWTH REGULATION

Size of cell population in adult tissue is determined by rate of cell proliferation, differentiation and apoptosis. Increased cell numbers may result from either increased proliferation or decreased cell death by apoptosis.

- Apoptosis is a physiologic process required for tissue homeostasis, but it can also be induced by a variety of pathologic stimuli (*refer* to Chapter 1).
- Hyperplasia is an increase in the number of cells of a tissue due to increased rate of cell division resulting from increased functional demand, hormonal stimulation. It may be physiologic, compensatory or pathologic conditions (*refer* to Chapter 1).
- Tissue healing occurs by removal of the damaged tissue by phagocytosis, lymphatic drainage of exudates, angiogenesis, and regeneration of residual stem cells, which restore the continuity of the injured tissue.
- Damage to residual stem cells leads to fibrosis resulting in malfunction of organs. Both platelet-derived growth factor (PDGF) and fibroblast growth factor (FGH) participate in migration, proliferation of fibroblasts leading to synthesis and deposition of extracellular matrix leading to wound healing. Most of the organs heal by regeneration and fibrosis.

TISSUE PROLIFERATIVE ACTIVITY

Tissue proliferative activity is a process of replacement of damaged components and returning to normal state, which requires preservation of the basement membrane, residual stem cells and intact extracellular matrix (collagen fibers type 3 and fibronectin). Regeneration is not possible if the stem cells are destroyed.

- Tissue healing involves two processes: (a) proliferation of residual stem cells to replace lost tissue; and (b) migration of residual stem cells into the vacant space.
- Regeneration of cells depends on the ability of cells to replicate (e.g. labile cells, stable cells and permanent cells) and growth factors such as VEGF, FGF-β, and EGF, which stimulate cell division resulting in restoration of tissue to normal.
- The tissues are divided into three groups based on proliferative activity of their cells in the context of the cell cycle, which include labile cells, stable cells and permanent cells. Role of labile, stable and permanent cells in tissue healing is shown in Fig. 2.50. Cells based on proliferative activity in the context of cell cycle are given in Table 2.56.

Labile Cells/Continuous Dividing Cells

Labile cells enter cell cycle continuously and proliferate throughout life. Regeneration of cells is excellent, if stem

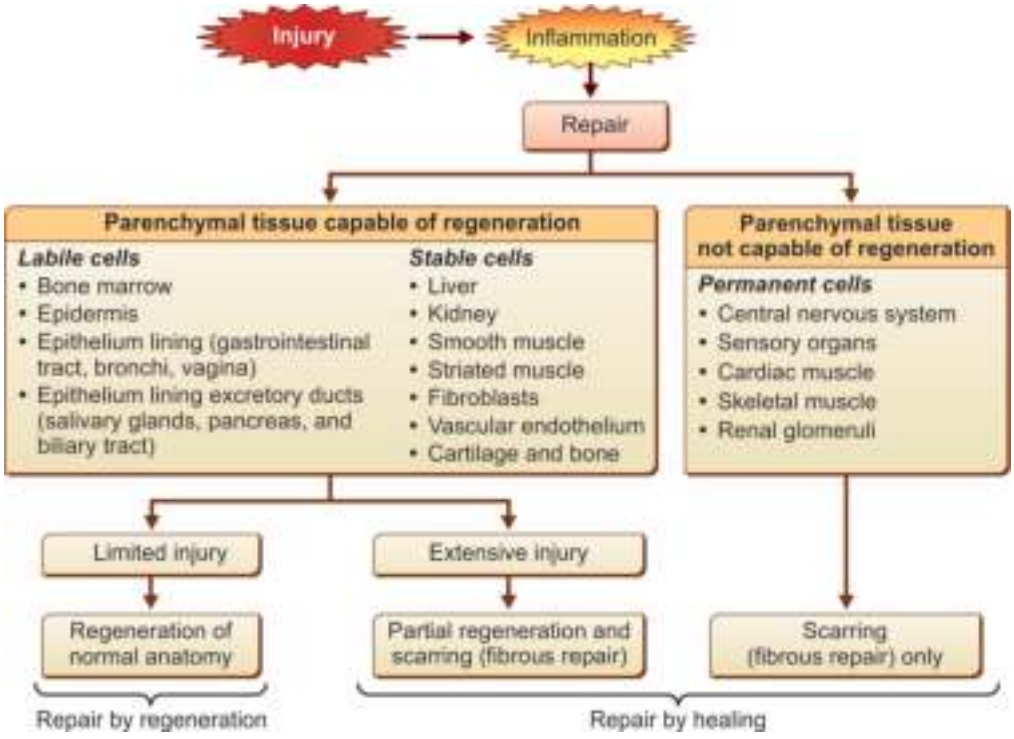


Fig. 2.50: Role of labile, stable and permanent cells in tissue healing. Labile cells in healing and repair process. Parenchymal tissue with labile cells contains stem cells that continuously regenerate new stem cells. Tissue with labile cells repairs by regeneration. Parenchymal tissue with stable cells contains stem cells that reproduce rapidly as a result of injury. Tissues with stable cells repair by regeneration. Tissues with permanent cells do not contain stem cells. Hence, injury to tissues with permanent cells repair by fibrosis only.

Table 2.56 Cells based on proliferative activity in the context of cell cycle

Labile Cells
Bone marrow cells
Epidermis
Epithelium lining gastrointestinal tract, bronchi and vagina
Epithelium lining excretory ducts of salivary glands, pancreas, and biliary tract
Stable Cells
Liver
Kidney
Smooth muscle
Fibroblasts
Vascular endothelium
Cartilage
Bone
Permanent Cells
Brain
Cardiac muscle
Skeletal muscle

cells are not destroyed. Examples of labile cells are bone marrow cells and epithelium lining epidermis, oral cavity, gastrointestinal tract, genitourinary tract, uterus, cervix and vagina, excretory ducts of salivary glands, pancreas, and biliary tract. Organs with labile cells heal by regeneration without fibrosis.

Stable Cells/Quiescent Cells (Facultative Mitotic Cells)

Stable cells normally have little proliferative activity but remain capable of more rapid cell division following injury, e.g. liver, renal tubular epithelium and smooth muscle, fibroblasts, vascular endothelium, cartilage and bone. That is the reason that stable cells regenerate from G0 cells (quiescent) when needed.

Permanent Cells/Nondividing Cells

Permanent cells are irreversibly postmitotic terminally differentiated cells, which are incapable of division and regeneration in postnatal life. Healing occurs by fibrosis. Examples of permanent cells are neurons of central nervous system, sensory organs, renal glomeruli, myocardial muscle, and striated muscle. Injury to adult neurons is replaced by glial cells (gliosis in CNS), cardiac muscle in acute myocardial infarction replaced by fibrosis, and skeletal muscle fibrosis through the differentiation of the satellite cells located beneath myocytes basal lamina.

STEM CELLS IN TISSUE HOMEOSTASIS

Stem cells possess self-renewal properties to generate differentiated cell lineages, which are essential to give rise to these lineages during life. Embryonic and adult stem cells can be used to repopulate damaged cells. Generation and differentiation of stem cells is shown in Fig. 2.51. Stem cell therapy in clinical practice is given in Table 2.57.

Embryonic Stem Cells

Human embryonic stem cells are pluripotent stem cells derived from the inner mass of the blastocyst, an early-stage pre-implanted embryo. Human embryo reaches the blastocyst stage 4–5 days of post fertilization, at which time blastocyst consists of 70–100 cells. The blastocyst, later gets implanted into uterine endometrium. The embryonic stem cells are cultured and used to repopulate damaged cells in various organs such as **β-**embryonic stem cells in diabetes mellitus, myocardial embryonic stem cells in acute myocardial infarction and hepatocyte embryonic stem cells in hepatocellular injury.

- **Totipotent cells:** Totipotent cells are obtained from a fertilized oocyte in a woman's fallopian tube, that can differentiate into embryonic and extraembryonic cells to create an entire human body, which normally results in the birth of new human being.
- **Pluripotent cells:** Pluripotent cells are derived from totipotent cells, which have the potential to differentiate into almost any type of cell (i.e. embryonic stem cells from blastocyst).
- **Multipotent cells:** Multipotent cells can give rise to multiple cell types. Hematopoietic stem cell can change into several types of blood cells.
- **Oligopotent cells:** Oligopotent cell can differentiate into a few cell types (i.e. myeloid or lymphoid progenitor cells).
- **Unipotent cells:** Unipotent cell can only make one cell type itself, which also has the power to self-renewal (i.e. hepatocytes).

Adult Stem Cells

Adult stem cells, also known as somatic cells, are undifferentiated cells that reside among differentiated cells in a tissue or organ throughout the body after development. Adult stem cells possess two distinctive properties: self-renewal and capacity to differentiate into multiple lineages.

- Unlike embryonic stem cells, adult stem cells are multipotent progenitor cells, which possess limited differentiation potential, responsible for replenishing damaged or dead cells due to injury or disease.
- Adult stem cells are located in specialized vascular microenvironments, referred to as the stem cell

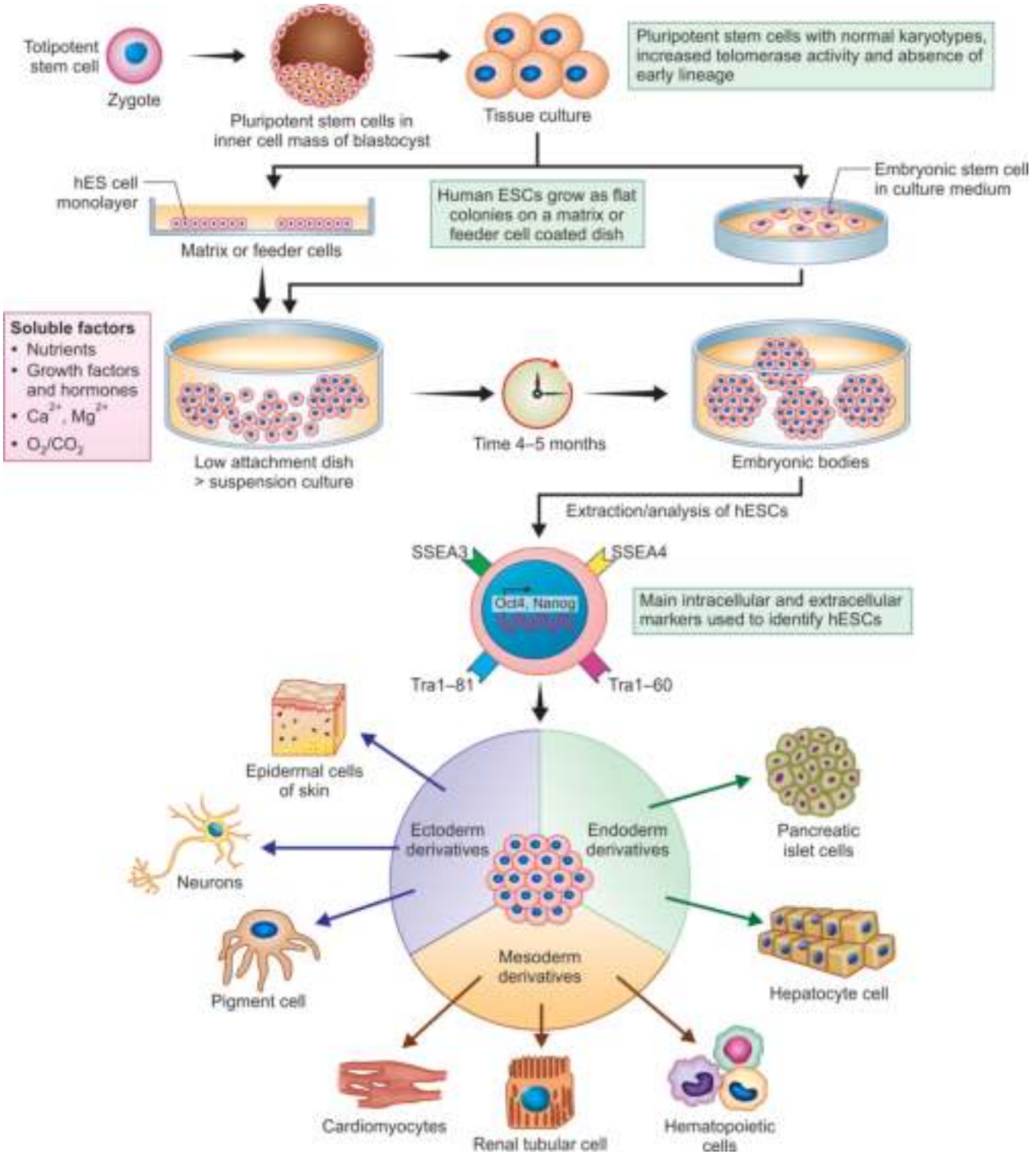


Fig. 2.51: Generation and differentiation of stem cells. Embryonic pluripotent stem cells obtained from inner cell mass of blastocyst are cultured and induced to obtain differentiated cells such as hepatocytes, pancreatic islets of Langerhans' cardiomyocyte and neurons.

niche: bone marrow, liver, epidermis, skeletal muscle, colon and cornea. These niche cells generate or transmit stimuli that regulate stem cell self-renewal and the generation of progeny cells.

- Adult stem cells can be reprogrammed into pluripotent cells, similar to embryonic stem cells, by the transduction of genes encoding embryonic stem cell transcription.

Table 2.57 Stem cell therapy in clinical practice

Disease	Stem Cell Therapy
Embryonic stem cells	
Diabetes mellitus	β-Embryonic stem cells
Myocardial infarction	Myocardial embryonic stem cells
Hepatocellular damage	Hepatocytic embryonic stem cells
Adult stem cells	
Restricted use skin, lining epithelia of gut, cornea and hemato-poietic tissue	Reprogramming of adult stem cells into pluripotent cells, similar to embryonic cells, by the transduction of genes encoding embryonic stem cell trans-cription

Pathology Pearls: Maintenance of Stem Cells

Maintenance of stem cells is achieved by two mechanisms: obligatory asymmetric replication and stochastic differentiation.

Obligatory Asymmetric Replication of Stem Cell

- Obligatory asymmetric replication is also known as symmetric cell division in which one cell is identical to the mother cells and the other cell is totally differentiated one.
- In obligatory asymmetric replication, each stem cell division produces two daughter cells with different cellular fates: one copy of the original stem cell retains self-renewal property, while second cell enters a differentiation pathway into a non-stem cell fate.
- Obligatory asymmetric replication is a key mechanism to ensure tissue homeostasis. In normal stem and progenitor cell, asymmetric cell division balances proliferation and self-renewal with cell cycle exit and differentiation.

Stochastic Differentiation of Stem Cell

- Stochastic differentiation refers to the process in which a father stem cell divides into two differentiated daughter cells.
- In order to maintain the population (stem cell reserve) in the body, a second father cell divides into two stem cells.

Stem Cell Therapy

Both embryonic stem (ES) and induced pluripotent stem (iPS) cells are capable of differentiating into various cell types. Adult stem cells, location and their clinical uses are given in Table 2.58.

- **Embryonic stem cell therapy:** Therapeutic cloning using embryonic stem cells is done by introducing diploid nucleus of an adult cell into an anucleate oocyte. The oocyte is activated, and the zygote divides to become a blastocyst that contains the donor DNA. The blastocyst is dissociated to obtain embryonic stem cells. Differentiated cells of adult tissues can be reprogrammed to become pluripotent by transferring their nucleus to an enucleated oocyte.
- **Induced pluripotent stem cell therapy:** The cells of a patient are placed in culture and transduced with genes encoding transcription factors, to generate induced pluripotent stem cells (iPS cells). The goal of stem cell therapy is to repopulate damaged organs of a patient or to correct a genetic defect, using the cells of the same patient to avoid immunological rejection.

Stem Cells in Tissue Homeostasis

Stem cells have an essential role in tissue homeostasis, repair and regeneration of a tissue or an organ. Stem cells are immature cells having unlimited ability of self-renewal and capacity to differentiate into the specialized cell types. The zygote gives rise to pluripotent cells in the embryo, which gives rise to multipotent tissue-specific stem cells that complete the process of organogenesis during fetal development.

- **Bone marrow stem cells:** Bone marrow consists of two types of stem cells: hematopoietic stem cells and marrow stromal cells.
  - **Hematopoietic stem cells:** Hematopoietic stem cells (HSCs) are pluripotent labile cells involved in the hematopoiesis. Labile cells enter cell cycle continuously and proliferate throughout life. Regeneration of cells is excellent, if stem cells are not destroyed. Hematopoietic stem cells are used in various hematologic disorders. These hematopoietic stem cells are obtained from bone marrow, umbilical cord and blood. Granulocyte macrophage colony stimulating factors administered to obtain hematopoietic stem cells.
  - **Marrow stromal cells:** Marrow stromal cells (MSCs) are multipotent, which do not participate in normal hemostasis. These marrow stromal cells are important for therapeutic applications to generate osteoblasts, chondroblasts, endothelial cells, myoblasts and adipocytes. Marrow stromal cells are obtained and administered to generate cells depending on the tissues.
- **Liver stem cells:** Liver stem cells are present in canals of Hering at the junction between parenchymal hepatocytes and biliary ductal system. Liver stems located in this niche may undergo transformation to bipotential progenitor 'oval cells', which are capable of differentiating into parenchymal hepatocytes and biliary ductular system. Oval cells are prominent in the patients with liver diseases such as recovery phase of fulminant hepatocellular failure, liver tumors, chronic hepatitis and advanced liver cirrhosis.
- **Neural stem cells in brain:** Brain contains neural stem cells (NSCs), also known as neural precursor cells, which are capable to generate neurons,

Table 2.58 Adult stem cells, location and their clinical uses

Location of Stem Cells	Clinical Use
Bone marrow stem cells	
Hematopoietic stem cells	Various hematological disorders
Marrow stromal cells	Generation of osteoblasts, chondroblasts, endothelial cells, myoblasts and adipocytes
Liver stem cells	
Canals of Hering	Oval cells prominent in recovery phase of fulminant hepatic failure, liver tumors, chronic hepatitis and cirrhosis
Neural stem cells	
Subventricular zone (SVZ) and dentate nuclei	Generating of neurons, astrocytes, oligodendrocytes in Parkinson's disease, Alzheimer disease and spinal cord injury
Epidermis stem cells	
Hair follicle bulge, interfollicular areas of epidermis and sebaceous glands	Wound healing
Skeletal muscle stem cells	
Satellite cells located beneath the myocyte basal lamina	Regeneration of injured skeletal muscle
Cardiac muscle stem cells	
Under debate	May be used in injured myocardium
Intestinal stem cells	
Located immediately above Paneth cells in the small intestine, or at the base of the crypts in the colon	Regeneration of crypts of small intestine
Limbal stem cells in cornea	
Located at the junction between the epithelium of the cornea and the conjunctiva	Corneal opacity and correction of photoreceptors

astrocytes and oligodendrocytes. Neural stem cells have been demonstrated in two areas of brain such as subventricular zone (SVZ) and dentate nuclei. Research is in progress for neural stem transplantation in patients suffering from Parkinson's disease, Alzheimer disease and spinal cord injury.

- **Skin epidermis stem cells:** Skin consists of stem cells in the epidermis regions: the hair follicle bulge, interfollicular areas of epidermis and sebaceous glands. Skin epidermis stem cells are regulated by two mechanisms: (a) Wnt pathway stimulates epidermis stem cells, and (b) bone morphogenic protein (BMP) inhibits epidermis stem cells. Bulge area of hair follicle is niche for stem cells. Epidermis stem cells are capable to generate all the cell lineages of the hair follicle. Human skin consists of labile cells capable of high turnover rate of about 4 weeks, which replenish surface epithelial cells in skin wound. These labile cells do not participate in normal hemostasis. Skin epidermis stem cells are shown in Fig. 2.52.
- **Intestinal epithelial stem cells:** Intestinal epithelial stem cells are located immediately above Paneth cells

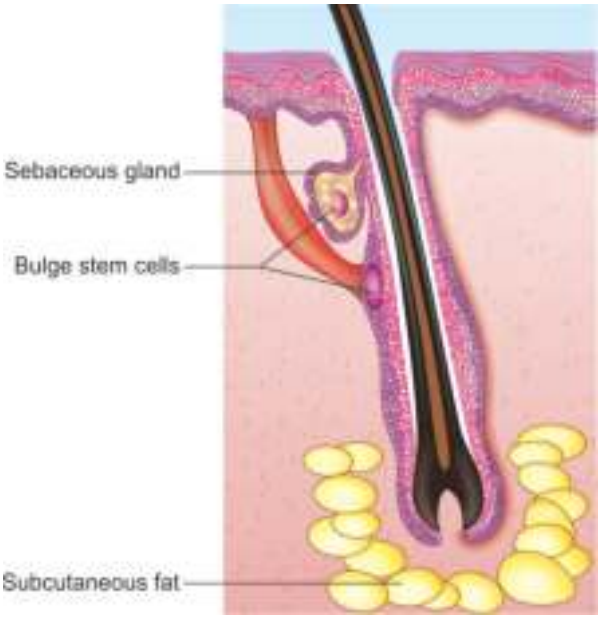


Fig. 2.52: Skin epidermis stem cells. Stem cells are located in the bulge area of the hair follicle, sebaceous glands and lower layer of the epidermis. Stem cells in skin replenish surface epithelial cells after skin wound.

in the small intestine, or at the base of the crypts in the colon. Intestinal stem cells are regulated by Wnt pathway and bone morphogenic protein similar to regulation seen in skin epidermis. Monoclonal proliferation of intestinal stem cell participates in formation of crypts in the villous structures of intestine. Stem cells in crypts of small intestine regenerate crypts in about 3–5 days.

- **Skeletal muscle satellite stem cells:** Skeletal muscle satellite cells are located beneath the myocyte basal lamina, which participate in regeneration of injured skeletal muscle. NOTCH signaling regulate skeletal muscle satellite stem cells by upregulation of δ-like ligands and angiogenesis.
- **Cardiac muscle stem cells:** Presence of cardiac muscle stem cells in myocardium is under debate. It has been proposed that myocardium may contain progenitor cells, which may repair the injured heart. There is no role of these progenitor cells during physiologic aging process.
- **Limbal stem cells in cornea:** The transparency of cornea depends on the integrity of the outermost corneal epithelium, which is maintained by limbal stem cells (LSCs). The limbal stem cells located at the junction between the epithelium of the cornea and the conjunctiva. Hereditary or acquired deficiency of limbal stem cells results in corneal opacity. It can be treated by transplantation of limbal stem cells. Limbal stem cells in cornea are shown in Fig. 2.53.

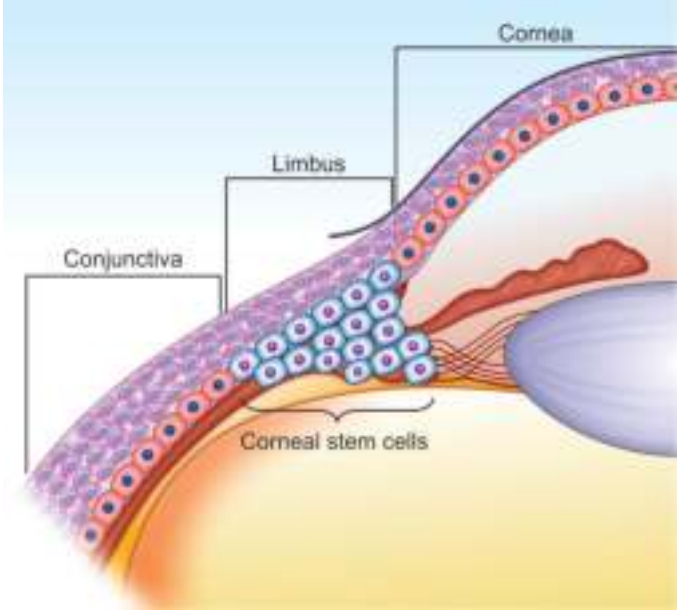


Fig. 2.53: Limbal stem cells in cornea. Limbal stem cells, also called corneal epithelial stem cells are located in the basal epithelial layer of the corneal limbus. These cells form the border between the cornea and sclera. Additionally, these cells also prevent the conjunctival epithelial cells from migrating onto the surface of cornea.

- **Retinal stem cells:** Retinal stem cells persist at the margin of retina, near the junction with the ciliary epithelium. Retinal stem cells transplantation may correct the loss of photoreceptors that occurs in degenerative diseases of the retina.

CELL CYCLE IN TISSUE HOMEOSTASIS

Before division, a cell must double its mass and content. This occurs during the growth phase, called interphase. Chromatin, the small, slender rods of the nucleus that give its granular appearance, begins to form. Replication and duplication of deoxyribonucleic acid occurs during the four phases of mitosis.

- During prophase, the chromosomes coil and shorten, and the nuclear membrane dissolves. Each chromosome is made up of a pair of strands called chromatids, which are connected by a spindle of fibers called a centromere.
- During metaphase, the centromeres divide, pulling the chromosomes apart. The centromeres then align themselves in the middle of the spindle.
- At the onset of anaphase, the centromeres begin to separate and pull the newly replicated chromosomes toward opposite sides of the cell. By the end of anaphase, 46 chromosomes are present on each side of the cell.
- Telophase is the final phase of mitosis—a new membrane forms around each set of 46 chromosomes. The spindle fibers disappear, cytokinesis occurs, and the cytoplasm divides, producing two identical new daughter cells.

CELL CYCLE PHASES

G0, G1, G2, S and M phases of cell cycle are shown in Fig. 2.54. Cell cycle phases comprising G0, G1, G2, and M phases are given in Table 2.59.

- **M (mitotic phase):** Mitotic phase describes the interval between the onset of prophase and conclusion of telophase. The cell undergoes mitosis and cytokinesis or equal division of chromosomes and cell membrane, cytoplasm and organelles between two new daughter cells. Ki-67 is expressed during active phases of cell cycle (G1/M).
- **G1 (pre-synthetic phase):** Following mitosis, the cell enters this phase. Synthesis of RNA, protein, organelles and cyclin-D occurs in G1 phase. The main difference between slowly and rapidly dividing cells is the length of the G1 phase of cell cycle.
- **S (synthetic phase):** Synthesis of DNA, RNA, and protein occurs in S phase. Labile cells undergo mitosis throughout life. Doubling of DNA occurs in this phase.

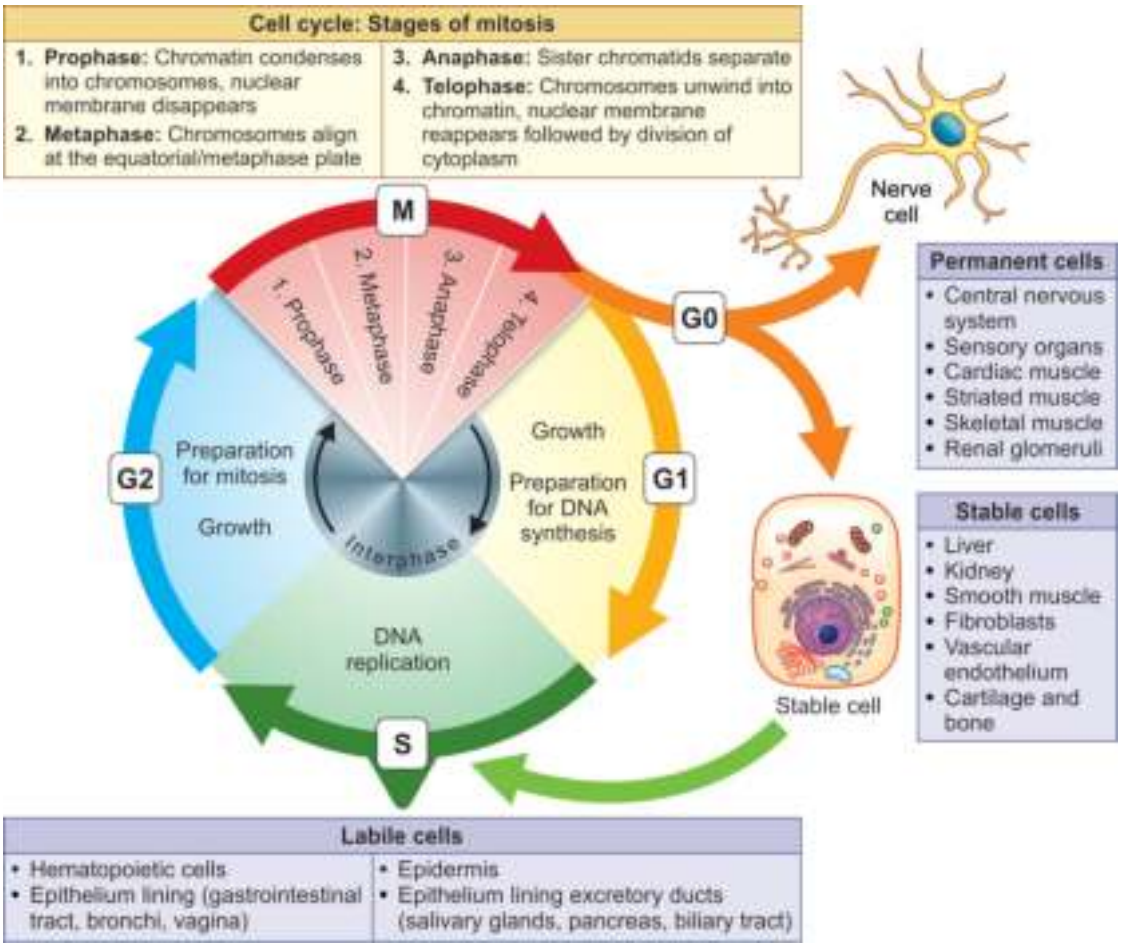


Fig. 2.54: G0, G1, G2, S and M phases of cell cycle. Location of the G1 is the restriction point. G1/S and G2/M are checkpoints of cell cycle. Cells from labile tissues such as bone marrow, epidermis, epithelial lining gastrointestinal tract, bronchi and vagina may cycle continuously.

Table 2.59 Cell cycle phases comprising G0, G1, G2 and M phases

Stage	Major Functions	
G0 phase of cell cycle		
G0 phase	Relatively inactive and nondividing stable state for cell cycle	
Interphase of cell cycle		
G1 phase	▪ Period of cell growth and preparation for DNA synthesis in the cell: G1/S checkpoint	▪ Ki-67 is expressed during active phases of cell cycle (G1/M)
S phase	▪ Period during which DNA is synthesized	▪ Ki-67 is expressed during active phases of cell cycle (G1/M)
G2 phase	▪ In G2 phase the cell grows and prepares for cell division: G2/M checkpoint	▪ Ki-67 is expressed during active phases of cell cycle (G1/M)
M phase of cell cycle		
Prophase	Chromosomes condense and mitotic spindle formed	
Prometaphase	Nuclear envelope disintegrates, spindle microtubules anchor to kinetochores	
Metaphase	Chromosomes align on metaphase plate; spindle-assembly checkpoint	
Anaphase	Sister chromatids separate, becoming individual chromosomes that migrate toward spindle poles	
Telophase	Chromosomes arrive at spindle pole. The nuclear envelope re-forms, and the condensed chromosomes relax	
Cytokinesis	Cytoplasm divides	

1. Ki-67 is expressed during active phases of cell cycle (G1/M). 2. In M phase, the cell undergoes mitosis and cytokinesis or equal division of chromosomes and cell membrane, cytoplasm and organelles between two daughter cells. 3. Proliferation of the cell is only appreciated once cytokinesis has occurred.

- **G2 (pre-mitotic phase):** After completion of nuclear DNA duplication, cell enters G2 phase. Synthesis of tubulin is necessary for formation of mitotic spindle.
- **G0 phase:** G0 phase is the resting phase of stable parenchymal cells. Quiescent cells that have not entered the cell cycle are in the G0 phase. This phase consists of stable and permanent cells. Stable cells may enter cell cycle. Permanent cells do not enter cell cycle.

CELL CYCLE REGULATION

Regulation of the G1, checkpoint (G1 to S) phase is the most critical phase of the cycle. It is controlled by cyclin D and cyclin-dependent kinase 4 (CDK-4). Growth factors activate nuclear transcribing proto-oncogenes to produce cyclin D and CDK-4.

- **Binding of cyclin D to CDK-4:** Cyclin D binds to CDK-4 to form a complex; that causes the cell to enter the S phase by phosphorylation of RB protein. RB proteins normally arrest the cell in the G1 phase. CDK-4 phosphorylates the RB protein causing the cell to enter the S phase.
- **TP53 suppressor gene:** TP53 protein arrests the cell at G2/M phase by inhibiting CDK-4. It prevents RB protein phosphorylation. TP53 protein may provide time for repair of damaged DNA in the cell.
- **BAX gene:** When there is excessive DNA damage, BAX gene is activated, which inhibits BCL-2 anti-apoptotic gene causing release of '**cytochrome c**' from the mitochondria and apoptosis of the cell.

Pathology Pearls: Cyclin-dependent Kinases in Cell Cycle

- Cyclin-dependent kinases (CDKs) are a family of related proteins that bind to specific cyclin proteins to form active protein complexes that can phosphorylates specific target molecules.
- CDKs play a critical role in cell replication, and different CDKs are sequentially activated and inactivated during G1, S, and M phases of the cell cycle.
- CDK4 binds cyclin D at the transition to activate it to phosphorylate the retinoblastoma protein (Rb).
- CDK2 binds cyclin E late in G1 and with cyclin A in S phase to mediate the G2/M transition.
- CDK1 binds cyclin B in G2/M.

CELL REGENERATION: REGULATION

Regeneration is controlled by stimulatory and inhibitory factors. Stimulation is a three-stage processes described as under.

- **Initiation:** Cells in growth arrested phase (G0) are primed for progression to cell division. Initiation is brought about by tissue specific growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). It is worth mentioning that growth factors play important role in cancers.
- **Potentiation:** Nonspecific growth factors such as insulin, hydrocortisone, growth hormone and ACTH stimulate cells which have already been primed by the appropriate initiator to enter S phase.
- **Signaling through integrin:** Signaling through integrin enhances contact of adjacent cells with basement membranes.

GROWTH FACTORS AND CYTOKINES INVOLVED IN CELL REGENERATION

Growth factors (polypeptides) synthesized by macrophages, damaged cells and platelets play important role in the healing process by cell regeneration. These growth factors are chemotactic for white blood cells, fibroblasts and endothelial cells, which also act as mitogens, which increase proliferation of cells in tissue healing process.

Growth factors bind to specific receptors, which deliver signals to the target cells and stimulate the transcription of genes that may be silent in resting cells.

Gene products (i.e. growth factors) activate cell cycle progression, enhance synthesis of cellular proteins and prevent apoptosis resulting in cell growth. Growth factors involved in tissue healing and repair are given in Table 2.60.

- **Platelet-derived growth factor:** Platelet-derived growth factor (PDGF) is synthesized by macrophages, smooth muscle cells, endothelial cells, keratinocytes and many tumor cells. PDGF is stored in platelet granules and released on platelet activation.
  - PDGF binds to specific cell-surface receptors (transmembrane proteins) and induces tyrosine kinase activity in their intracellular domains resulting in conformational changes.
  - PDGF causes migration (chemotaxis) and proliferation of fibroblasts, smooth muscle cells and monocytes to the site of tissue injury.
  - PDGF participates in healing skin wounds by process of wound contraction. It activates hepatic stellate cells in the initial steps of liver fibrosis.
- **Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α):** EGF and TGF-α share common biologic activities.
  - EGF is synthesized by macrophages, keratinocytes, and other inflammatory cells during healing of skin wounds.
  - EGF participates in formation of granulation tissue by promoting the growth of endothelial cells and fibroblasts.
  - EGF stimulates keratinocytes. Amplification of EGFR1 occurs in glioblastoma and cancers of the lung, head and neck, and breast.

Table 2.60 Growth factors involved in tissue healing and repair

Growth Factor Source/Synthesis	Functions
Platelet-derived growth factor (PDGF stored in platelets)	
Macrophages, smooth muscle cells, endothelial cells, keratinocytes synthesize	Migration and proliferation of smooth muscle cells, fibroblasts, endothelial cells and monocytes
EGF and TGF-α (both have similar actions)	
Macrophages, keratinocytes and other inflammatory cells	▪ Stimulation of keratinocytes ▪ Granulation tissue formation as a result of proliferation of fibroblasts and endothelial cells
Fibroblast growth factor-β (FGF-β)	
Macrophages, T cells, endothelial cells, platelets, many other cells	▪ Angiogenesis ▪ Synthesis of extracellular matrix proteins
Transforming growth factor-β (TGF-β)	
Macrophages, T cells, endothelial cells, platelets	▪ Chemotaxis of fibroblasts, macrophages, and lymphocytes ▪ Stimulation of fibroblasts to synthesize collagen fibers ▪ Modulation of repair process by inhibiting collagen degradation ▪ Decreasing the synthesis of metalloproteinase enzymes and enhances protease inhibitor activities
Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α)	
Macrophages	▪ Proliferation of fibroblasts, smooth muscle cells, and endothelial cells in wound healing ▪ Chemotaxis of neutrophils ▪ Regeneration of hepatocytes ▪ Synthesis of metalloproteinases and acute-phase reactants
Fibronectin	
▪ Blood plasma	▪ Chemotaxis of fibroblasts and endothelial cells ▪ Angiogenesis and extracellular matrix deposition
Vascular endothelial growth factor (VEGF)	
Endothelial cells and many other cells	▪ Angiogenesis in chronic inflammation and tumors ▪ Healing of wounds
Hepatocyte growth factor (HGF)	
Fibroblasts, endothelial cells, and mesenchymal cells of liver	Proliferation of hepatocytes, epithelia of biliary tract, lungs, kidney, mammary gland and skin
Keratinocyte growth factor(KGF)/fibroblast growth factor 7 (FGF-7)	
Fibroblasts	Migration, proliferation and differentiation of keratinocytes
Insulin growth factor 1 (IGF-1)	
Liver	▪ Synthesis of collagen fibers ▪ Migration of keratinocyte

  - Transforming growth factor-α (TGF-α) is involved in epithelial cell proliferation in embryos and adults. TGF-α also participates in transformation of normal cells to cancer stem cells.
- **Fibroblastic growth factor-β:** Fibroblastic growth factor-β (FGF-β) is synthesized by macrophages, endothelial cells and many other cells.
  - FGF-β stimulates fibroblasts to synthesize extracellular matrix proteins including fibronectin, and epithelialization of skin.
  - FGF-β stimulates endothelial cells to synthesize new vessels (angiogenesis) and participates in development of skeletal and cardiac muscles, and lung maturation.
  - FGF-β differentiates specific lineages of hematopoietic stem cells (HSCs).
- **Transforming growth factor-β:** Transforming growth factor-β (TGF-β) is synthesized by macrophages, T cells, endothelial cells, platelets and many other cells.

- TGF-β is chemotactic for fibroblasts, macrophages and lymphocytes.
- TGF-β stimulates fibroblasts and enhances synthesis of collagen fibers, fibronectin and proteoglycans.
- TGF-β also modulates the repair tissue process by inhibiting collagen degradation by downregulating metalloproteinase enzymes and upregulating protease inhibitor activities. TGF-β causes fibrosis of the lung, kidney, and liver during chronic inflammation.

- **Interleukin-1 and tumor necrosis factor-α:** Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) are synthesized by macrophages.
  - IL-1 and TNF-α promote the proliferation of fibroblasts, smooth muscle cells and endothelial cells.
  - IL-1 and TNF-α are chemotactic for neutrophils at the site of injury.
  - IL-1 and TNF-α are involved in the initiation of liver regeneration.
  - IL-1 stimulates synthesis of metalloproteinases and acute phase reactants.
- **Fibronectin:** Fibronectin is a glycoprotein that links extracellular matrix components (e.g. collagen fibers and proteoglycans) and macromolecules (e.g. heparin and fibrin) to cell surface integrins.
  - Fibronectin participates in chemotaxis of fibroblasts and endothelial cells.
  - Fibronectin promotes angiogenesis (new vessel formation).
- **Vascular endothelial growth factor:** Vascular endothelial growth factor B (VEGF-B) is located in endothelial cells and many other cells.
  - VEGF family comprises VEGF-C and VEGF-D. VEGF-C and VEGF-D bind to VEGF-C and signal through three tyrosine receptors.
  - VEGF is potent inducer of new blood formation during intrauterine life.
  - VEGF plays central role in angiogenesis (capillaries and lymphatic channels) in adults.
  - VEGF promotes angiogenesis in chronic inflammation, healing of wounds, and malignant tumors.
- **Insulin-like growth factor 1:** Insulin-like growth factor 1 (IGF-1) is synthesized by liver. IGF-1 participates in proliferation of collagen fibers and migration of keratinocytes.
- **Hepatocyte growth factor:** Hepatocyte growth factor (HGF) is synthesized by fibroblasts, endothelial cells and mesenchymal cells of liver.
  - HGF participates in proliferation of hepatocytes, epithelia of biliary tract, lungs, kidney, mammary gland and skin.
  - HGF promotes cell scattering, migration, and enhances survival of hepatocytes.

Pathology Pearls: Properties of Vascular Endothelial Growth Factor (VEGF)

- Capillary and lymph duct formation
- Monocyte migration
- Hematopoiesis
- Recruitment of hematopoietic progenitor cells from bone marrow
- Regulation of the endothelial cell pool during development
- Capillary permeability

VEGF and its cognate receptors play multiple roles in the angiogenesis and hematopoietic process.

SIGNALING MECHANISMS OF CELL GROWTH

The process of receptor-mediated signal transduction is activated by the binding of ligands such as growth factors, and cytokines to specific receptors. It leads to expression of specific genes. Biochemical pathways and transcriptional regulation mediate growth factor activity.

Modes of Cell Signaling

According to the source of the ligand and the location of its receptors, there are four modes of cell signaling, i.e. autocrine (in the same cell), paracrine (adjacent cells), endocrine (distant cells) and juxtacrine (cell to cell or cell to extracellular matrix). General patterns of intercellular signaling demonstrating autocrine, paracrine, endocrine, contact-dependent and neurocrine signaling are shown in Fig. 2.55A to E. Signaling mechanisms involved in cell growth in the organs are shown in Table 2.61.

- **Autocrine signaling:** Cells respond to signaling molecules due to synthesis of growth factors, which act on the same cells, so establishing an autocrine loop. Autocrine signaling participates in liver regeneration and proliferation of antigen-stimulated lymphocytes. Cancer cells frequently overproduce growth factors and their receptors, thus stimulating proliferation of own cells through an autocrine loop.
- **Paracrine signaling:** One cell type synthesizes the ligand, which acts on adjacent target cells that express the appropriate receptor. The responding cells are in close proximity to the ligand-producing cells generally of a different type. Paracrine stimulation is common in connective tissue repair of healing wounds, in which a growth factor produced by one cell (e.g. macrophage) has growth effect on adjacent cell (e.g. a fibroblast). Paracrinal signaling is necessary for hepatocyte replication during liver regeneration, and NOTCH effects in embryonic development, wound healing, and renewing tissues.

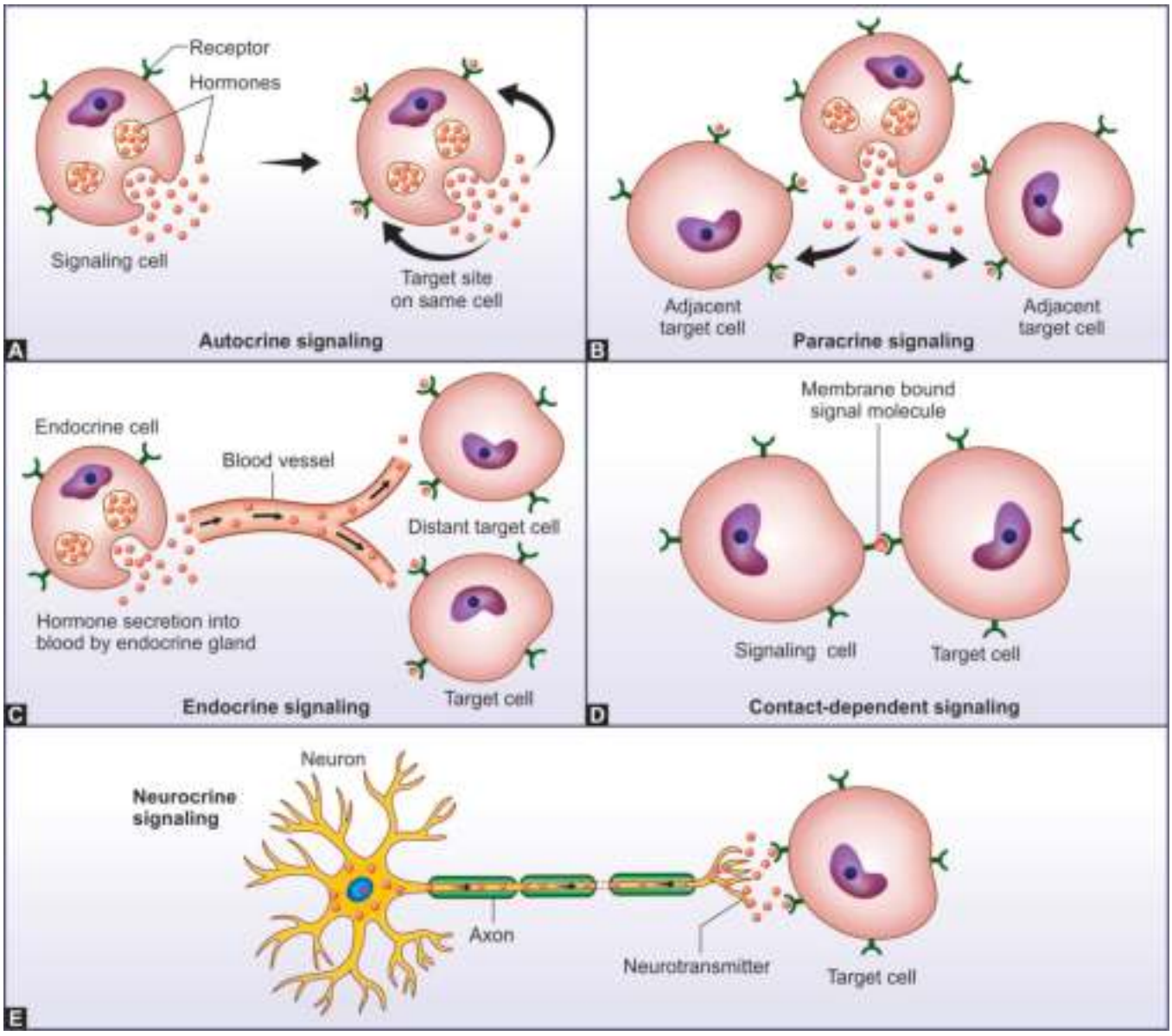


Fig. 2.55A to E: General patterns of intercellular signaling demonstrating autocrine, paracrine, endocrine, contact-dependent and neurocrine signaling.

- **Endocrine signaling:** Hormone synthesized by cells of endocrine organs act on target cells at distant sites from their site of synthesis, being usually carried by blood. Growth factors may also circulate and act at distant sites. Cytokines also act in similar fashion and produce systemic effects of inflammation.
- **Juxtacrine signaling:** Juxtacrine signaling requires close contact in multicellular organisms. There are three types of juxtacrine signaling described as under: (a) Two adjacent cells interact with the help of membrane ligand and membrane protein. (b) Intracellular compartment of two adjacent cells interacts via communicating junction permit relatively small molecules. (c) Two adjacent cells interact via extracellular matrix glycoprotein and membrane protein.

Receptors and Signaling Transduction Pathways

Binding of a ligand to its receptor on cell induces a series of events by which extracellular signals into the cell leading to expression of genes. Behavior of cells in tissue healing process are initiated by three receptor systems that share integrated intracellular signaling pathways: (a) protein tyrosine kinase receptors for peptide growth receptors, (b) seven transmembrane G protein-coupled receptors for chemokines and other factors, and (c) integrin receptors for extracellular matrix. Cell surface receptors and their principal signal transduction pathways are shown in Fig. 2.56.

- **Receptors with intrinsic tyrosine kinase activity:** Receptor tyrosine kinases (RTKs) are enzyme-linked receptors localized on the plasma membrane containing an extracellular ligand-binding domain, a

Table 2.61 Signaling mechanisms involved in cell growth in the organs

Cell Signaling	Mechanism	Regulation of Cell Growth
Autocrine signaling	Cells synthesize molecules, which act on same cells forming autocrine loop	▪ Liver regeneration ▪ Proliferation of antigen stimulated lymphocytes
Paracrine signaling	Cells synthesize molecules, which act on adjacent cells	▪ Connective tissue repair of wound healing ▪ Hepatocyte replication during regeneration ▪ NOTCH effects in embryonic development, wound healing and renewing tissues
Endocrine signaling	Cells synthesize hormones, which act on distant target cells from their site of synthesis	▪ Endocrine glands synthesize hormones, which act on distant target cells ▪ Hepatocyte growth factor acting at distant target cells ▪ Several cytokines produce systemic effects in inflammation
Juxtacrine signaling	Known as contact-dependent signaling (cell to cell or cell to extracellular matrix signaling)	▪ Two adjacent cells interacting with the help of membrane ligand membrane protein ▪ Intracellular compartment of two adjacent cells interacting via communicating junction permit relatively small molecules ▪ Two adjacent cells interacting via extracellular matrix glycoprotein and membrane protein

transmembrane domain, and an intracellular protein-tyrosine kinase domain. The tyrosine kinase receptors are proteins that span cell membrane surfaces, which have critical roles in transducing extracellular signals to the cytoplasm. The tyrosine kinase receptors bind to growth factors such as EGF, TGF-α, HGF, PDGF, VEGF, FGH, c-KIT and insulin and activate tyrosine kinase domain phosphorylates tyrosine amino acid residues. Following intracellular pathways initiate a cascade of events leading to several signaling pathways.

- **MAP kinase signaling pathway:** One of the most common intracellular signaling pathways triggered by RTKs is known as the mitogen-activated protein (MAP) kinase cascade. RTKs can activate RAS, a protein that is tethered to the plasma membrane, by causing it to bind guanosine triphosphate (GTP). Once activated, RAS activates an enzymatic cascade of MAP kinases. This results in potent changes in the cell, such as the alteration of key proteins and changes in gene transcription leading to synthesis of transcriptional factors such as Fos and Jun.
- **RAS phosphatidylinositol 3-kinase signaling pathway:** RAS phosphatidylinositol 3-kinase (PI3) signaling pathway increases concentration of calcium results in activation of serine threonine kinase protein C. Net result is activation of transcription factors.
- **Phosphatidylinsositol 3-kinase (PI3-kinase) signaling pathway:** Phosphatidylinositol 3-kinase (PI3) phosphorylates a membrane phospholipid results in generation of product that activates kinase Akt (also known as protein kinase B). Net result is cell proliferation and survival through inhibiting apoptosis.

- **Seven transmembrane G protein-coupled receptors:** Chemokines, vasopressin, serotonin, histamine, epinephrine, norepinephrine, calcitonin, glucagon, parathormone, corticotropin and rhodopsin bind to seven transmembrane G protein-coupled receptors and trigger cyclic AMP leading to multiple cellular effects.
- **Receptors without tyrosine kinase activity:** Erythropoietin, granulocyte stimulating factor and growth hormone bind to receptors without tyrosine kinase activity and transmit extracellular signals to the nucleus by activating JAK-STAT (Janus kinase) pathway. JAK-STAT pathway activates cytoplasmic transcription factors known as STAT (signal transducers and activation of transcription). Cytokines may also activate MAP kinase intracellular signaling pathway.
- **Nuclear steroid receptors:** Steroid receptors are most often located in the nucleus, which function as ligand-dependent transcription factors. These bind to steroid hormones, thyroid hormone, vitamin D and retinoids. Activated nuclear steroid receptors then bind to specific DNA sequences known as hormone response elements involved in inflammation, atherosclerosis and adipogenesis.
- **Integrin receptors:** Integrin receptors transmit chemical and physical information in the extracellular matrix, which create attachment sites for the actin filaments of cytoskeleton. These integrin receptors activate PI3 kinase and MAP kinase intracellular signaling pathways leading to proliferation and differentiation of cells.

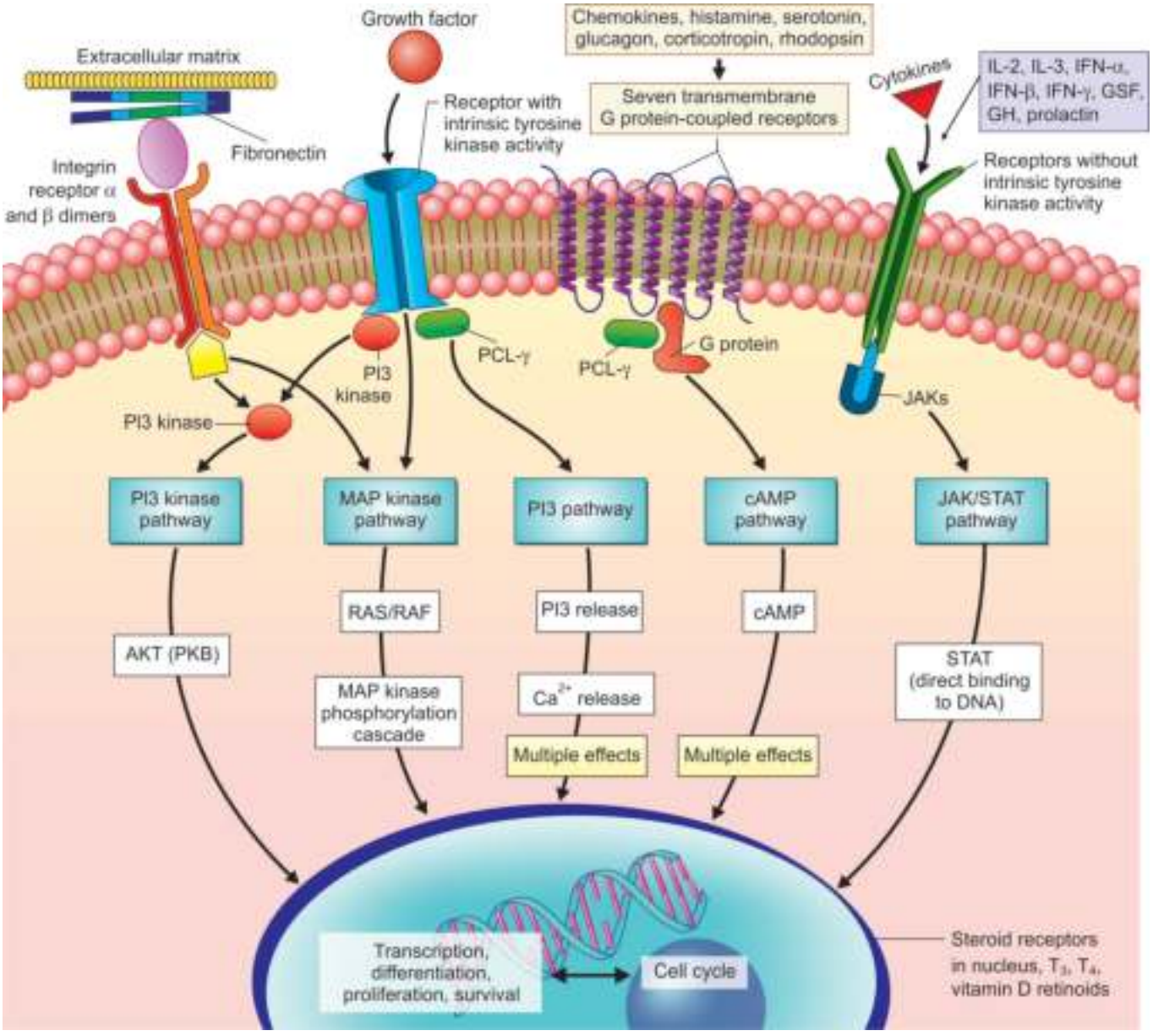


Fig. 2.56: Cell surface receptors and their principal signal transduction pathways. It shows receptors with intrinsic tyrosine kinase activity, seven transmembrane G proteins, and receptors without tyrosine kinase activity. Cyclic adenosine monophosphate (cAMP): Inositol triphosphate (IP3); Janus kinase (JAK); Mitogen-activated protein (MAP) kinase; Signal transducers and activators of transcription (STAT).

Transcriptional Pathway

Transducing signals transmit the information to the nucleus and modulate gene transcription through the activity of transcriptional factors. Transcriptional factors regulating cell proliferation include products of growth promoting genes such as c-Jun and c-Myc, and cell cycle-suppressing TP53.

EXTRACELLULAR MATRIX

Spaces between cells are filled with extracellular matrix (ECM). Epithelial cells rest on a dense sheet of ECM are called the basement membrane. Mesenchymal cells are surrounded by a diffuse ECM. Extracellular matrix plays important role in tissue repair. Extracellular matrix is essential for wound healing, which provides framework for cell migration, cell shape, gene expression and differentiation. ECM also participates in angiogenesis and maintenance of correct polarity for the re-assembly of multicellular structures.

- The ECM is composed of two main classes of interlocking macromolecules: (a) structural fibrous proteins including collagen, elastin, fibronectin and laminin, which have both structural and adhesive functions, and (b) amorphous ground substance is composed of polysaccharide chains of the class

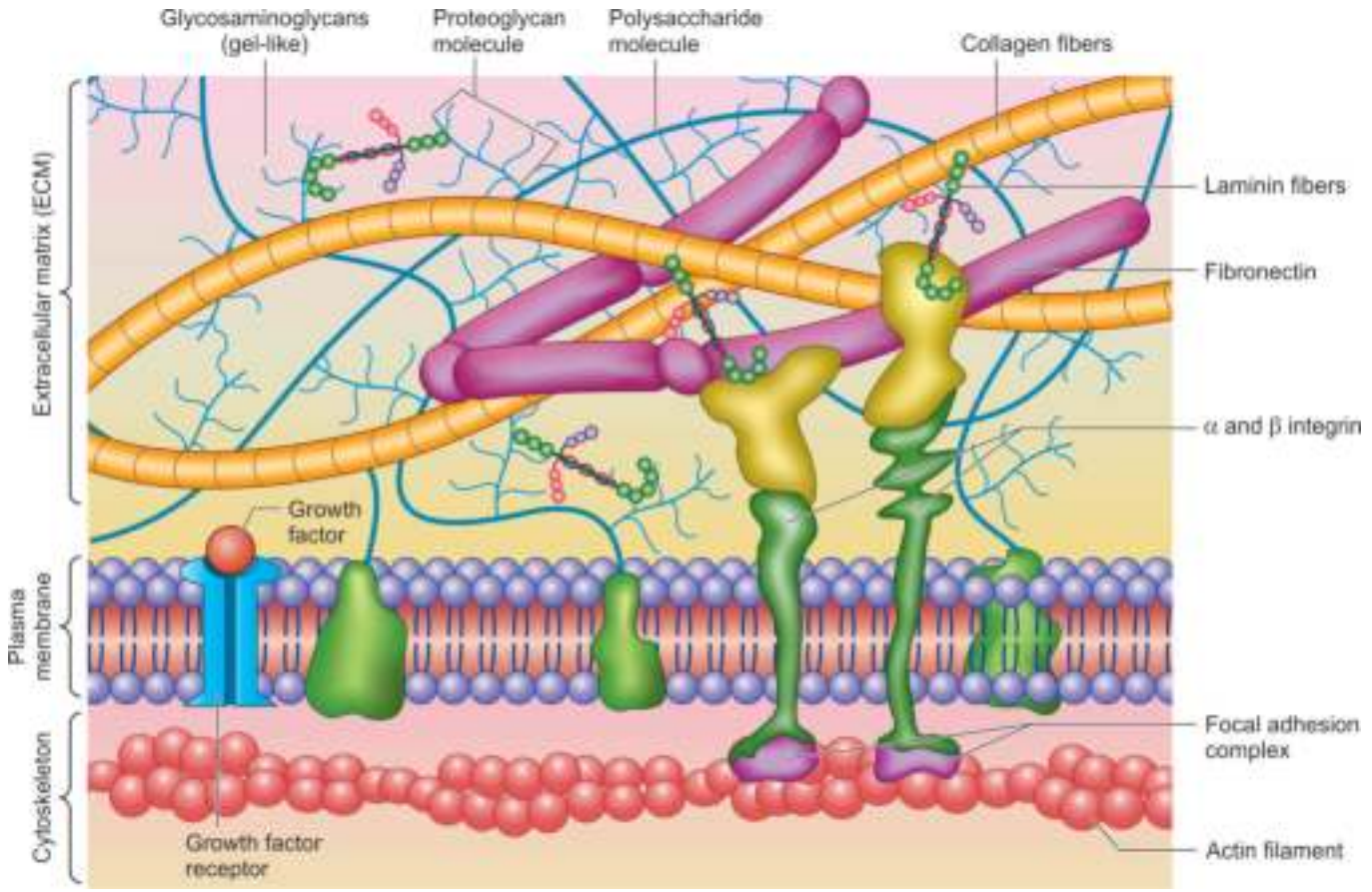


Fig. 2.57: Composition of extracellular matrix (ECM). ECM consists of structural fibrous glycoproteins and amorphous ground substance. Structural fibrous glycoproteins are composed of collagen fibers, fibronectin, laminin, and elastin. These reinforce the ground substance. Amorphous ground substance is a gel-like material composed of glycosaminoglycans (hyaluronic acid, chondroitin sulfate, heparin, and heparan sulfate) and proteoglycans (carbohydrate and proteins). Both epithelial and mesenchymal cells interact with extracellular matrix via integrins.

called glycosaminoglycans (GAGs), which are usually found covalently linked to protein in the form of proteoglycans. Specialized proteins include fibronectin and laminin, which form mesh of fibers embedded in proteoglycans. Hyaluronic acid, dermatan sulfate, keratan sulfate and heparan sulfate belong to the group of sulfated GAGs. Composition of extracellular matrix is shown in Fig. 2.57.

- Four groups of GAGs are distinguished according to their sugars, the type of linkage between sugars and the number and location of sulfate groups: (a) hyaluronan, (b) chondroid sulfate and dermatan sulfate, (c) heparan sulfate, and (d) keratan sulfate.
- ECM macromolecules are secreted locally by fibroblasts and assembled into an organized meshwork in close association with the surface of the cell that synthesizes them.
- Cell surface receptors transduce signals into cells from ECM, which regulate diverse cellular functions, such as cell growth, proliferation, migration, differentiation, survival and some vital role in maintaining cells homeostasis. Functions of extracellular matrix are given in Table 2.62.

Table 2.62 Extracellular matrix functions

Mechanical Support
▪ Cell anchorage ▪ Migration ▪ Maintenance of cell integrity
Control of Growth
Regulation of cell proliferation by signaling mechanism via cellular receptors of the integrin family
Maintenance of Differentiation
Regulation of differentiation of the cells in tissues via acting cell-surface integrin
Scaffoldings for Tissue Renewal
▪ Regeneration of labile and stable cells if intact ECM ▪ Scar formation due to disruption of ECM
Establishment of Tissue Microenvironment
Forming boundary between epithelial cells and underlying connective tissue
Storage of Regulatory Molecules
Storage of growth factors in ECM during regeneration of cells at injured site

STRUCTURAL FIBROUS PROTEINS

Collagen fibers are the most abundant glycoprotein protein in humans (>25% total protein). Variety of genes encode different collagen molecules. Mnemonic, collagen fibers go from hard to soft types in various organs.

- Collagen fibers provide strength and help in organization of extracellular matrix. Rubber-like elastic fibers provide stretchability and resilience to the ECM. Fibronectin has binding sites for cells and other ECM proteins, which links cells to the ECM.
- Sequence of fibronectin such as arginine, glycine, and aspartate binds to cells avidly. Laminin is abundant in basement membranes where it promotes adhesion of many types of cells. Laminin participates in attachment of endothelial and visceral cells to glomerular basement membrane.

Collagen Fibers

Collagen fibers may be fibrillar or nonfibrillar (amorphous) types. Fibrillar collagen types 1, 2, 3; while non-fibrillar collagen (amorphous) types 4–18. Collagen fibers are the most abundant protein in the body, which form the major structural component of many organs and provide tensile strength of healing wounds. Collagen synthesis is shown in Fig. 2.58. Collagen fibrils and their distribution are given in Table 2.63.

- **Biosynthesis:** Various growth factors such as PDGF, EGF, TGF-α, FGF-β, IGF-1, IL-1 and TGF-β participate in proliferation of fibroblasts resulting in synthesis of collagen fibers in early wound healing and tissue repair (3 to 5 days). Some of the fibroblasts also acquire features of smooth muscle cells, including the presence of actin filaments called myofibroblasts.
- **Synthesis of α-chains of collagen fibers:** DNA sends signal to messenger RNA, where splicing occurs, where α-chains of collagen fibrils are formed.

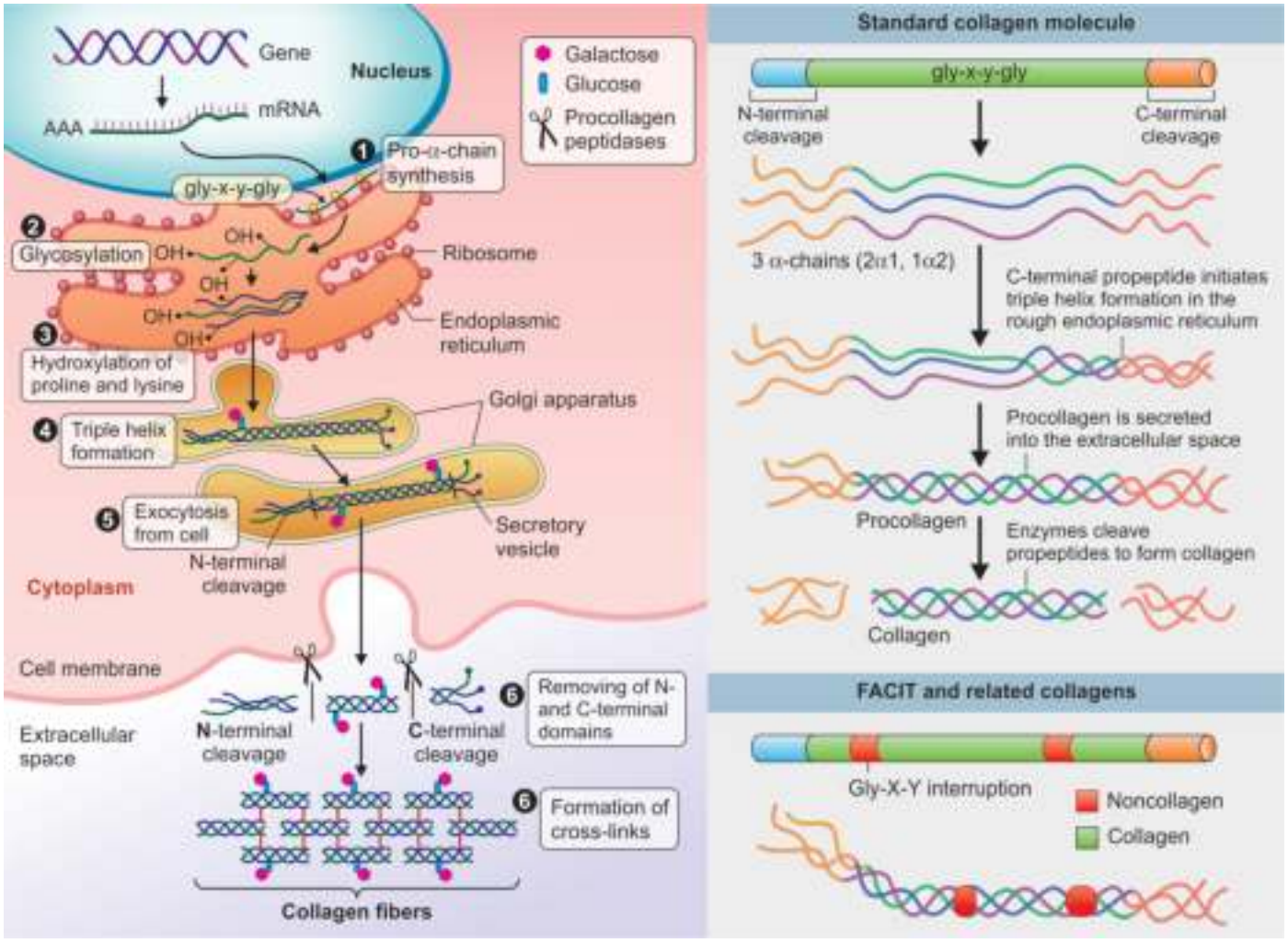


Fig. 2.58: Collagen synthesis. Principal steps in biosynthesis of interstitial collagens include synthesis of pro-α-chains in rough endoplasmic reticulum, aggregation of three pro-α-chains, hydroxylation of lysin and proline residues, secretion of procollagen molecule, cleavage of propeptides, alignment of collagen molecules to form fibrils, and aggregation of fibrils to form collagen fibers.

Table 2.63 Collagen fibrils and their distribution

Collagen Fibrils	Property	Distribution	Genetic Disorders
Fibrillar collagen			
Type 1	Greatest tensile strength	Bones, tendons and skin (90%)	▪ Osteogenesis imperfecta ▪ Ehlers-Danlos syndrome ▪ Arthrochalasia type 1
Type 2	Thin collagen fibrils	Cartilage (50%) and vitreous humor	Achondrogenesis type 2
Type 3	Thin collagen fibrils	Blood vessels, uterus, skin (10%) and granulation tissue	Vascular Ehlers-Danlos syndrome
Type 5	Amorphous fibrils	Cell surfaces, hair and placenta	Classical Ehlers-Danlos syndrome
Type 9	Amorphous fibrils	Soft tissue, blood vessels, cartilage, intervertebral discs	▪ Stickler syndrome ▪ Multiple epiphyseal dysplasia
Basement membrane			
Type 4	Amorphous fibrils	Basement membranes (glomerular basement membrane) and basal lamina	Alport's syndrome
Other molecules			
Type 6	Amorphous microfibrils	Various organs	Bethlem myopathy
Type 7	Anchoring fibrils at dermal-epidermal junctions	Skin	Dystrophic epidermolysis bullosa
Type 9	Amorphous microfibrils	Cartilage and intervertebral disc	Multiple epiphyseal dysplasia
Type 17	Transmembrane collagen	Skin	Benign atrophic generalized epidermolysis bullosa
Types 15, 18	Endostatin-forming collagen	Endothelial cells	Knobloch syndrome type 18 collagen

Ehlers-Danlos syndrome (EDS) is characterized by defects of type 1 and type 3 collagen synthesis and structure. Patients present with hypermobile joints, aortic dissection (most common cause of death), bleeding into the skin (ecchymoses), rupture of the bowel, and poor wound healing.

- **Assembly of collagen fibrils:** Structural unit of collagen is tropocollagen (collagen fibrils) comprising α-chains. Collagen molecules consist of three polypeptide chains arranged in a triple helix, known as α-chains, which combine with proline. Hydroxylation reactions in the rough endoplasmic reticulum convert proline to hydroxyproline; and lysine to hydroxylysine. Ascorbic acid is required in hydroxylation reactions, which leads to assembly of triple helix of α-chains.
- **Stabilization of triple helix of α-chains:** Hydroxyproline residues produce bonds that stabilize the triple helix in the tropocollagen molecule.
- **Cross linkage of collagen fibers:** Transportation of **triple helix α-chains** through Golgi apparatus results in formation of true fibrils in extracellular space.
  - **Oxidation of hydroxylysine** by lysyl oxidase (MMP enzyme) results in cross linkage of α-chains occurs. Lysyl oxidase is a metalloproteinase enzyme containing copper. Fibrils are arranged in 'quarter stagger' mode to form insoluble fibers. Inhibition of lysine oxidation results in malformation of skeleton, and blood vessels seen in Marfan's syndrome.
  - **Cross linkage of collagen fibrils** provide structural stability to tropocollagen, which are relatively resistant to general proteases; slow remodeling by specific collagenases. Decreased cross-linking (e.g. vitamin C deficiency) reduces the tensile strength of collagen. In vitamin C deficiency, the structurally weakened collagen fibers are responsible for a bleeding diathesis (e.g. bleeding into skin and joints) and wound healing and tissue repair. Decreased cross-linking with advancing age leads to decreased elasticity of skin, joints, and blood vessels.

Elastin Fibers

Blood vessels, lungs, skin and uterus require elasticity to perform functions. Collagen fibers provide tensile strength to the tissues. On the other hand, elastic fibers can stretch up to 1.5 times their length and snap back to their original length when relaxed.

- **Composition of elastic fibers:** Elastic fibers consist of a central core made of elastin, surrounded by network of fibrils. Elastic fibers are synthesized by fibroblasts and smooth muscle cells in arteries. Substantial

quantity of elastin is found in aorta, skin, ligaments and uterus.

- **Biosynthesis of elastic fibers:** Elastic fibers are derived from elastic microfibril, which consists of numerous proteins such as microfibrillar-associated glycoproteins, fibrillin and fibrullin. Elastic fibers are also derived from amorphous elastin. Microfibrils scaffold and organize the deposition of amorphous elastin, which also influence the availability of active TGF-β in the ECM. Genetic defect in synthesis of fibrillin causes Marfan's syndrome. Inhibition of lysine oxidation results in malformation of skeleton, and blood vessels seen in Marfan's syndrome.

Fibronectin

Fibronectin is a ligand member of integrin receptor family that links extracellular matrix with the intracellular cytoskeleton. Fibronectin has many functions, which allow it to interact with many extracellular matrix proteins such as collagen, fibrin and heparin, and with specific membrane receptors in respective cells.

- Fibronectin is adhesive glycoproteins that is widely distributed in stromal connective tissue and deposited at the site of tissue injury.
- During the initial phase of tissue healing, fibronectin in the extravasated plasma is cross-linked to fibrin, collagen, and other extracellular matrix components by the action of transglutaminases. This cross-linking of fibronectin to various components provides a provisional stabilization of the wound during the first several hours. Fibronectin, cell debris, and bacterial products are chemoattractants for a variety of cells that are recruited to the wound site over the next several days.

Laminin

Laminin is cell adhesion molecule that interacts with receptors anchored in the plasma membrane of cells adjacent to the basement membranes. Laminin regulates multiple cellular activities and signaling pathways.

AMORPHOUS GROUND SUBSTANCE

Amorphous ground substance is composed of polysaccharide chains of the class called glycosaminoglycans **(GAGs)**, which are usually found covalently linked to protein in the form of proteoglycans. Proteoglycans attract Na^+ and water and expand to form gels that occupy space between cells, which also bind and selectively release growth factors.

- **Glycosaminoglycans (GAGs):** Glycosaminoglycans are long unbranched polysaccharide chains composed of repeating units of disaccharides, e.g. an amino sugar (n-acetyl glucosamine) and a glucuronic acid. Four groups of GAGs are distinguished according to their sugars, the type of linkage between sugars and the number and location of sulfate groups: (a) hyaluronan, (b) chondroid sulfate and dermatan sulfate, (c) heparan sulfate, and (d) keratan sulfate.
- **Proteoglycans:** Proteoglycan molecules in connective tissue form a highly hydrated gel-like amorphous ground substance in which the structural fibrous proteins are embedded. Proteoglycans contain (>50% carbohydrate with some protein, which possess an extracellular and intracellular domain. Proteoglycans interact with collagen, fibronectin, and other ECM molecules. Hydrated gel-like amorphous ground substance absorbs water and also binds and selectively release growth factors. Polysaccharide gel resists compressive forces on the extracellular matrix while permitting the rapid diffusion of nutrients, metabolites, and hormones between the blood and tissue cells.

CELL ADHESION MOLECULES

Cell adhesion molecules enable cells to contact and specifically interact with each other in multicellular organisms. Specialized cell–cell and cell–matrix interactions permit communication between cells and their surrounding environment necessary for development and functional activity. Several different families of receptors mediate these interactions.

Pathology Pearls: Categories of Cell Adhesion Molecules

Cell Junctions

Cell junctions are formed slowly but generate very stable and durable connections such as tight junctions, desmosomes, and gap junctions.

Cell Surface Adhesion Molecules

- Cell surface adhesion molecules include selectins, immunoglobulin-like cell adhesive molecules, and integrins. Their function is adhesion of cells to extracellular matrix or to neighboring cells, rather than cell activation.
- Cell surface adhesion molecules play an important role in acute inflammation, which ensure arrival of cells to the required locations. These are selective and quickly formed. Cell surface adhesion molecules are relatively weak in comparison to cell junctions.

Substrate Adhesion Molecules

Substrate adhesion molecules consist of extracellular matrix molecules and matched receptors, which are expressed on the cell surface.

Family of Cell Surface Adhesion Molecules

Families of cell surface adhesion molecules include selectins, integrins, immunoglobulin (Ig) super family members and cadherins, which are critical in embryonic development, differentiation, migration, inflammation, and wound healing and cancer metastases.

- **Selectin family:** Selectins are cell adhesion molecules mediating initial adhesion of leukocytes (primarily neutrophils) to endothelial cells during acute inflammation.
  - **E-selectins (CD62E):** E-selectins are stored in Weibel-Palade bodies of resting endothelial cells. Upon activation, E-selectins are redistributed along the luminal surface of the endothelial cells, where they mediate the initial adhesion and rolling of leukocytes.
  - **P-selectins (CD62P):** P-selectins are stored in Weibel-Palade bodies of endothelium and platelet α-granules, relocate to the plasma membrane after stimulation by chemical mediators such as histamine and thrombin.
  - **L-selectins (CD62L):** L-selectins are expressed on neutrophils, which bind to endothelial mucin-like molecules such as GlyCam-1.
- **Lectins:** Lectins are expressed on cells and endothelial cells. Glycosyltransferases are lectin-related cell adhesive molecules (CAMs), which transfer monosaccharides to an oligosaccharide chain on an adjacent cell.
- **Immunoglobulin family of cell adhesion molecules:** Intercellular adhesion molecules (e.g. ICAM-1 and ICAM-2) are expressed on the endothelial cells, which bind to integrin expressed on leukocytes.
- **Integrins:** Integrins contain 2α and 2β chains, which belong to immunoglobulin supergene family. The α-chain has ligand binding sites for Ca^{++} and Mg^{++} which are needed for integrin to adhere. During inflammation, integrin participates in adhesion of leukocytes to vascular endothelium leading to chemotaxis of leukocytes to the injury site. Integrin family of proteins is given in Table 2.64.

Table 2.64 Integrin family of proteins

Integrins	Mechanism of Actions
Integrin β_1	▪ Binding of cells by integrin β_1 to extracellular matrix ▪ β-Chain using CD29
Integrin β_2 (Leu CAMs)	▪ Leukocyte adhesion to endothelium by integrin β_2 during inflammation ▪ β-Chain using CD18
Integrin β_3 (cytoadhesion)	Interactions of platelets and neutrophils at inflammatory sites or sites of vascular damage

- **Cadherins:** Cadherins participate in forming stable adhesion, which are expressed on all cells forming solid organs. Cadherin family includes E-cadherin, P-cadherin, N-cadherin and L-cadherin members. E-cadherin is present in desmosomes of epithelial cells. P-cadherin is expressed in placenta. N-cadherin is present in neural tissue. L-cadherin is demonstrated in liver. In adults, cadherins are responsible for tight cell-to-cell associations within tissues. Cadherins are intimately associated with the cytoskeleton, interacting via other proteins with both microfilaments and intermediate filaments, which mediate homotype interactions in zonula adherens, tight junctions, gap junctions, and desmosomes. Cadherins are rapidly degraded by proteases in the absence of Ca^{++}.
- **Cadherin-catenin linkage:** Catenin family includes α-catenin, β-catenin and γ-catenin. The β-catenin is a membrane protein associated with cell adhesion molecules, which link cadherin to the cytoskeleton. If cadherin-catenin linkage is disturbed, cadherin does not work, which leads to disruption of embryonic development especially of brain neural tissue.

WOUND HEALING AND TISSUE REPAIR

Steps of wound healing and tissue repair are acute inflammation, granulation tissue formation, re-epithelialization, collagen accumulation, regression of vascular channels, replacement of granulation tissue with scar formation and wound contraction. Inflammatory infiltrate is removed within 36 hours to 3–4 weeks.

- Granulation tissue becomes a scar as a result of proliferating fibroblasts by laying down collagen which accumulates in the location of the eventual scar. Therefore, granulation tissue is a combination of capillary loops and myofibroblasts involved in wound-healing and tissue repair phenomenon.
- Wound healing and tissue repair occurs in four phases under normal conditions in adults: (a) hemostasis (scab formation), (b) inflammatory phase (inflammation and edema formation), (c) proliferative phase (granulation tissue formation), and (d) remodeling phase and wound contraction with scar formation.
- Granulation tissue is an important component of wound healing and tissue repair process. Wounds can heal by primary intention and secondary intention. Wound edges approximate easily in tissue healing by primary intention. Wound edges do not approximate in tissue healing by secondary intention.
- Tissue repair by connective tissue involves the influx of debris-removing inflammatory cells, formation of

granulation tissue, maturation of granulation tissue, migration and proliferation of fibroblasts forming, fibrosis tissue that is remodeled over time to form a fibrous scar.

- Tissue repair depends on growth factor activity and interaction between cells and extracellular matrix (**ECM**). Extracellular matrix serves many functions: providing support, anchorage for cells, segregating tissues from one other and regulating intercellular communication.
- Tissue repair by connective tissue involves five mechanisms: removal of injured tissue by inflammation, angiogenesis, migration and proliferation of fibroblasts, scar formation and remodeling of connective tissue. Organization refers to replacement of dead tissue or hematoma by granulation tissue, which is seen in hematoma (wound and bone fracture), thrombi, infarcts and fibrinous exudates. Phases of wound healing tissue repair process are given in Table 2.65 and Fig. 2.59. Growth factors, enzymes and other factors regulating wound healing and tissue repair are given in Table 2.66.

HEMOSTASIS PHASE

Hemostasis starts when blood leaks out of the body. The initial phase of tissue repair process, which typically begins with hemorrhage, involves the coagulation of blood resulting from the aggregation and degranulation of platelets and formation of fibrin network within seconds and minutes that fills the gap created by the wound.

- A thrombus, referred to as a scab after drying out, forms on the wounded skin as a barrier to invading microorganisms, which also prevents the loss of plasma and tissue fluid. Thrombus contains fibronectin and growth factors.
- Fibrin network is the basis for a blood clot and serves as a temporary matrix for migratory cells. Platelets release cytokines and growth factors that serve as proinflammatory signals to attract the immune cells to the site of the wound.

INFLAMMATORY PHASE

Inflammation is the second phase of wound healing and tissue repair, that begins immediately after the tissue injury, when injured blood vessels leak transudate causing swelling. Immune cells such as neutrophils and monocytes/macrophages play important role in inflammatory phase of wound healing and repair. Chemical mediators released by injured cells, plasma proteins, tissue macrophages; mast cells, platelets and endothelial cells increase vascular permeability resulting in formation of inflammatory exudates. Polymorphonuclear cells liquefy the injured tissue and remove dead cellular debris. Inflammatory phase of wound healing and tissue repair takes about 72 hours to complete.

- **Neutrophilic infiltration:** Neutrophils are produced from hematopoietic stem cells in the bone marrow and undergo maturation in 1–2 weeks. Majority of neutrophils are released into peripheral blood circulation in the segmented forms. About 50% of

Table 2.65 Phases of wound healing and tissue repair process

Hemostatic Phase	
▪ Platelet aggregation	▪ Coagulation system cascade
Inflammatory Phase	
▪ Polymorphonuclear cell migration	▪ Removal of necrotic debris
Proliferative Phase	
▪ Endothelial proliferation results in granulation tissue formation and angiogenesis ▪ Keratinocyte proliferation results in re-epithelialization of the wound	▪ Fibroblast proliferation results in synthesis of type 3 collagen and extracellular matrix
Remodeling Phase and Wound Contraction (3 Weeks–36 Months)	
▪ Resolution of acute inflammation and dissolution of fibrin clot ▪ Maturation of granulation tissue produced by fibroblasts and endothelial cells that completely fill wound space ▪ Maturation of extracellular matrix (ECM) ▪ Fibroblasts lay down vertically oriented collagen fibers at the wound margin ▪ Metalloproteinases (MMPs) activity can be minimized by binding to specific proteinase inhibitors such as α_1-antitrypsin and α_2-macroglobulin	▪ Progressive re-epithelialization, devascularization, type 1 collagen fibers deposition over next two to three weeks resulting in a dense white scar ▪ Remodeling increases the tensile strength of scar tissue that involves two processes: (a) collagen fibers remodeling by growing in both size and strength to replace fibronectin and hyaluronic acid, (b) vascular maturation and repression ▪ Myofibroblasts are responsible for the contractile process in wound closure

Cytokines and growth factors (e.g. PDGF, VEGF, FGF-2, TGF-α, TGF-β) play key role in wound healing. Phases of wound healing: hemostatic and inflammatory phase (0–3 days), proliferative phase (3–14 days) and remodeling phase and wound contraction (3 weeks–36 months).

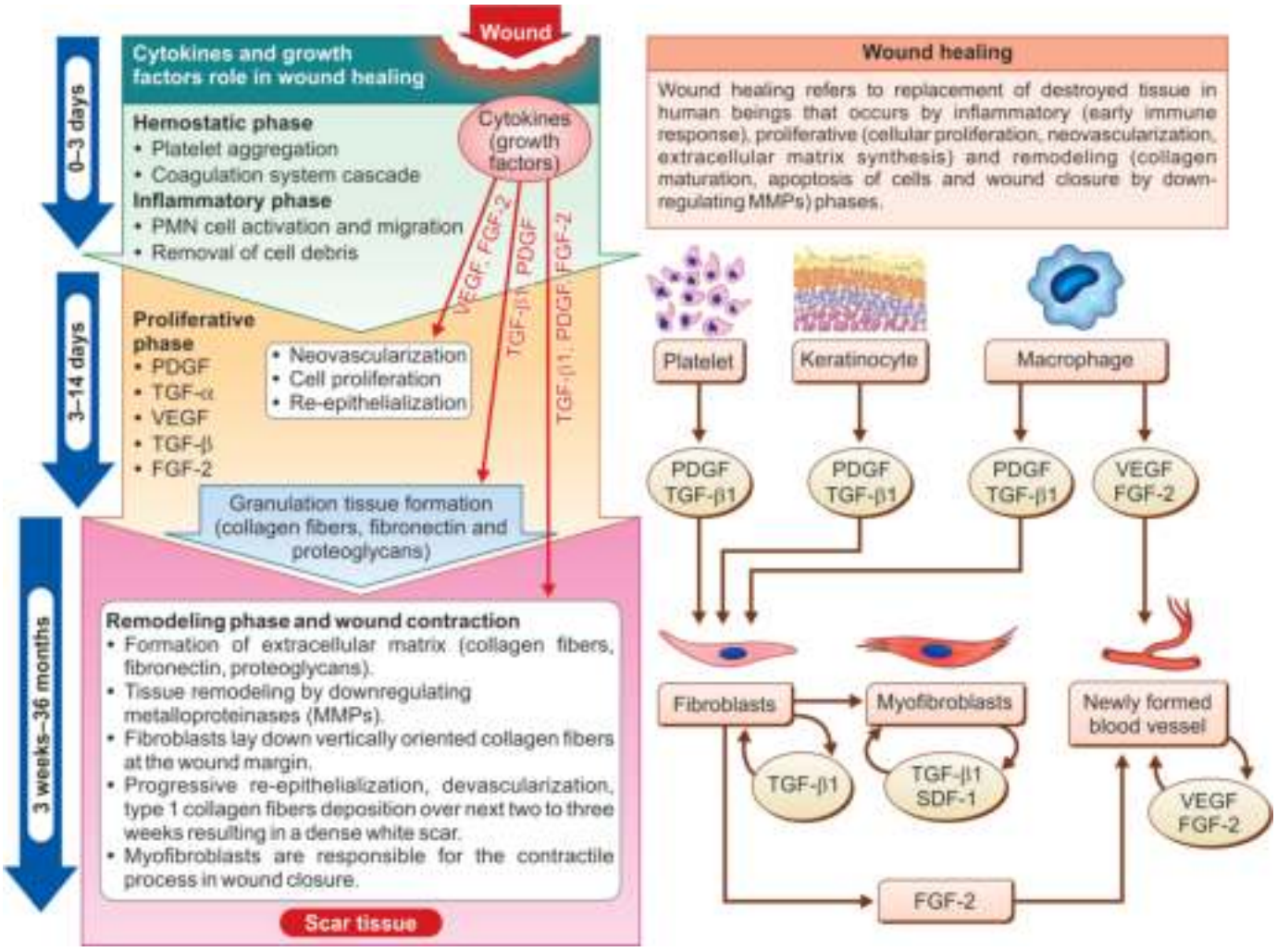


Fig. 2.59: Phases of wound healing and tissue repair process. Wound healing involves influx of necrotic debris removing inflammatory cells, formation of granulation consisting of fibroblasts and delicate capillaries in a loose extracellular matrix, and conversion of said granulation tissue into fibrous tissue that undergoes remodeling over time to form a scar. Numerous cytokines and growth factors are involved in every stage of wound healing however, it appears that transforming growth factor-β is a major factor in matrix protein synthesis and granulation tissue formation.

Table 2.66 Growth factors, enzymes and other factors regulating tissue wound healing and tissue repair

Functions	Growth Factors and Enzymes
Chemotaxis of monocytes/macrophages	PDGF, FGF-β, TGF-β, MCP-1 (CCL2)
Migration of fibroblasts	PDGF, FGF-β, TGF-β, EGFs, CTGF, SDF-1
Proliferation of fibroblasts	PDGF, FGF-β, EGFs, IGF, CTGF, TGF-β
Maturation of new blood vessels during angiogenesis	VEGF-A, VEGF-B, VEGF-C, VEGF-D, angiopoietin 1 and angiopoietin 2, FGFs, HGF
Endothelial cell migration and survival during angiogenesis and lymphangiogenesis	Integrin-α and integrin-β
Inhibiting proliferation of endothelial cells and angiogenesis	ECM protein such as endostatin
Collagen synthesis	TGF-β, PDGFs, IGH, CTGF
Collagen secretion	PDGFs, FGFs, CTGF
Collagen cross-linking and maturation	Lysyl oxidase
Epithelial cell migration and proliferation	KGF, TGF-α, HGH, IGF
Tissue remodeling by movement of surface and stromal cells	Metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-13)
Resolution of tissue repair	IP-8 (CXCL11), IP-10 (CXCL10)

the neutrophils in the peripheral blood circulation are in the marginating pool; and rest 50% circulate in the central column. Neutrophils spend <10 hours in the peripheral blood before marginating and reaching tissues, where they spend 1–5 days. Neutrophils arrive at the wound site within few minutes and continue to accumulate for several days. Neutrophils entrap the microorganisms at the wound site from the beginning of the tissue injury. Neutrophils also amplify the proinflammatory response by releasing their own chemical mediators.

- **Monocytes/macrophages infiltration:** Monocytes arrive at the site of tissue injury within two days, where these cells differentiate into macrophages. Macrophages play key role in phagocytosis of necrotic debris, pathogens and apoptotic neutrophils. Macrophages also produce chemical mediators, e.g. transforming growth factor-β and vascular endothelial growth factor (VEGF) that severe to recruit fibroblasts and endothelial cells that mark the next phase of wound healing and tissue repair.

PROLIFERATIVE PHASE

In proliferative phase of wound healing and tissue repair, wound is rebuilt with new tissue made up of collagen and extracellular matrix.

- Proliferative phase of wound healing and tissue repair includes three components: (a) keratinocytes proliferation and re-epithelialization, (b) endothelial cell proliferation involved in granulation tissue formation and angiogenesis, (c) fibroblasts proliferation involved in collagen synthesis and extracellular matrix formation.
- During proliferative phase of wound healing and tissue repair, re-epithelialization, and the replacement of the blood clot by granulation tissue formation occurs spontaneously. In proliferative stage, specific cells migrate, proliferate and fill the gap created by tissue destruction. It occurs by formation of granulation tissue and angiogenesis. The early extracellular matrix of granulation tissue contains proteoglycans, glycoproteins, and type 3 collagen.
- Granulation tissue is a reddish connective tissue that forms on the surface of a wound, when wound is healing, in which fibroblasts and vascular endothelial cells proliferate and form granulation tissue. Granulation tissue is characterized histologically by the presence and proliferation of fibroblasts, keratinocytes, endothelial cells, formation of new thin-walled capillaries, and inflammatory cell infiltration of the extracellular matrix.
- Angiogenesis is the formation of new blood vessels from the existing vasculature, that is required to support a healing wound environment.

Keratinocytes and Re-epithelialization

Keratinocytes play multiple roles essential for skin repair. Keratinocytes proliferate in the basal layer of the epidermis and start differentiating on their way to the surface, undergoing gradual differentiation, execution of re-epithelialization process by various cytokines and growth factors (epidermal growth factor, transforming growth factor-α and keratinocyte growth factor produced by keratinocytes and fibroblasts) and restoration of epidermal barrier.

- During this process, keratinocytes profoundly change their morphology and start to produce keratin, cytokines, growth factors, interleukins and complement proteins.
- In an autocrine and paracrine fashion, keratinocytes produce signals that regulate more keratinocytes activation that alters gene expression, migration of keratocytes and stimulate nearby fibroblasts.

Endothelial Cells Involved in Granulation Tissue Formation and Angiogenesis

During proliferative phase, the wound defect is filled with highly vascular connective tissue, commonly referred as 'granulation tissue'.

- Granulation tissue typically grows from the base of a wound and is able to fill wounds almost any size.
- The endothelial cells are responsible for revascularization/angiogenesis at the site of wound.
  - Initially, quiescent resident endothelial cells become activated by many angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), angiopoietin and transforming growth factors α and β.
  - Once activated, the endothelial cells undergo four events to occur in the formation of new blood vessels (angiogenesis): (a) protease production that breakdown extracellular matrix, (b) chemotaxis, (c) proliferation and (d) remodeling and differentiation.
- Angiogenesis is important for the restoration of the flow of nutrients and oxygen to the wound site, removing waste products, and transportation of leukocytes to the wound site.
- Any errors in the formation of granulation tissue can result in persistence of chronic wound. Foreign bodies at wound site can also result in persistent granulation tissue formation and poor wound healing with excess of macrophages, fibroblasts and capillaries around the foreign material.

Granulation Tissue Formation

Granulation tissue is soft, pink and highly vascular young immature tissue in 3–7 days seen in chronic

inflammation, which fills defects created by liquefaction of cellular debris. Granulation tissue serves as the foundation for scar tissue development. Due to presence of numerous, newly formed capillaries in granulation tissue are fragile. These capillaries bleed easily and permit leakage of plasma proteins and white blood cells into the tissues.

- Granulation tissue is formed by proliferation of fibroblasts, myofibroblasts delicate collagen fibrils, macrophages and vascular endothelial cells. Capillaries arise from adjacent blood vessels by division of endothelial cells in a process termed angiogenesis. Newly formed blood vessels supply blood to the granulation tissue. Granulation tissue supplies antibacterial antibodies and growth factors.
- Macrophages synthesize growth factors and remove cellular debris. If the tissue injury affects deeper layers of tissue, fibroblasts proliferate resulting in synthesis of collagen fibers. Granulation tissue can mature into fibrous tissue (scar formation).
- Healthy granulation tissue appears pink to red due to new blood vessels formation, which is soft to touch, moist in appearance and painless. On the other hand, unhealthy granulation appears dark red in color, painful, easy to bleed with minimum contact, which may be covered by avascular shiny white or yellow fibrous tissue, that impedes wound healing.

Angiogenesis

Angiogenesis is a process of new blood vessels development from existing blood vessels. It is essential in tissue development, and wound healing. Vasculogenesis is the process of blood vessel formation in the embryo occurring by a *de novo* production of endothelial cells by angioblasts (endothelial cell precursors). Mechanism of angiogenesis is shown in Fig. 2.60. The main angiogenic proteins involved in angiogenesis are given in Table 2.67.

- Angiogenesis is regulated by various angiogenic cytokines (e.g. VEGF, FGF).
- Angiogenesis occurs as a result of mobilization of endothelial precursor cells (EPCs) from bone marrow and preexisting vessels at the site of tissue injury.
- Bone marrow EPCs migrate to a site of tissue injury and proliferate to form a mature network by linking with preexisting vessels. Endothelial cells from preexisting blood vessels become motile and proliferate to form capillary sprouts.
- Regardless of the mechanism of angiogenesis, blood vessel maturation requires the recruitment of pericytes and smooth muscle cells to form the periendothelial layer. Mechanism of angiogenesis by following steps is described below:
  - **Sprouting of vessel:** It occurs by separation of pericytes. NOTCH signaling pathway regulates sprouting and branching of new vessels. Growth factors synthesized by extracellular proteins participate in sprouting of vessel process.
  - **Chemotaxis of endothelial cells:** Fibronectin participates in recruitment of endothelial cells toward the site of tissue injury.
  - **Proliferation of endothelial cells:** Endothelial cells proliferate just behind the leading front (tip) of the migrating cells. This function is mediated by growth factors such as VEGF, PDGF, EGF, TGF-α FGF-β, fibronectin, IL-1 and TNF-α. Vascular endothelial growth factor acts on VEGF-1, VEFG-2 and VEGF-3 receptors. Targeted mutations in these receptors result in lack of angiogenesis.
  - **Remodeling and extension of the vascular tube:** Matrix metalloproteinases synthesized by extracellular matrix degrade ECM to permit remodeling and extension of the vascular tube.
  - **Formation of mature blood vessels:** Recruitment of periendothelial cells form the mature blood vessels. This function is mediated by proteins such as fibronectin, VEGF-A, VEGF-B, VEGF-C, VEGF-D, angiopoietin 1 and angiopoietin 2. VEGF-C especially induces hyperplasia of lymphatic vasculature. Targeted mutations in these VEGFs result in lack of angiogenesis.
  - **Modulation of tissue repair:** TGF-β modulation of tissue repair the repair process by inhibiting proliferation and chemotaxis of endothelial cells. TGF-β also suppresses deposition of basement membrane.

Regulation of Angiogenesis by ECM Proteins

Angiogenesis is required by extracellular matrix (ECM) proteins.

- Integrin-α and integrin-β play key role in angiogenesis and its maintenance.
- Extracellular matrix proteins such as thrombospindin 1, SPARC and tenascin destabilize cell–ECM interactions, thus leading to angiogenesis.
- Matrix metalloproteinases and plasminogen activators are essential in tissue remodeling.
- Matrix metalloproteinases and plasminogen activators cleave ECM proteins and release VEGF and FGF-2 leading to induction of angiogenesis.
- On the other hand, ECM protein such as endostatin inhibits proliferation of endothelial cells and angiogenesis.

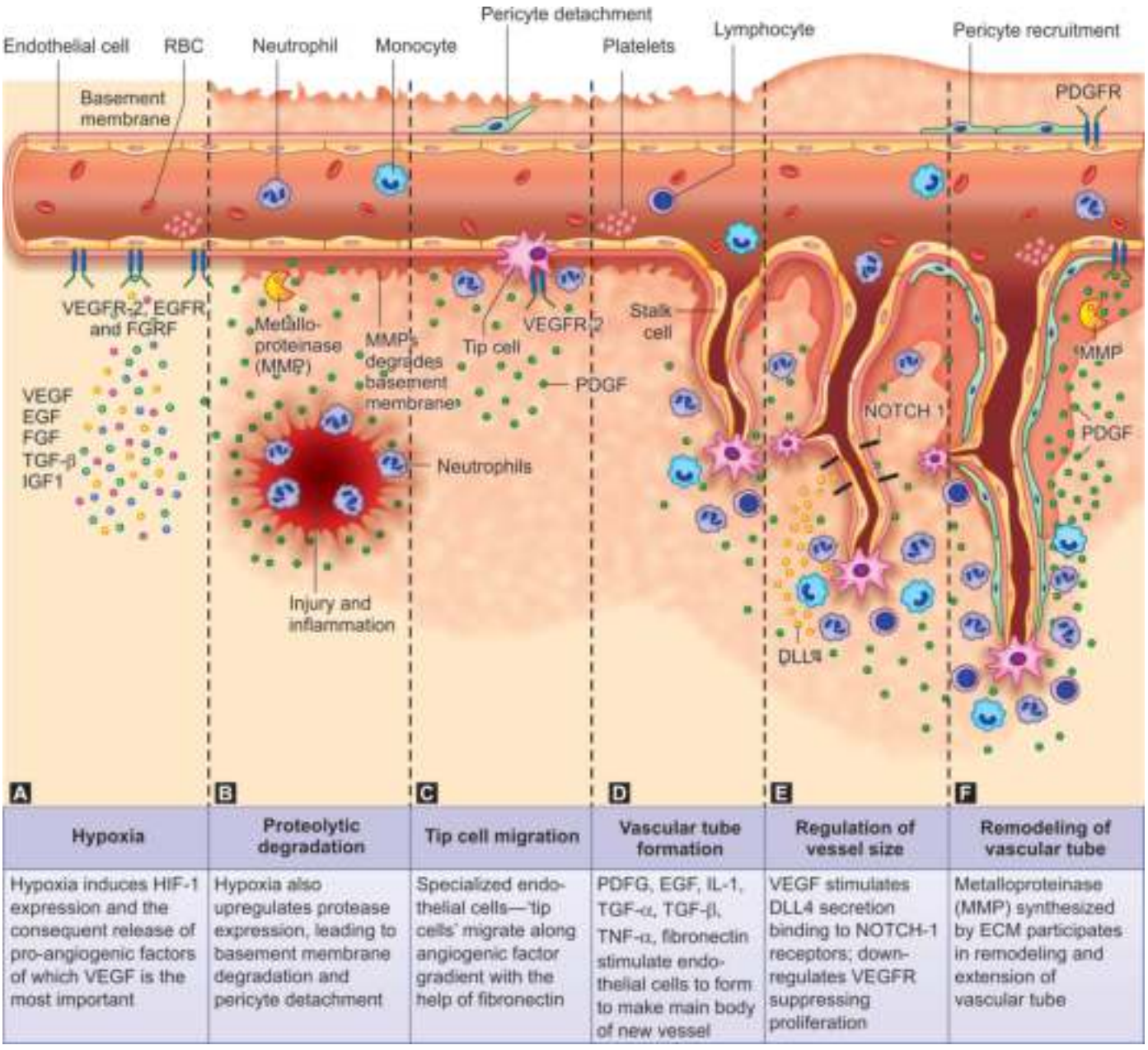


Fig. 2.60: Mechanism of angiogenesis. Endothelial precursor cells are mobilized from bone marrow and pre-existing capillaries. Endothelial cells become motile, proliferate to form capillary sprouts. It is followed by maturation (stabilization) by recruitment of pericytes and smooth muscle to form periendothelial layer. Blockage of DLL4 leads to sprouting, proliferation of endothelial cells, decrease in vascular lumen size and organization. Blockage of VEGF leads to decrease in sprouting, proliferation of endothelial cells, decrease in vascular lumen size and organization.

Interactions between DLL4 and Vascular Endothelial Growth Factor

Vascular endothelial growth factor induces delta 4 (DLL4) that inhibits VEGF signaling. Blockage of DLL4 leads to sprouting, proliferation of endothelial cells, decrease in vascular lumen size and organization. Blockage of VEGF leads to decrease in sprouting, proliferation of endothelial cells, decrease in vascular lumen size and organization.

Fibroblasts Involved in Synthesis of Collagen Fibers and Extracellular Matrix

The primary function of fibroblasts is the maintenance of structural integrity within the connective tissue, granulation tissue and extracellular matrix (ECM) formation. Fibroblasts synthesize transforming growth factor-β that maintains structural integrity within the connective tissue, granulation tissue and ECM synthesis.

Table 2.67 The main angiogenic proteins involved in angiogenesis

Angiogenic Protein Family	Angiogenic Proteins	Protein Receptors
Vascular endothelial growth factor (VEGF) protein family	VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PIGF	VEGFR1 (FLT1), VEGF (KDR, FLK1), VEGFR3 (FLT4)
Fibroblastic growth factor (FGF) protein family	FGF-1 (acidic FGF), FGF-2 (basic FGF)	FGF-1, FGF-2, FGF-3, integrins, heparan sulfate proteoglycans
Platelet-derived growth factor (PDGF) protein family	PDGF-A, PDGF-B, PDGF-C, PDGF-D	PDGF-A, PDGF-B
Angiopoietin (Ang) protein family	Ang-1, Ang-2, Ang-3, Ang-4	Tie-2, Tie-1
Hepatocyte growth factor (HGF) protein family	HGF	c-Met
Hypoxia-inducible factor (HIF) protein family	HIF-α, HIF-β	Aryl hydrocarbon receptor nuclear translocator
Insulin growth factor (IGF) protein family	IGF	IGF-1R, IGF-2
Transforming growth factor-β (TGF-β) protein family	TGF-β1, TGF-β2, TGF-β3	TGFBR1, TGFBR2
Metalloproteinases (MMPs) protein family	MMP	MMP1-MMP23
Tumor necrosis factor (TNF) protein family	TNF	TNF1-TNF18

Angiogenesis inhibitors include thrombospondin 1, thrombospondin 2, interferon-α, interferon-β, angiostatin, endostatin and collage IV fragments.

- Fibroblasts produce and secrete all components of ECM, including structural proteins (collagen fibers, elastic fibers, fibronectin, laminin), adhesive proteins and a space-filling ground substance composed of glycosaminoglycans (GAGs) and proteoglycans.
- Activated fibroblasts, myofibroblasts, and capillary sprouts are abundant in wound healing and tissue repair 3–5 days following injury. These fibroblasts change shape from oval to bipolar as they begin to form collagen and synthesize a variety of extracellular matrix proteins. Neutrophils accumulate in the wound 12–24 hours after injury.
- The extracellular matrix initially consists of weaker form of type 3 collagen during wound healing and tissue repair within 5–7 days that is ultimately replaced by stronger type 1 collagen at the end of wound healing and scar formation. As the amount of collagen in granulation tissue progressively increases, the tissue becomes gradually less vascular and less cellular. Cellularity and edema are diminished with formation of an avascular, hypocellular scar. Mature scar tissue would be visible 2 weeks following injury.

REMODELING PHASE

Remodeling phase, also known as maturation phase of wound healing and tissue repair, is the final phase of the tissue healing process in which granulation tissue and extracellular matrix mature into scar formation leading to increase in tissue tensile strength.

- Sequence of events in scar formation in remodeling phase of wound healing and tissue repair include: (a) resolution of acute inflammation and dissolution of fibrin clot, (b) maturation of granulation tissue produced by fibroblasts and endothelial cells filling wound space, (c) maturation of ECM, (d) fibroblasts lay down vertically oriented collagen fibers at the wound margin, and (e) progressive re-epithelialization, devascularization, collagen deposition over next two to three weeks resulting in a dense white scar.
- Collagen fibers become denser and align according to force as seen in muscle, tendon or ligament. It is known as remodeling. Remodeling increases the tensile strength of scar tissue. Remodeling phase involves two processes: (a) collagen fibers remodeling by growing in size and strength replacing fibronectin and hyaluronic acid, (b) vascular maturation and repression.
- In remodeling phase in wound healing and tissue reapir, fibroblasts regulate the process of wound matrix breakdown by matrix metalloproteinases (MMPs) and synthesis of new ECM. Immature type 3 collagen fibers are replaced by mature type 1 collagen fibers, which are critical to scar formation, integrity and strength. Remodeling phase can continue from 21 days to two years.
- Myofibroblasts are responsible for the contractile process in wound closure.
  - During granulation tissue formation, fibroblasts slowly modulate into myofibroblasts, characterized by bundles of actin microfilaments along their cell's plasma membrane.
  - Prostaglandins, bradykinin, epinephrine and norepinephrine play important role in modulation of contraction by myofibroblasts.

- Eventually, actin binds to the extracellular matrix component fibronectin, attaches to the collagen fibers, retracts and draws collagen fibers toward it. The net result is wound contraction and closure.

- Errors during remodeling phase can cause excessive wound healing leading to formation of hypertrophic scar or keloid or a persistent granulation tissue in chronic wound.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are crucial components in wound healing and tissue repair because enable cells to migrate by degrading extracellular matrix (ECM) proteins. MMPs are synthesized by polymorphonuclear cells, macrophages, fibroblasts, synovial cells and some epithelial cells.

- In addition to cell migration, MMPs can also disrupt cell–cell adhesions and release bioactive molecules stored in the ECM. MMPs degrade collagen fibers type 3 and get replaced by type 1 collagen fibers, and increasing tensile strength to approximately 80% of the original. MMPs cut triple helix collagen into two unequal fragments; which are susceptible to digestion by other macrophages.
- MMPs activity can be minimized by binding to specific proteinase inhibitors such as **α_1-antitrypsin** and **α_2-macroglobulin**. Metalloproteinases synthesized by various cells are given in Table 2.68.

Wound Contraction

Proliferation of myofibroblasts causes progressive wound contraction and deformity. Myofibroblasts are bound together by tight junctions forming syncytia, which express smooth muscle actin, desmin, and vimentin. On the other hand, fibroblasts tend to be spindle cells, surrounded by collagen fibers.

- A mature scar is composed of type 1 collagen fibers. Further changes in scars occur such as cicatrization—a late diminution in size resulting in deformity, calcification and ossification. Fibronectin attaches fibroblasts to collagen fibers.
- Chondronectin binds chondrocytes to type 2 collagen, the matrix of cartilage.
- Laminin binds epithelial cells to the type 4 collagen of basement membranes.
- Osteonectin binds hydroxyapatite and calcium ions to type 1 collagen (bone matrix) and initiates mineralization.

CUTANEOUS WOUND HEALING AND TISSUE REPAIR

Type of wound healing process in the skin depends on the extent of tissue damage. Minimal cutaneous tissue loss involves wound healing by primary intention. Extensive cutaneous tissue loss involves wound healing by secondary intention. Differences between primary and secondary intention of cutaneous wound healing and tissue repair are given in Table 2.69.

- Cutaneous wound healing by primary intention indicates that wound edges have been mechanically brought together. Fibrin occupies the small residual space and initiates steps of wound healing and tissue repair. Surgical clean incised wound with closely apposed margins heals by primary intention leading to minimal scar formation. Collagen deposition is the ultimate source of strength for the healed wound.
- Cutaneous wound healing by secondary intention indicates in which edges are not approximated, requires more extensive granulation tissue growth that fills the defect; myofibroblasts then contract wound, forming a scar and subsequently, more wound contracture is likely to occur.
- There are no fundamental differences between healing by primary and secondary intention, they merely differ in the degree to which the various stages apply. Escape of blood and exudate results

Table 2.68 Metalloproteinases synthesized by various cells

Cell Types	MMPs Synthesis
Proliferating keratinocytes	MMP3, MMP19, MMP28
Migrating keratinocytes	MMP1, MMP10, MMP9, MMP26
Fibroblasts	MMP1, MMP2, MMP3, MMP19, MT1-MMP (membrane-type 1 matrix metalloproteinase)
Endothelial cells	MMP9, MMP2, MMP19, MT1-MMP
Neutrophils	MMP8, MMP9
Macrophages	MMP12, MMP19

MMP1 strongly influences keratinocyte migration at the wound edges. MMP7 regulates neutrophil recruitment. The wound induced by angiogenesis, result of migration and proliferation of endothelial cells, is driven by vascular endothelial growth factor (VEGF) and tumor necrosis factor-α (TNF-α) release through the gelatinases MMP2 and MMP9. MMP-1 is overexpressed in different malignant tumors and is associated with invasion of epithelial ovarian cancer cells, lymph node involvement and metastases.

Table 2.69 Differences between primary and secondary intention of cutaneous wound healing and tissue repair

Parameters	Primary Intention	Secondary Intention
Site	Clean, uninfected surgical incision approximated by surgical sutures	In all natural, open wounds like, abscess, surface wounds in road accidents. Infection may be present
Tissue injury	Limited to superficial layer of epithelium and connective tissues	Extensive, causing cells death and necrosis in larger area; larger defect
Inflammatory reaction	Less intense	More intense for clearing large amount of fibrin, necrotic debris and exudate
Granulomatous tissue	Small amount of granulation tissue is formed	Larger amount of granulation tissue is formed to fill large defect
Wound contraction	Absent	Present due to presence of fibroblasts
Scarring	Less scarring	More scarring
Keloid	Absent	▪ May be formed later transforming into squamous cell carcinoma ▪ Skin appendages destroyed do not regenerate in either type of wound healing

Skin appendages destroyed do not regenerate in either type of wound healing.

in acute inflammatory response at the margins, scab formation and removal of necrotic debris by macrophages, which is followed by epidermal proliferation, contraction of the wound, progressive increase in collagen fibers and loss of vascularity and shrinkage of the scar.

- Cutaneous wound healing and tissue repair is inhibited by superadded infection, foreign body acting as a nidus for inflammation, protein depletion, vitamins depletion, vitamin C deficiency, ischemia and hydrocortisone.

CUTANEOUS WOUND HEALING AND TISSUE REPAIR BY PRIMARY INTENTION

Cutaneous wound healing and tissue repair by primary intention occurs in clean surgical superficial skin wounds with closely apposed edges and minimal tissue loss. Normally, maturation of the epidermis requires an intact layer of basal cells that are in direct contact with one another. Disruption of this contact activates basal cells resulting in migration, proliferation and epithelialization of skin wound. Cutaneous wound healing and tissue repair by primary intention is shown in Fig. 2.61A to D.

- **Contact inhibition of growth and motility:** When epithelial continuity is re-established, migration and cell division cease, and the epidermis resumes its normal cell cycle of maturation and shedding. This process of epithelial growth regulation is referred to as 'contact inhibition of growth and motility'. Such a wound requires only minimal cell proliferation and neovascularization to heal, and formation of a small scar.
- **Wound healing in surgical wound:** Suturing of apposed edges by sutures used for clean surgical wounds. Surgical clean wound with opposed margins heals by primary intention. Epithelial regeneration predominates over fibrosis. Wound healing in surgical wound is fast with minimal scarring/infection. There is regeneration of epidermis. Dermis undergoes fibrous repair. Sutures are taken out on 5–10 days after surgery. Surgical scar provides 80% normal strength. Minimal surgical scarring gives good strength. Maturation of scar continues up to 2 years. Risk of trapping infection under skin produces abscess.

Pathology Pearls: Wound Healing and Tissue Repair by Primary Intention

- **Within 24 hours:** Wound is filled with blood clot. Neutrophils appear at the margin of wound.
- **By third day:** Neutrophils are replaced by macrophages with progressive granulation tissue and collagen deposition at the margins of the incision and epidermal layer thickening due to epithelial cell proliferation and migration along incised wound margin.
- **By fifth day:** Wound incision space is filled with granulation tissue. There is maximum neovascularization. Collagen fibers become abundant and bridge the wound incisional gap. Differentiation of surface cells yields mature epidermal architecture.
- **By first week:** Wound shows continued accumulation of collagen fibers and fibroblast proliferation and regression of vascularity, edema and inflammation.
- **End of first month:** Connective tissue is cellular with intact epidermis. Wound is devoid of inflammatory infiltrate. There is gradual gain in tensile strength overtime. Epidermal appendages do not regenerate.

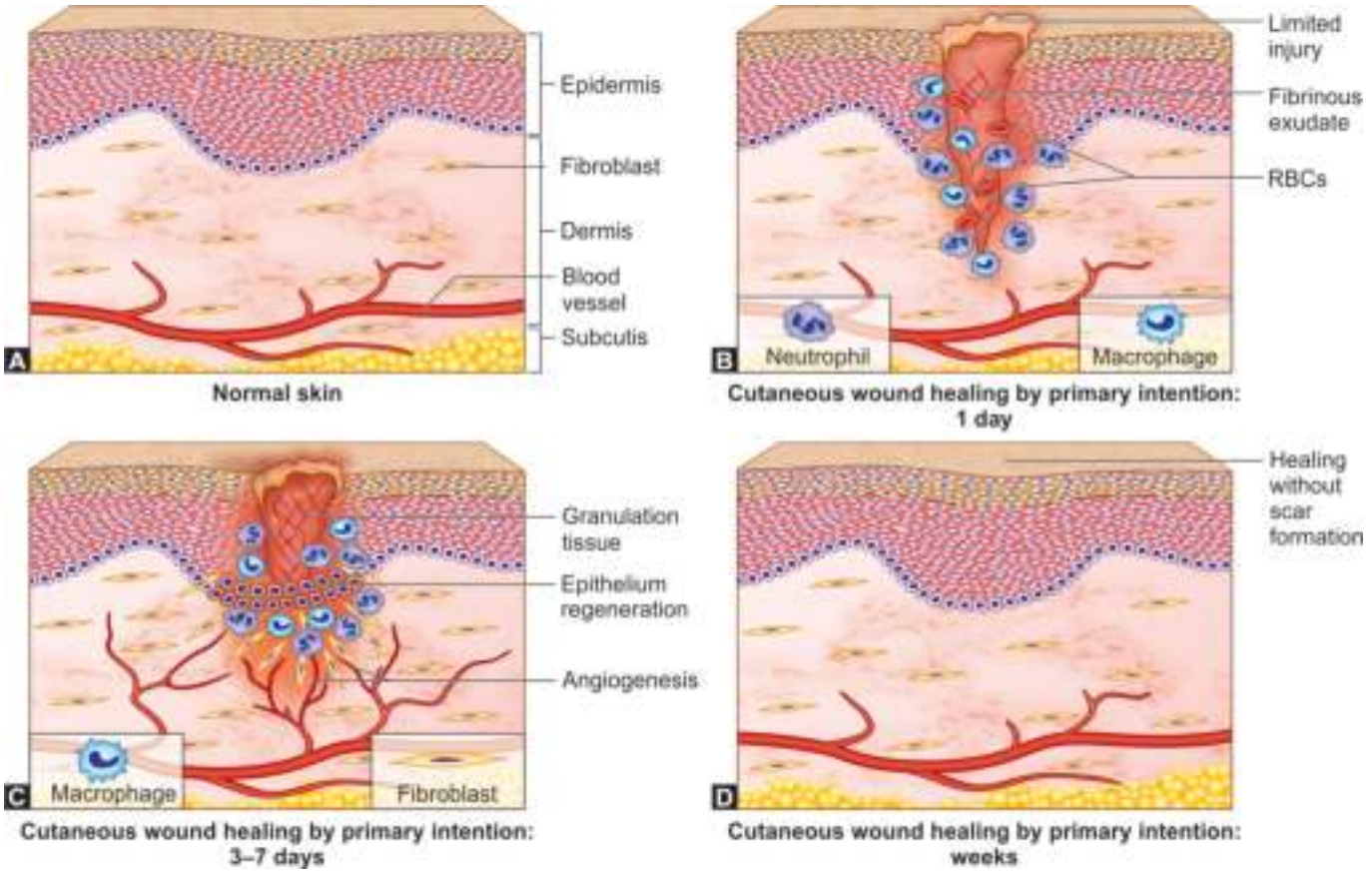


Fig. 2.61A to D: Cutaneous wound healing and tissue repair of skin by primary intention. It occurs in surgical wound with closely opposed edges with minimum tissue loss. Healing requires only minimal proliferation of cells and neovascularization with formation of small scar.

CUTANEOUS WOUND HEALING AND TISSUE REPAIR BY SECONDARY INTENTION

Cutaneous wound healing and tissue repair by secondary intention occurs in open gaping or infected wounds, in which the edges are far apart and in which there is substantial tissue loss. Cutaneous wound healing and tissue repair by secondary intention is shown in Fig. 2.62A to D.

- Healing of cutaneous wound requires extensive cell proliferation and granulation tissue formation. Resorption of granulation tissue is replaced by deposition of collagen-rich scar tissue in settings of open gaping or infected wounds, large third-degree burns, ulcers, tooth extraction, and external bevel gingivectomies. Tissues in the third stage burn do not restore to normal owing to loss of skin, basement membrane and connective tissue infrastructure.
- Extensive proliferation of epithelial cells and formation of granulation tissue are essential for healing of such wounds by secondary intention.
- Cutaneous wound healing and tissue repair is slower in open gaping or infected wounds. Fibrosis predominates over epithelial regeneration. Resorption of granulation tissue is replaced by deposition of collagen-rich scar tissue. There is more inflammatory exudate to remove necrotic tissue. Wound contraction is necessary for tissue repair. Initially, wound contraction occurs, then clot dries to form a 'scab'. Epidermis regenerates in cutaneous wound.
- There is increased susceptibility to infection in the presence of granulation tissue. Cutaneous wound healing and tissue repair by secondary intention takes longer time, which produces a larger scar; not necessarily weaker resulting in fibrosis.

FACTORS AFFECTING WOUND HEALING AND TISSUE REPAIR

Factors accelerating wound healing and tissue repair include ultraviolet light, administration of anabolic steroids, deoxycorticosterone acetate, and growth hormone, rise in temperature and hyperbaric oxygen. Wound healing and tissue repair can be delayed by factors local to the wound itself, including infection, desiccation or abnormal bacterial presence, maceration, trauma, necrosis, pressure and edema. Systemic factors adversely affecting wound healing and tissue repair include age, body type, chronic disease,

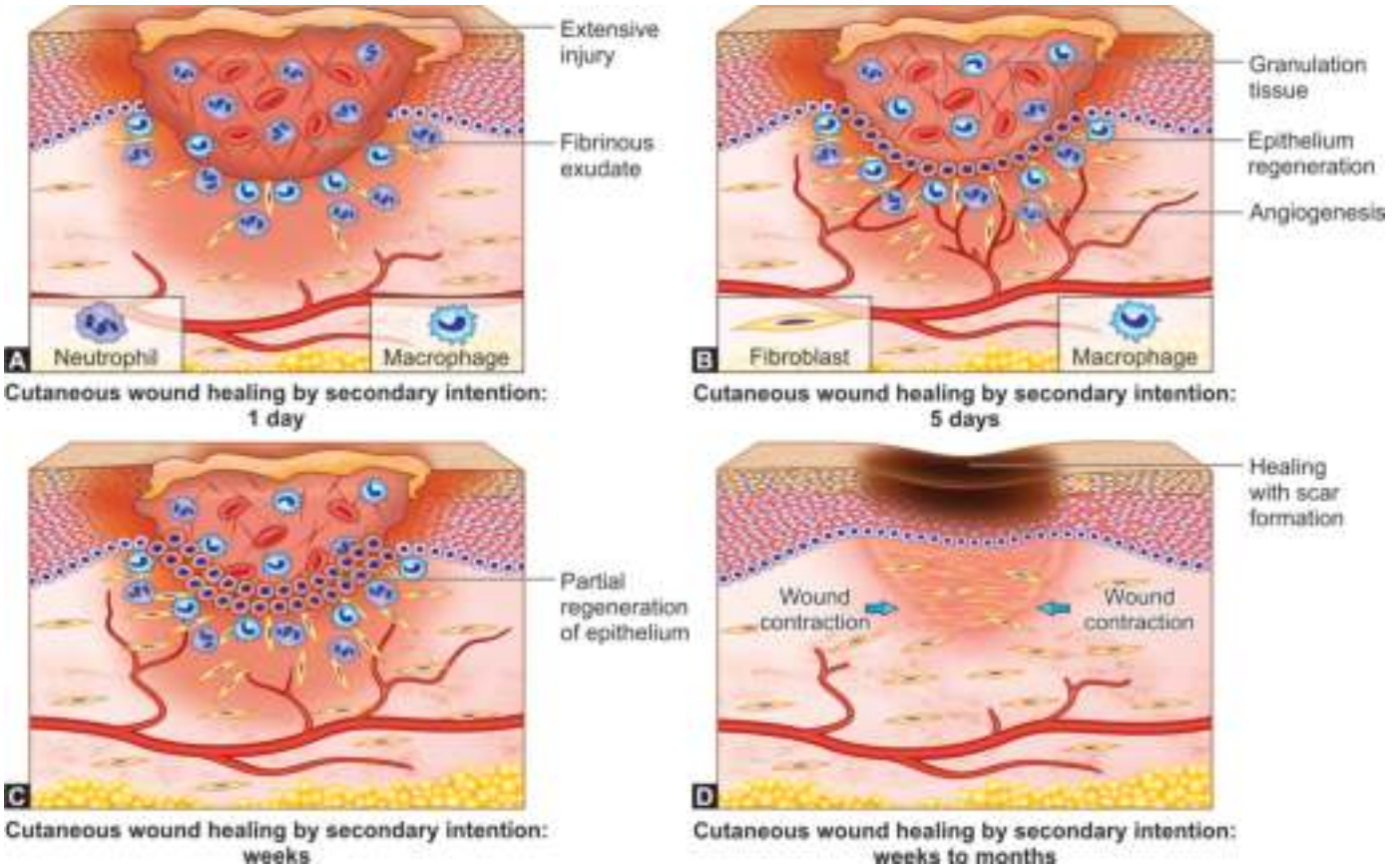


Fig. 2.62A to D: Cutaneous wound healing and tissue repair by secondary intention. It occurs in wound with far apart edges with substantial tissue loss. The wound requires contraction, extensive proliferation of cells and neovascularization. Re-epithelialization of wound starts from the margins with deposition of collagen fibers and granulation tissue leading to resorption of granulation tissue and formation of large scar.

immunosuppression, nutritional status, radiation therapy and vascular insufficiencies.

LOCAL FACTORS AFFECTING WOUND HEALING

Local factors can affect wound healing and tissue repair.

- **Decreased blood supply:** Molecular oxygen is required for collagen synthesis. It has been shown that even a temporary lack of oxygen due to cardiovascular pathology can result in the formation of less stable collagen fibers. Failure of proper collagen synthesis leads to delayed wound healing and weak scars.
- **Type of wound:** Wound healing and tissue repair can be delayed by factors local to the wound itself, including infection, foreign bodies, desiccation, maceration, necrosis, trauma, edema and type of open wound. Large wounds take longer time to heal and linear wound heals faster than circular or rectangular. In addition, wound healing is slower when wounds have infection, necrotic tissue, desiccation (dry), and foreign bodies.
- **Type of injurious agent:** Blunt, crushing or tearing of tissues adversely affect wound healing.
- **Poor apposition of wound margins:** Due to poor opposition of wound margins leads to large hematoma formation, which retards wound healing and tissue repair.
- **Persistent infection in wound:** Wounds in ischemic tissue become infected more frequently. Polymorphonuclear cells and macrophages require oxygen for destruction of microorganisms. *Staphylococcus aureus* is the most common cause of impaired wound healing. Gangrene, suppuration and secondary hemorrhage delayed wound healing and tissue repair.
- **Retention of debris:** Retention of foreign bodies, dirt, sutures, hematoma, and necrotic tissue delayed wound healing and tissue repair.
- **Denervation:** The skin is densely innervated with an intricate network of cutaneous nerves, neurotransmitters and specific receptors which influence a variety if physiological and disease processes. Denervated skin could result in impaired wound healing and tissue repair, such as decubitus ulcers and diabetic foot ulcers.

SYSTEMIC FACTORS AFFECTING WOUND HEALING

Wound healing and tissue repair can be delayed due to systemic factors such as age, body type, nutritional

status, chronic disease, immunosuppression, radiation therapy and vascular insufficiencies.

- **Age of patient:** There are many changes in wound healing capacity that are related to age. Wound healing and tissue repair is delayed in elderly people over the age of 60 years. Due to advancing age, there is decrease in the body's inflammatory response, a delay in angiogenesis and slow the process of epithelialization. Some visible changes to the skin are related to the alteration in melanocytes and decreased function of sebaceous glands. Decreased collagen synthesis is attributed to slower scar formation in the wound healing and tissue repair process.
- **Nutritional status:** Proteins rich in sulfur amino acids are essential for wound healing. Protein deficiency leads to delayed wound healing. In geriatric patient or chronic ailment, poor nutrition can cause delayed wound healing and tissue repair. Inadequate nutrition can occur due to infections. Large pressure injuries induced ulcers require large quantity of protein and calorie. Insufficient protein and calorie intake delayed wound healing and tissue repair.
  - **Vitamins and trace elements deficiency:** Vitamin and trace elements deficiency delayed wound healing.
  - **Vitamin C (ascorbic acid):** It is a powerful, biologic reducing agent. It is necessary for the hydroxylation of proline residues in collagen. Deficiency of vitamin forms an abnormal collagen that lacks tensile strength. Patients with vitamin C deficiency exhibit poor wound healing and tissue repair.
  - **Vitamin A:** Vitamin A stimulates epithelialization, capillary formation, and collagen synthesis. Deficiency of vitamin A retards wound healing.
  - **Vitamin K:** Vitamin K plays an indirect role in wound healing and tissue repair by preventing bleeding disorders. Deficiency of vitamin K retards wound healing and tissue repair.
  - **Vitamin B complex:** Vitamin B complex components are important cofactors in enzymatic reactions that contribute to the wound-healing process. Deficiency of vitamin B complex retards wound healing and tissue repair.
  - **Zinc deficiency:** Zinc leads to defects in removal of type 3 collagen in wound remodeling and causes bleeding in wound resulting to delayed wound healing and tissue injury.
- **Immunosuppression:** Glucocorticoids interfere with collagen formation and thus decrease tensile strength. It is worth mentioning that glucocorticoids along with antibiotic are occasionally used to prevent scar formation (e.g. bacterial meningitis).
- **Vascular insufficiencies:** Blood delivers the necessary components to tissue for wound healing process. Blocked or narrowed blood vessels or diseases of the heart, kidneys and lungs can also cause issues in the body delivering vital wound healing components, including adequate oxygen and white blood cells to wounded tissues.
- **Chronic disease:** Diabetes mellitus is associated with susceptibility to infection, impaired circulation and increasing tissue levels of glucose. Diabetic patients affect circulatory system that may delay wound healing. In renal failure, uremia impairs platelet function leading to diminished hemostasis, the first stage in wound healing.
- **Genetic disorders:** Marfan syndrome and Ehlers-Danlos disease adversely affect wound healing process. Marfan syndrome is a genetic disorder of the connective tissue affecting most notably the skeletal system, cardiovascular system and skin. Ehlers-Danlos disease is characterized by wound dehiscence, increased bleeding tendency due to the blood vessel fragility and delayed wound healing and tissue repair process.

COMPLICATIONS OF WOUND HEALING AND TISSUE REPAIR

The complications of wound healing and tissue repair can be grouped into four categories: (a) inadequate granulation tissue, (b) excessive fibrosis, (c) exuberant granulation tissue, and (d) other complications.

INADEQUATE GRANULATION TISSUE

Granulation tissue is an important component in the wound-healing process. Proliferation of fibroblasts and endothelial cells generates granulation tissue near the wound. The new capillaries make the tissue pink and granular. Cutaneous wounds can heal by primary intention and secondary intention. Granulation tissue matrix fills cutaneous wound that heals by secondary intention. Inadequate granulation tissue can occur due to multiple factors, that cause delayed wound healing and tissue repair.

Wound Dehiscence

Wound dehiscence occurs when a surgical incision following abdominal or cardiothoracic procedures associated with superadded infection reopens either internally or externally within 3–10 days of surgery.

- Several risk factors for wound dehiscence include obesity, malnutrition, tobacco smoking, peripheral vascular disease, cardiopulmonary diseases, anemia, diabetes mellitus, hypertension, cancer, infection, hematoma formation and elderly persons.

- A clean wound will have minimal space between the edges of the wound and commonly form a straight line. One can see holes in the wound dehiscence.
- Wound dehiscence can become life-threatening. Patient has a feeling that wound is ripping apart or giving away with leaking pink or yellow fluid.
- Signs of infection at wound site shows yellow or green pus, swelling, redness or warmth and fever.

Ulceration in Wound

Common causes of ulceration in wound are caused by venous hypertension, dependent edema, peripheral vascular disease, uncontrolled diabetes mellitus, cutaneous necrosis, vasculitis, pyogenic gangrenosum, skin disorders and connective tissue disorders such as rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, and systemic sclerosis.

- Ulceration in rheumatoid arthritis is usually of rapid onset, associated with pain, tender margin, fever, arthralgia and myalgia.
- Pyoderma gangrenosum is characterized by the appearance of lesions at the site of trauma. Surgical debridement of pyoderma gangrenosum often results in worsening of the ulceration.
- Abnormities of coagulation factors associated with skin necrosis occur due to deficiencies of protein C, protein S, antithrombin III, heparin cofactor 2 including homocysteinemia, increased prothrombin concentration and factor V Leiden mutation.
- *Staphylococcus aureus*, β-hemolytic streptococci, *Streptococcus pyogenes* and opportunistic infections in AIDS can complicate existing ulceration.

Incisional Hernia

Incisional hernia is caused by an incompletely-healed surgical wound. All abdominal surgeries carry higher risk of incisional hernia leading to protrusion of intestine, organ and other tissues. Since, median incisions in the abdomen are frequent in patients undergoing abdominal exploratory surgery, patients develop ventral incisional hernias within three to six months post-surgery due to many reasons such as excessive physical activity, gain considerable weight, pregnancy and increase in abdominal pressure.

- Patient presents with constipation, protrusion in the abdomen at or near the site of a previous incision, nausea, vomiting, fever, pain and abdomen around the protrusion.
- If the protrusion portion of intestine has become trapped within the abdominal wall, the blood supply to the intestine can get cut off leading to necrosis. Imaging techniques are helpful in detection of blockage or actual location of the intestinal protrusion.

EXCESSIVE FIBROSIS

Excessive fibrosis leads to cosmetic scarring, hypertrophic scar and keloid formation. In hypertrophic scar, excessive connective tissue is formed within the original wound and stays within that area. With keloid scars, excessive connective tissue that forms within original wound extends beyond the original wound area. Fibrosis induced by transforming growth factor-β in various organs is given in Table 2.70.

Hypertrophic Scar

A hypertrophic scar is thick, wide and raised scar formed within the original wound and stays within that area due to an abnormal response to wound healing. Hypertrophic scar commonly occurs due to lot of tension around wound in taut skin areas following skin trauma, burns or surgical incisions. Hypertrophic scar can be treated by medication, freezing, injections, lasers and surgery.

Keloid

Keloid is exuberant scar that tends to progress beyond the site of initial injury and recurs after excision, which is thick raised, smooth and hard area much larger than the original wound. Keloid is characterized by changes

Table 2.70 Fibrosis induced by transforming growth factor-β in various organs

Fibrosis in Various Organs	Disorder
Eyes	▪ Graves' disease ▪ Conjunctival cicatrization
Lung	▪ Pulmonary fibrosis ▪ Pulmonary sarcoidosis
Liver	▪ Cirrhosis ▪ Primary biliary cirrhosis
Kidney	▪ Glomerulosclerosis ▪ Interstitial fibrosis
Pancreas	▪ Chronic of fibrosing pancreatitis
Skin	▪ Keloid ▪ Hypertrophic scar ▪ Scleroderma
Endometrium	▪ Endometriosis
Peritoneum	▪ Post-surgical adhesions ▪ Sclerosing peritonitis
Retroperitoneum	▪ Retroperitoneum fibrosis
Bone	▪ Renal osteodystrophy
Bone marrow	▪ Myelofibrosis
Skeletal muscle	▪ Muscular dystrophy ▪ Polymyositis ▪ Dermatomyositis ▪ Eosinophilia myositis

in the ratio of type 3 to type 1 collagen, suggesting a 'maturation arrest' in the healing process.

- Dark-skinned persons are more frequently affected by keloids than light-skinned people. Keloid is formed due to excessive scar tissue as a result of synthesis of collagen in the settings of burns, ear piercing, severe acne scar, scratches and surgical incision site.
- Keloid is most commonly found on the chest, shoulders, earlobes and cheeks including any part of the body. Keloid tends to grow slowly with raised pink, pink red, purple margin causing pain, itching and tenderness. Histologic examination of keloid reveals irregular, thick collagen bundles that extend beyond the confines of the original injury. Histology of keloid is shown in Fig. 2.63.

EXUBERANT GRANULATION TISSUE

Granulation tissue fills the dead space during normal wound healing. Exuberant granulation tissue is an overgrowth of granulation tissue. The proliferation of excess granulation prevents proper wound healing and tissue repair. There are various causes and predisposing factors of exuberant granulation tissue that include infections, and chronic inflammation induced by implanted material (e.g. bandages, casts).

Pyogenicum Granuloma

Pyogenicum granuloma is sometimes also called granuloma pyogenicum. It is a common benign vascular neoplasm that arises in skin and mucous membranes (e.g. lips, eyelids, genital, inside mouth). Scientifically, accurate term for this entity is the lobular capillary hemangioma. Patient presents with grossly visible, 2 cm size solitary, red, friable pedunculated papule that has tendency to bleed.

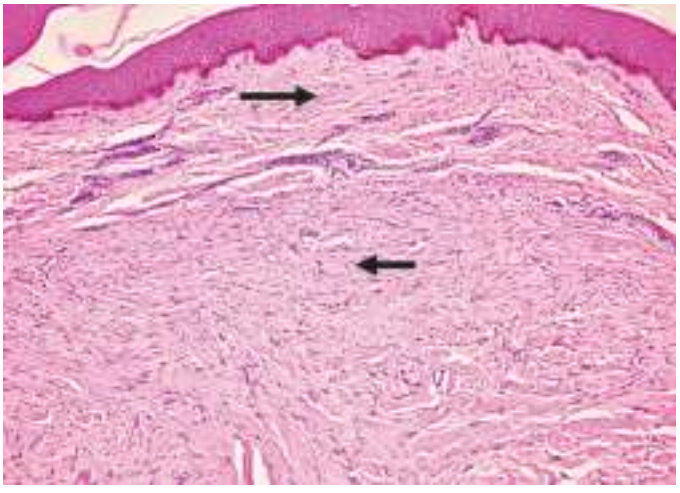


Fig. 2.63: Histology of keloid. Keloid is an exaggerated response to injury that produces abundant collagenous soft tissue, forming a large nodular scar. It often follows trauma to the skin, such as ear-piercing or surgical wounds. It most often occurs in dark-skinned persons especially of African lineage. It tends to recur after resection. Keloid is composed of haphazard fascicles of dense, hyalinized collagen that appear edematous due to dermal mucosubstances in dermis. There is lack adnexal structures. Scattered fibroblasts and myofibroblasts and widely scattered blood vessels are present (arrows) (400X).

Desmoid Tumor

Desmoid tumor is known as aggressive fibromatosis. develop from skeletal muscle, connective tissue, fasciae and aponeuroses in the abdomen as well as neck, shoulders, upper arms and thighs. Desmoid tumor is a low-grade malignancy that occurs in sporadic form and in familial neoplastic syndromes. In about 10–15% of patients with familial adenomatous polyposis (FAP) syndrome, aggressive fibromatosis is a potential manifestation of FAP due to APC gene mutation.

Wound Contracture

Wound contracture can occur during wound healing that leads to severe physical deformity of the wound and surrounding tissues wound contracture is characterized by skin constriction and functional limitations of movements. Wound contracture may be observed after serious burns, crushing injuries, obvious lacerations and avulsion injuries that may occur on the palms, souls and the anterior thorax, which can be severe enough to compromise the movement of joints. Wound contracture results from the proliferation of the specialized fibroblasts termed myofibroblasts.

Proud Flesh

Exuberant granulation tissue, also known as proud flesh, is a frustrated complication of wound healing as a result of extensive growth of the granulation tissue leading to delayed or inhibition of healing process. In severe cases, excessive granulation tissue can lead to the development of significant elevation above the wound edges giving cauliflower-like appearance causing inhibition of epithelial migration.

OTHER COMPLICATIONS

Other complications of cutaneous wound healing and tissue repair include wound infection, epidermal inclusion cyst, hyperpigmentation and malignant transformation.

Wound Infection

Infected wound is a localized defect of the skin or underlying soft tissue in which pathogenic organisms have invaded into viable tissue. Signs of wound infection include yellow-green foul smelling pus discharge, spreading redness around wound edges, increased pain, swelling, fever and lymphadenopathy.

Epidermal Inclusion Cyst

Epidermal inclusion cyst is also called sebaceous, keratin or epidermoid cysts. An epidermal inclusion cyst is lined by stratified squamous epithelium, that appears as a slow-growing mobile, dome-shaped lump filled with keratin material and located just below the surface of skin. It can be located anywhere, but most commonly on the face, followed by the chest or back, scalp, neck, legs, and/or genitalia. It can range in size from 0.5 cm to several centimeters. It has a 'punctum' small-dark-colored opening on the surface of epidermal inclusion cyst, which connects to the cyst located below the skin's surface. Patient can present with pain, redness, swelling and/or drainage. Keratin material can be drained out through the punctum by dermatologist.

Hyperpigmentation over Healed Wound

Hyperpigmentation occurs when the skin affected by an inflammatory disorder and healed scar left after a wound induced by a deep cut or skin burn. Tissue damage also frequently leads to alterations in skin pigmentation in particular to wound hyperpigmentation. The skin remains more pigmented than the normal surrounding skin due to immune cell recruitment to wounds and subsequent recruitment of melanoblasts and melanocytes.

Wound Healing and Malignant Transformation

The relationship between wound healing and development of cancer has been recognized. The mechanisms that regulate wound healing can promote transformation of normal cell to cancer stem cell.

- In addition, chronic inflammation has been associated with malignant transformation in many tissues by enhancing cancer stem cell (CSC) populations, which are highly resistant to current treatments.
- Proinflammatory cytokines and development of signaling pathways are involved in tissue repair, whose deregulation in the tumor microenvironment may promote growth and survival of CSCs.
- Presence of myofibroblastic cells has emerged as common hallmark of wound healing and repair including malignant transformation.
- Both epithelial–mesenchymal transition (EMT) and myofibroblastic differentiation are common occurrences during wound healing, inflammation and fibrosis as well as tumor progression. EMT and myofibroblastic differentiation are regulated by growth factors and signaling pathways.

HEALING OF BONE FRACTURE

Bone fracture is usually accompanied by trauma to adjacent soft tissues with hemorrhage. Healing of bone fracture occurs by the process of organization, while the bone is repaired by regeneration. Pathologic fractures may occur due to osteoporosis, especially steroid induced, metastatic tumors, primary tumors (benign and malignant), Paget's disease, and bone lesions of hyperparathyroidism and osteogenesis imperfecta.

TYPE OF BONE FRACTURES

There are several types of bone fractures. Following terminology is being used in describing basic principles of bone fractures: open, closed, incomplete or partial, complete, stable and displaced bone fracture.

- **Open bone fracture:** An open bone fracture is also called a compound fracture in which there is an open wound, that exposes the bone through the skin near the site of broken bone. Compound bone fractures are extremely painful and patients be shifted to emergency. If not properly treated, open bone fractures can lead to nonlethal, long-term complications such as osteomyelitis and problems in proper bone fracture healing.
- **Closed bone fracture:** A closed bone fracture is a broken bone that does not penetrate the skin. This is also called simple bone fracture. Common causes of closed bone fracture include trauma, motor vehicle accidents, direct blow and repetitive forces such as running. Repetitive forces can induce stress fractures of foot, ankle, tibia or hip bones.
- **Incomplete or partial bone fracture:** This is a crack in the bone that does not completely break the bone into two or more pieces. Incomplete or partial bone fracture occurs as a result of a direct hit to the body or repetitive motions on the muscles that mounts pressure on the bones. Bones are weakest when they are twisted hence prone to partial fracture.
- **Complete bone fracture:** In complete bone fracture, the break goes completely through the bone separating into two or more pieces.
- **Stable bone fracture:** Stable bone fracture occurs when an injury causes the bone to break clean with its segments in alignment and the bone maintains its original position. Stable bone fracture can be induced by physical trauma, osteoporosis and aging process that weaken the bones.
- **Displaced bone fracture:** There is gap between the broken ends of the bone. Repairing a displaced bone fracture may require surgery.

Clinical Pearls: Type of Bone Fractures

Transverse Bone Fracture

A transverse bone fracture occurs when the fracture straight line is perpendicular to the shaft of long bone. This type of bone fracture may be caused by trauma or fall.

Spiral Bone Fracture

In spiral bone fracture, the broken bone resembles a corkscrew or winding staircase because the break happens diagonally across a bone that is longer than it is wide. Spiral; fractures occur in the long bones, usually femur, tibia or fibula, humerus, radius or ulna. Spiral bone fractures are caused by twisting injuries sustained during sports and/or physical trauma or automobile accident.

Greenstick Bone Fracture

- A greenstick bone fracture is a partial fracture that occurs in infants and children as their bones are softer and more flexible.
- The bone bends and breaks but does not separate into two separate pieces.

Stress Bone Fracture

Stress bone fracture is also called hairline fracture, which is tiny crack in the weight-bearing bones of the lower leg and foot caused by repetitive motions such as running. It can be difficult to diagnose with a regular radiograph.

Compression Bone Fracture

- Crushing of bone is called a compression fracture. The broken bone occurs most often in the spine and can cause vertebral body to collapse making it shorter in height.
- Compression bone fracture can lead to slowly worsening severe pain, deformity and restricted movements (bending or twisting). Osteoporosis is the most common cause of compression bone fracture.

Oblique Bone Fracture

An oblique bone fracture occurs when the bone breaks at an angle across the bone, which tends to occur most often in femur or tibia as a result of a sharp blow that comes from an angle due to a fall or trauma. This type of injury causes a visible bone deformity beneath the skin.

Impacted Bone Fracture

An impacted bone fracture occurs when the broken ends of the bone are jammed together by the force of the injury that caused the fracture.

Comminuted Bone Fracture

- A comminuted bone fracture is one in which the broken ends of the bone are shattered into three or multiple pieces.
- Comminuted bone fracture occurs when there is high-impact trauma, such as an automobile accident.

Segmental Bone Fracture

- Segmental bone fractures occur when bone is broken in at least two places, leaving a segment of bone totally separated by two fracture lines.
- Segmental bone fracture can affect any long bone such as femur, tibia, fibula, humerus, radius and ulna, which may take longer time to heal and cause complications.

Avulsion Bone Fracture

An avulsion bone fracture occurs in a bone where a tendon or ligament attaches to the bone that leads to pulling of the bone by a tendon or ligament, which is more common in children than adults. Sometimes a child's ligament can pull hard enough to cause a growth plate to fracture.

STAGES OF BONE FRACTURE HEALING

There are four stages of bone fracture healing: (a) hematoma formation and inflammatory stage, (b) fibrocollagenous callus formation stage, (c) bone callus formation stage, and (d) bone remodeling stage. Healing of bone fracture is shown in Fig. 2.64A to F.

Hematoma Formation and Inflammatory Stage

Initial phase of the bone fracture healing process is considered to be one of the critical determinants of the healing outcome. Hematoma formation and inflammatory stage begins immediately following the bone fracture within 1–5 days.

- The blood vessels supplying the bone and periosteum are ruptured during the bone fracture causing hematoma formation around the bone fracture site. Hematoma clots and forms the temporary frame for subsequent healing of bone fracture.
- With the onset of inflammation, regeneration begins by secretion of proinflammatory cytokines like interleukins (IL-1, IL-6, IL-11, IL-23), tumor necrosis factor-α (TNF-α) and bone morphogenic proteins (BMPs), which stimulate migration of mesenchymal stem cells, endothelial cells and immune cells (monocytes/macrophages and lymphocytes) towards the bone fracture site.
- The immune cells act together to remove necrotic debris, which is followed by early organization due to proliferation of fibroblasts and new capillaries formation (angiogenesis) by vascular endothelial growth factor (VEGF) at the bone fracture site.

Fibrocollagenous Callus Formation Stage

Fibrocollagenous callus formation stage is formed within 5–11 days of bone fracture. Secretion of VEGF leads to angiogenesis at the bone fracture site and within hematoma.

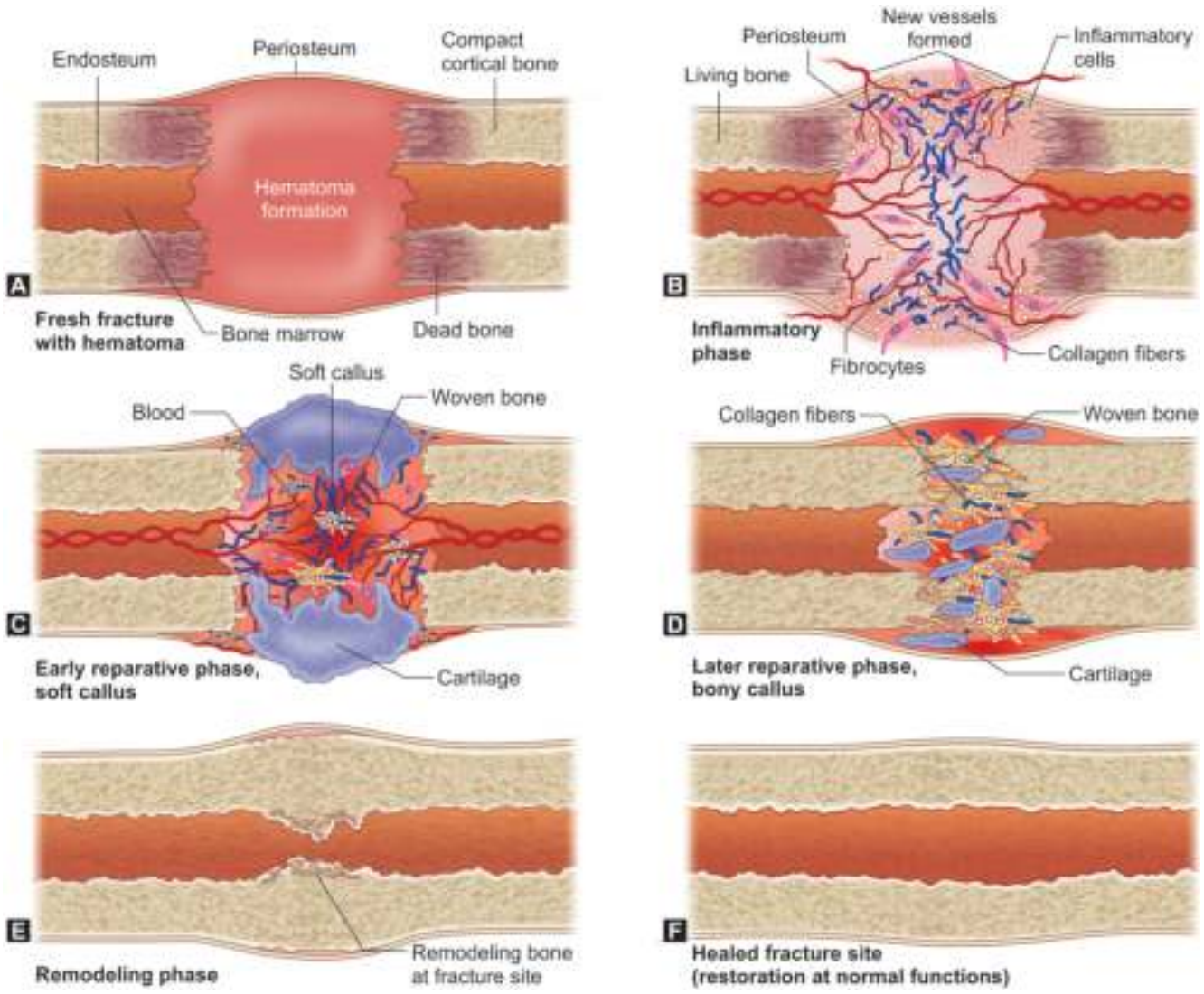


Fig. 2.64: Healing of bone fracture. (A) Hematoma is formed in recent fracture, (B) during inflammatory phase (2–7 days), neovascularization, early fibrosis and woven bone occur, (C) during reparative phase (2–6 weeks), there is gradual decrease in inflammation and granulation tissue. Soft callus is formed. Abundant woven bone is formed. Cartilage appears at the periphery, (D) during later reparative phase, bony callus is formed, (E) during remodeling phase in later months, linear stress causes alignment of new bone and formation of compact bone joining new bone to original bone, (F) bone formation at fracture site and restoration of function occur.

- Fibrin-rich granulation tissue begins to develop. Further mesenchymal stem cells are recruited to the bone fracture site and begin to differentiate to fibroblasts, chondroblasts and osteoblasts driven BMPs.
- As a result, chondrogenesis begins that leads to laying down a collagen-rich fibrocollagenous network spanning the bone fracture ends with a surrounding hyaline cartilage after one week of bone fracture.
- At the same time, adjacent to the periosteum layers, a layer of woven bone is laid down by the osteoprogenitor cells, that bridges the gap.

Bony Callus Formation Stage

The cartilaginous callus begins to undergo endochondral ossification from three weeks onwards at the bone fracture site. RANKL, a transmembrane protein of the TNF is expressed on the surface of osteoblasts and osteoclasts by MMP-7, that stimulates differentiation of chondroblasts, osteoblasts and osteoclasts.

- As a result, the cartilaginous callus is reabsorbed and begins to calcify. Woven bone continues to be laid down in subperiosteal region.
- The newly formed blood vessels continue to proliferate, that allows further migration of mesenchymal stem cells. At the end of this phase, a hard calcified callus of immature bone is formed.

Bone Remodeling Stage

With the continued migration of osteoblasts and osteoclasts, hard calcified callus of immature bone undergoes

repeated bone remodeling termed coupled remodeling within weeks to months of bone fracture.

- The coupled bone remodeling is balance of resorption by osteoclasts and new bone formation by osteoblasts. The center of the callus is ultimately replaced by compact bone, while the callus edges become replaced by laying down of lamellar bone.
- Subsequently, remodeling of the vasculature occurs alongside these changes. The process of bone remodeling lasts for many months ultimately leading to regeneration of the normal bone structure.
- Endochondral ossification process continues, that converts cartilage to bone leading to formation of bony callus, in which the newly formed collagen-rich cartilaginous callus gets replaced by immature bone.
- Final reconstruction in healing of bone fracture occurs months later. Bone fracture site may be almost invisible.

Clinical Pearls: Endochondral and Intramembranous Ossification in Fetus
Endochondral Ossification
Endochondral ossification process plays key role in the formation of bones in the fetus, in which bony skeleton replaces the hyaline cartilage.
Intramembranous Ossification
Intramembranous ossification process in the fetus converts mesenchymal tissue to bone without cartilage formation. This process takes place in the flat bones of the skull in the fetus.

CLINICAL SIGNIFICANCE

Primary bone fracture healing is the reestablishment of the cortex without formation of callus in patients by adequate fixation through reduction, immobilization and rehabilitation. On the other hand, secondary bone fracture healing occurs through inflammatory, fibrocollagenous callus formation, bone callus formation and subsequent bone remodeling.

Factors Adversely Affecting Bone Fracture Healing

Multiple factors adversely affect bone fracture healing, which can broadly categorize into local and systemic categories.

- **Local factors:** Presence of any of local factors predisposes delayed bone fracture healing, which include bone fracture characteristics (i.e. excessive movement, misalignment, extensive damage, large hematoma at bone fracture site, and soft tissue caught within fracture ends), superadded infection (open bone fractures), inadequate blood supply, and avascular necrosis of head of femur and scaphoid, large hematoma and imperfect immobilization.
- **Systemic factors:** Presence of any of systemic factors predisposes to poor bone fracture healing, which include advancing age, obesity, malnutrition, anemia, diabetes mellitus, parathyroid diseases (decreased calcium and phosphate levels), menopause, steroid administration, tobacco smoking and systemic infections.

Complications of Bone Fracture Healing

Early life-threatening complications of bone fracture occur during the first hours or days after the bone fracture include neurovascular injury, visceral injury, hemarthrosis, pneumothorax, rhabdomyolysis, fat embolism, infection, compartment syndrome, deep vein thrombosis, thromboembolism and disseminated intravascular coagulation (DIC). Major complications of bone fracture include wound infection, osteomyelitis, delayed union, nonunion, malunion, premature physeal closure, and fracture associated sarcoma. Consideration of complications of bone fracture healing, it is essential to evaluate and manage these patients in preoperative and postoperative states.

- **Wound infection:** If the overlying skin is breached in any way, the bone fracture is compound, the risk of infection is greatly increased; which is an important adverse factor in the healing process. Patient can develop osteomyelitis.
- **Compartment syndrome:** Compartment syndrome occurs in a case of bone fracture when excessive pressure builds up inside an enclosed anatomical muscle space in the body, which temporarily or permanently compromises the circulation and function of the tissues within that space. Primary symptom of the compartment syndrome is the pain, especially when muscle is stretched, which can be acute or chronic. Anterior compartment of the leg is most common site in acute compartment syndrome. Chronic compartment syndrome is musculoskeletal condition brought on by exercise, that causes recurrent pain and disability.
- **Fat embolic syndrome:** Fat embolic syndrome (FES) is a life-threatening complication in patients with orthopneic trauma especially of long bone fractures due to entry of fat microglobules from the bone marrow cavity into the torn ends of veins and capillaries of the lungs and other sites leading to acute respiratory distress syndrome, cerebral dysfunction and a petechial rash.
  - FES follows closed bone fracture, orthopedic procedures such as intramedullary nailing of the long bones during replacement, severe burns, bone marrow trephine biopsy and liposuction.
  - Patient presents with breathlessness, petechial rash, cerebral dysfunction, oliguria, fever and drowsiness within 24–72 hours between injury and onset.

- Major diagnostic criteria of FES include respiratory insufficiency, cerebral dysfunction and petechial rash; and minor diagnostic criteria of FES include tachycardia, fever, confusion, sustained respiratory rate, retinal hemorrhages, jaundice, renal changes, thrombocytopenia, diffuse alveolar infiltrate and macroglobulinemia.

- **Late complications:** Late complications of bone fracture healing include excess callus formation, delayed union of fracture, malunion of bone fracture (angulation and shortening), nonunion, joint stiffness, myositis ossificans, avascular necrosis, osteomyelitis, septicemia and pseudoarthrosis. Nonunion occurs, if soft tissues such as muscle or fat are interposed between the severed ends. Fibrous union of bone fracture results from excessive movement, infection and ischemia.

REGENERATION OF SPECIFIC ORGANS

The liver has the greatest regenerative capacity of any organ in the body. The nervous system is the only tissue in the body that does not regenerate.

REGENERATION OF LIVER

Liver regeneration is activated spontaneously after hepatocellular injury and can be further stimulated by cell therapy with hepatocytes, hematopoietic stem cells, or mesenchymal cells. Liver regeneration occurs by two mechanisms: proliferation of remaining hepatocytes and repopulation from progenitor cells.

Proliferation of Remaining Hepatocytes

The compensatory proliferation of remaining hepatocytes is the main mechanism of liver regeneration following acute and chronic hepatocellular injury.

- **Priming phase:** IL-6 synthesized by Kupffer cells acts on the uninjured hepatocytes that makes the hepatocytes to receive and respond to growth factor.
- **Growth factor action:** Hepatocyte growth factor (HGF) and TGF-α act on primed hepatocytes. Hepatocytes replication is followed by replication of nonparenchymal cells.
- **Termination phase:** The hepatocytes turn to quiescence. The nature of the stop signal is poorly understood, which may be due to synthesis of TGF-β that inhibits further proliferation of liver parenchyma.

Repopulation from Progenitor Cells

Fulminant hepatic necrosis usually regenerates. Progenitor cells in the liver contribute to repopulation. Patient suffering from chronic viral hepatitis develops cirrhosis. There is development of broad collagenous scars within the hepatic parenchyma. Hepatocytes form regenerative nodules that lack central veins and expand to obstruct blood vessels and bile flow.

REGENERATION OF NERVOUS SYSTEM

Regeneration of nervous system involves the regrowth or repair of nervous tissues, cells or cell products.

- **Regeneration of central nervous system:** Central nervous system comprises permanent cells. In spinal cord injuries, axonal regeneration can be seen up to 2 weeks after injury. Damage to the brain or spinal cord results in proliferation of capillaries and gliosis (permanent scar formation). Gliosis occurs due to proliferation of astrocytes and microglia. After two weeks, axonal regeneration occurs only in the hypothalamohypophyseal portal region, because capillary and glial barriers do not interfere with axonal regeneration. Axonal regeneration seems to require contact with extracellular fluid containing plasma proteins.
- **Regeneration of peripheral nervous system:** Neurons in the peripheral nervous system can regenerate their axons. Patient develops a bulbous lesion known as traumatic neuroma, if cut ends are not in perfect alignment. Light microscopy shows disorganized axons and proliferating Schwann cells and fibroblasts. The nerve is surrounded by dense collagenous tissue, which appears dark blue in this trichrome stain.

CHAPTER 3

Hemodynamic Disorders, Thrombosis, Embolism and Shock

Vinay Kamal, Anubhav and Vigyat

LEARNING OBJECTIVES

EDEMA AND EFFUSIONS

- Pathophysiology of edema
  - Edema caused by sodium and water retention
  - Edema caused by increased capillary hydrostatic pressure
  - Edema caused by reduced capillary plasma oncotic pressure
  - Edema caused by increased capillary permeability
  - Edema caused by lymphatic obstruction
- Composition of the edema fluid
  - Transudate
  - Exudate
  - Lymphedema
  - Non-pitting edema
- Edema: clinical significance
  - Subcutaneous edema
  - Pulmonary edema
  - Ascites
  - Pericardial effusion
  - Hydrothorax
  - Cerebral edema

HYPEREMIA AND CONGESTION

- Hyperemia
- Venous congestion

HEMOSTASIS

- Hemostasis: components
  - Vascular endothelium
  - Platelets
  - Coagulation system cascade
- Hemostasis: stages
  - Primary hemostasis (platelet hemostatic plug formation)
  - Secondary hemostasis (stable hemostatic plug formation)
  - Blood clot retraction
  - Blood clot dissolution (thrombolysis)

HEMORRHAGIC DISORDERS

- Defects in hemostasis
  - Defects of primary hemostasis (platelet defects and von Willebrand disease)
  - Defects of secondary hemostasis (coagulation factor defects)
  - Defects in blood vessels

THROMBOSIS

- Thrombogenesis
  - Vascular endothelial injury
  - Alterations of blood flow
  - Role of platelets
  - Hypercoagulable state
- Thrombotic disorders
  - Inherited thrombotic disorders
  - Acquired thrombotic disorders
- Fate of thrombus
  - Propagation of thrombus
  - Dissolution of thrombus
  - Organization of thrombus
  - Recanalization of thrombus
  - Thrombus embolization
- Thromboembolism
  - Venous thromboembolism
  - Arterial thromboembolism

EMBOLISM

- Pathophysiology of embolism
  - Fat embolism syndrome
  - Air embolism
  - Amniotic fluid embolism
  - Embolism due to exogenous and endogenous materials

INFARCTION

- Factors influencing infarct formation
  - Anatomical pattern of arterial supply
  - Vulnerability of tissues to hypoxia
  - Blood oxygen content
  - Rapid development of infarct
- Types of infarct
  - Pale/white infarct
  - Hemorrhagic/red infarct
  - Brain infarct

SHOCK

- Categories of shock
  - Hypovolemic shock
  - Cardiogenic shock
  - Septic shock
  - Anaphylactic shock
  - Neurogenic shock
- Stages of shock
  - Compensated shock stage
  - Nonprogressive shock stage
  - Progressive decompensated shock stage
  - Irreversible decompensated shock stage

EDEMA AND EFFUSIONS

Edema is swelling caused by accumulation of excessive fluid in body's interstitial tissues. Edema most commonly occurs in the feet, ankles and legs and/or hands where it is referred to as **peripheral edema**. Edema of the feet is sometimes called **pedal edema**. In medical terminology, an **effusion** refers to accumulation of fluid in an anatomic space (**body cavity**) such as pleural cavity between two layers of membrane covering the lungs (pleural effusion) and peritoneal cavity (ascites). Pulmonary edema occurs as a result of excess fluid in the alveoli.

- Excess fluid accumulation occurs due to disorders, which impair functions of cardiovascular system,

liver and kidneys. Edema can occur by four mechnisms: (a) elevated capillary hydrostatic pressure, (b) decreased capillary colloidal osmotic pressure, (c) increased capillary permeability, and (d) obstruction of lymphatic flow.

- Edema can present in numerous forms including unilateral, bilateral, localized or generalized edema.
- In normal health, most of the body's fluids are stored in blood vessels and interstitial spaces outside the cells. In various disorders, excess fluid can accumulate either in one compartment or multiple compartments.
- Anasarca also known as extreme generalized edema is severe, widespread accumulation of fluid in the tissues and cavities of the body at the same time. Causes of edema are given in Table 3.1.

Pathology Pearls: Terminology of Edema and Effusions

- **Peripheral edema:** It occurs due to accumulation of excessive fluid in the subcutaneous interstitial tissues of legs and feet. It is caused by salt retention, cellulitis, congestive heart failure and side effects of medicines. Persistent identification of a swollen leg after pressure from a finger is known as 'pitting edema'.
- **Dependent edema:** It is edema of the legs and lower body affected by gravity which is dependent on a person's position (i.e. edema legs while standing, edema in buttocks and hands while lying down).
- **Cerebral edema:** It refers to accumulation of excessive fluid in the brain.
- **Angioedema:** It occurs due to accumulation of excessive fluid in the deeper layers underneath the skin and on the face. In contrast, hives affect the surface of the skin.
- **Hereditary angioedema:** It is genetic disorder that causes the capillaries to release excessive fluid into the surrounding tissue leading to edema.
- **Papilledema:** It is swelling of the optic nerve as a result of increased intracranial pressure.
- **Macular edema:** It is swelling of the portion of the eyes that perceives central detailed vision (the macula).
- **Lymphedema:** It refers to tissue swelling of arms, legs, genitals, face, neck, chest wall, and oral cavity due to accumulation of protein-rich fluid due to obstruction of lymphatics and lymphatic flow in the settings of tumor emboli, surgical excision of lymph nodes in breast cancer, and 'Milroy' disease.
- **Edema due to venous insufficiency:** Edema can occur in the settings of varicose veins and thrombophlebitis (inflammation of the deep veins of the legs).
- **Edema during pregnancy:** Mild swelling of face, hands or legs can be a sign of **preeclampsia of pregnancy**.
- **Non-pitting edema in myxedema:** Non-pitting edema occurs in myxedema due to accumulation of mucopolysaccharides secondary to overproduction of fibroblasts, which creates a suction force due to enhanced elastic recoil of the extracellular matrix that creates a high negative interstitial fluid pressure.

Table 3.1 Causes of edema

Mechanism	Causes of Edema	
Increased capillary hydrostatic pressure		
Increased vascular volume	▪ Cardiac failure ▪ Renal disease ▪ Pregnancy	▪ Premenstrual sodium retention ▪ Environmental heat stress ▪ Thiazolidinedione therapy
Venous obstruction	▪ Venous thrombosis (thrombophlebitis) ▪ Acute pulmonary edema	▪ Liver disease with portal hypertension
Decreased arteriolar resistance	Calcium channel-blocking drug response	
Decreased capillary colloidal osmotic pressure		
Increased loss of plasma proteins	▪ Protein losing renal disease	▪ Extensive skin burns
Decreased synthesis of plasma proteins	▪ Liver disease ▪ Malnutrition	▪ Starvation
Increased capillary permeability		
Inflammation	Increased vascular permeability	
Tissue injury and burns	Increased vascular permeability	
Allergic reactions	Hives	
Malignancies	▪ Malignant ascites	▪ Malignant pleural effusion
Obstruction of lymphatic flow		
Tumor emboli	Obstructing lymphatic channels and lymphatic flow	
Surgical excision of lymph nodes	Obstructing lymphatic flow	
Milroy's disease	Congenital absence of lymphatic channels	

PATHOPHYSIOLOGY OF EDEMA

Several factors regulate the direction of flow of interstitial fluid including capillary hydrostatic pressure, plasma oncotic pressure, endothelial integrity and the lymphatic system. These factors are thought to driven by Starling's law, which describes fluid movement across capillaries being proportional to capillary permeability, transcapillary hydrostatic pressure differences and transcapillary oncotic pressure differences.

- Normal hydrostatic pressure is 32 mm Hg in capillaries at arterial end, and 12 mm Hg at venous end. Oncotic pressure exerted by plasma proteins is 25 mm Hg throughout length of capillaries. Due to pressure gradient, hydrostatic pressure forces water out of capillaries at the arterial end. At the venous end, 90% of extravasated fluid is drained back in capillaries at venous end due to constant 25 mm Hg oncotic pressure exerted by plasma albumin. Remaining 10% fluid in interstitial tissue is drained via lymphatic channels into venous circulation. Normal fluid exchange between blood and extracellular fluid is shown in Fig. 3.1.
- When the balance of Starling forces is altered, the net result is accumulation of fluid in the interstitial spaces (i.e. edema). Edema can occur due to elevated capillary hydrostatic pressure, reduced capillary plasma oncotic pressure, sodium and water retention, and lymphatic obstruction.

EDEMA CAUSED BY SODIUM AND WATER RETENTION

Total body sodium is the principal determinant of extracellular fluid volume because it is the major cation in the extracellular fluid. The kidneys play an essential role in the regulation of sodium and water. The functioning of the kidneys is efficiently monitored and regulated by hormonal feedback mechanisms involving hypothalamus, juxtaglomerular apparatus and to a certain extent, the heart.

- Renin-angiotensin-aldosterone system (RAAS), antidiuretic hormone (ADH), and atrial natriuretic factor (ANF) provide an elaborate system of checks and balances that regulate body fluid osmolarity, salt concentrations, blood pressure and blood volume.

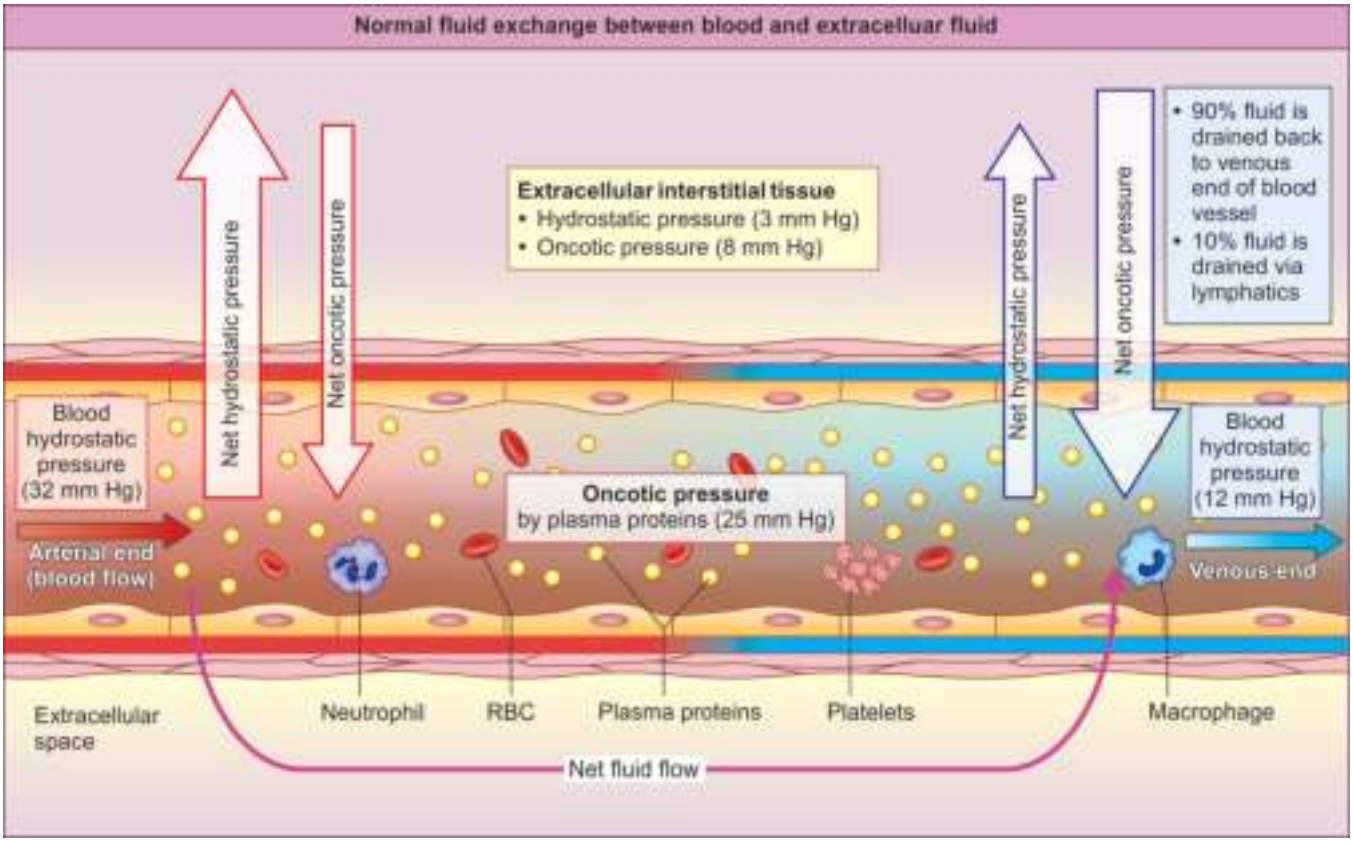


Fig. 3.1: Normal fluid exchange between blood and extracellular fluid. Normal hydrostatic pressure at arterial end of capillary is 32 mm Hg and 12 mm at venous end therefore fluid leaves blood vessel. Oncotic pressure exerted by plasma proteins is 25 mm Hg. At venous end, hydrostatic pressure is less than plasma oncotic pressure, so fluid enters blood vessel. Approximately 90% of fluid is drained back to capillary, while 10% of excess interstitial fluid is drained by lymphatic channels to venous circulation.

- Salt retention occurs whenever renal function is impaired in the settings of primary glomerulopathy and cardiovascular disorders resulting in hypoperfusion of kidneys. When the blood pressure falls, juxtaglomerular cells synthesize renin, which stimulate liver to synthesize angiotensinogen. Lungs convert angiotensinogen to angiotensin I, which is converted to angiotensin II by kidneys.
- Angiotensin II is a potent arteriolar constrictor and it also stimulates adrenal cortex to synthesize aldosterone. Angiotensin II increases peripheral resistance and blood volume leading to regulation of blood pressure via the renin-angiotensin-aldosterone system. Dilution effect of albumin results in decreased plasma oncotic pressure. Atrial natriuretic factor (ANF) is a peptide hormone, which opposes the regulation by RAAS.
- Increased salt retention and obligate water retention causes both elevated capillary hydrostatic pressure as a result of expansion of blood volume, and reduced plasma oncotic pressure leading to edema.

EDEMA CAUSED BY INCREASED CAPILLARY HYDROSTATIC PRESSURE

Increased hydrostatic pressure in vessels exceeds that of plasma oncotic pressure and so that water remains in the tissues in congestive cardiac failure.

- Hydrostatic pressure in capillary has autoregulatory capacity allowing alterations in resistance at the precapillary sphincter and thus determines the arterial pressure forced onto the capillary.
- In contrast, venous end of the capillary has poor autoregulation, and as a result, venous pressure alterations lead to parallel changes in capillary hydrostatic pressure. Venous pressure can enhance expansion of blood volume and obstruction at the venous end.
- Cardiac failure and renal disease lead to expansion of blood volume, while venous blood flow obstruction occurs due to right heart failure ultimately resulting in edema. Local venous obstruction increases capillary hydrostatic pressure in the settings of deep vein thrombosis (DVT) of legs, external compression of deep vein and superior vena cava obstruction.
- Patient develops pulmonary edema due to left-sided heart failure (e.g. systemic hypertension, aortic valve stenosis in rheumatic heart disease). Peripheral edema occurs in right-sided heart failure. Portal hypertension in cirrhosis leads to accumulation of fluid in peritoneal cavity known as '**ascites**'. Clinical manifestations of congestive heart failure are shown in Fig. 3.2.
- Excess fluid accumulation in interstitial tissues is a transudate, which is straw-colored ultrafiltrate of blood plasma composed of mainly water, dissolved electrolytes, and low-protein with specific gravity <1.012.

EDEMA CAUSED BY REDUCED CAPILLARY PLASMA ONCOTIC PRESSURE

Decreased plasma albumin concentration reduces the plasma oncotic pressure in the capillaries, so that water cannot be drained back into the capillary bed at the venous end. Excess fluid accumulation in the interstitial tissue results in transudate formation.

- Reduced plasma oncotic pressure in the capillaries typically occurs due to hypoalbuminemia resulting from massive proteinuria (i.e. nephrotic syndrome), decreased synthesis of albumin in liver in cirrhosis and inadequate albumin in the settings of malabsorption syndrome and kwashiorkor.
- Massive proteinuria, inadequate albumin intake and synthesis can lead to reduced oncotic pressure and ultimately edema. Patient develops anasarca (generalized edema) in nephrotic syndrome.

EDEMA CAUSED BY INCREASED CAPILLARY PERMEABILITY

In normal health, the wall of the normal venules is sealed by tight junctions between adjacent endothelial cells. Normally, fluid leaving and entering microvessels is in equilibrium.

- During acute inflammation, chemical mediators widen interendothelial space resulting in increased vascular permeability in postcapillary venules. Extravasation of inflammatory exudate is accumulated at injury site, which is responsible for swelling (edema), pain, and impaired function.
- Exudate contains proteins content with specific gravity >1020, particularly fibrinogen/fibrin, immunoglobulins, salts, water and neutrophils. Severe injury to microvasculature in severe burns is life-threatening.

EDEMA CAUSED BY LYMPHATIC OBSTRUCTION

In normal health, approximately 90% of extravasated fluid is drained back in capillaries at venous end, and remaining 10% of fluid in interstitial tissue is drained via lymphatics into circulation.

- Lymphatic obstruction prevents drainage of lymph fluid from the tissues into the venous circulation leading to lymphedema and tissue swelling as a result of excess accumulation of protein-rich fluid. Lymphedema and tissue swelling can occur

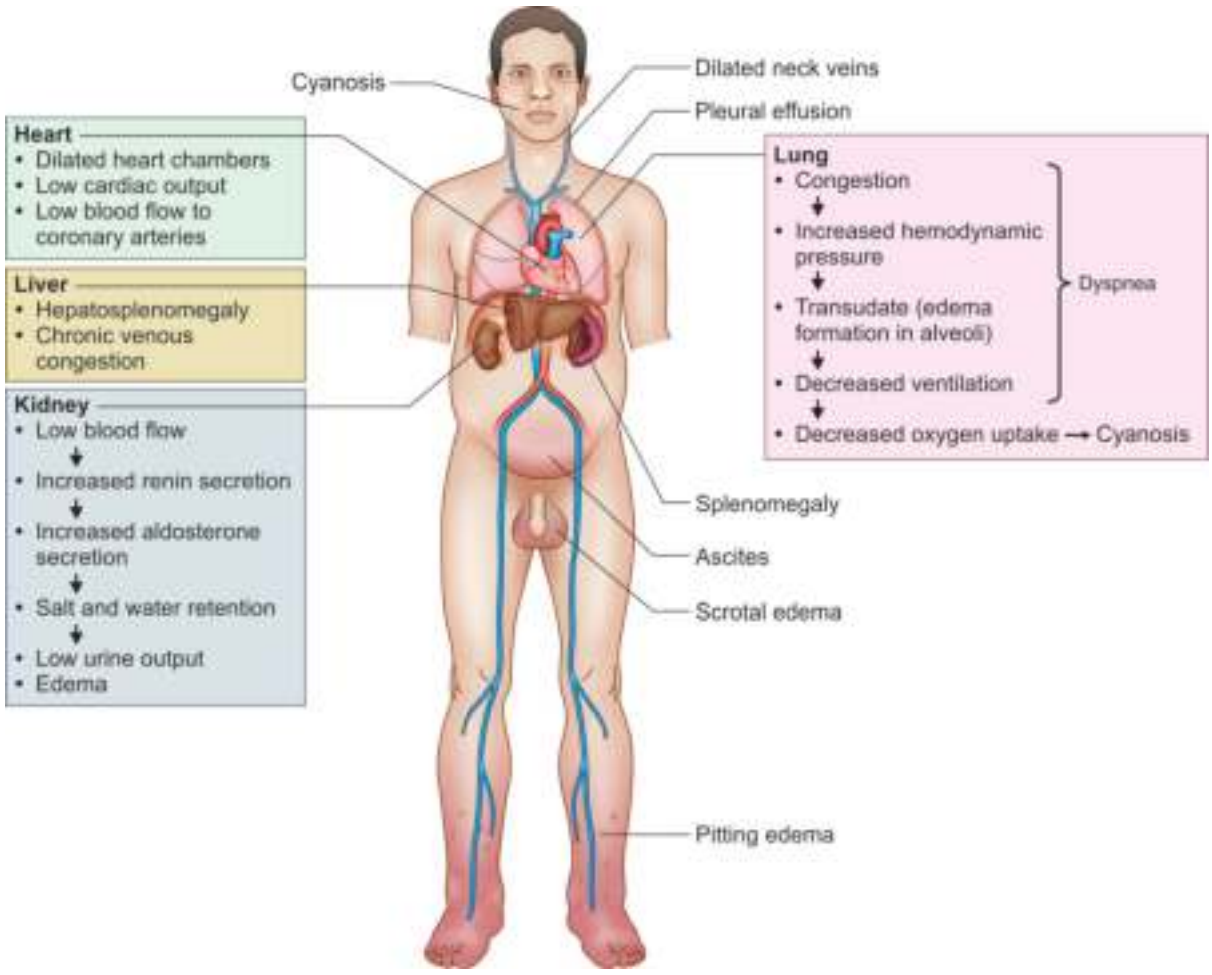


Fig. 3.2: Clinical manifestations of congestive heart failure. Left-sided heart failure results in pulmonary congestion, dyspnea on exertion, dyspnea on lying down (orthopnea), awakening from sleep (paroxysmal nocturnal dyspnea) and cough with frothy sputum. Right-sided heart failure results in systemic venous congestion, peripheral edema over ankles, hepatosplenomegaly and raised jugular venous pressure.

in arms, legs, genitals, face, neck, chest wall, oral cavity. Lymphedema can be either pitting or non-pitting.

- Most common causes of secondary lymphedema include oncologic surgical procedures such as excision of breast cancer along with axillary lymph node dissection, cancer stem cells blocking lymphatic vessels, radiation treatment of surgery (inducing inflammation and fibrosis), and *Wuchereria bancrofti* infestation. *Wuchereria bancrofti* can result in lymphatic obstruction that produces massive lymphedema of the scrotum and lower extremities (elephantiasis). Lymph fluid has high protein content, which may be fibrogenic stimulus in the formation of dermal fibrosis.
- Primary lymphedema is an inherited disorder (Milroy disease) or congenital disorder that causes malformation of lymphatic system, which can be subdivided into three categories: (a) congenital lymphedema is present at birth or recognized within two years of birth, (b) lymphedema praecox occurs at puberty or beginning of third decade, and (c) lymphedema tarda, which begins after 35 years of age. Primary lymphedema is marked by hyperplasia, hypoplasia or aplasia of lymphatic channels.

COMPOSITION OF THE EDEMA FLUID

Edema is a common clinical sign indicating expansion of the interstitial fluid volume. The edema fluid contains predominantly water, but protein and cell-rich fluid can accumulate in cases of infection or lymphatic obstruction. Factors that regulate the size of interstitial fluid compartment include capillary fluid dynamics, interstitial fluid pressure, lymph flow, interstitial compartment compliance, all of which are delicately balanced. Disruption of this delicate balance is caused by common disease processes, which can lead to a better understanding of clinical edema.

TRANSUDATE

A transudate is ultrafiltrate of plasma that occurs due to alterations in Starling's pressure (hydrostatic pressure or oncotic pressure). Permeability of vascular endothelium is usually normal. The noninflammatory edema fluid results from altered capillary hydrostatic or osmotic pressure. Transudate is straw-colored ultrafiltrate of blood plasma with low protein content (<3 g/dl), water, dissolved electrolytes, and specific gravity <1.012, which produces edema in dependent parts (pitting) and body cavity effusions. Formation of transudate due to fluid exchange between blood and extracellular fluid is shown in Fig. 3.3.

EXUDATE

An exudate occurs due to increased permeability of postcapillary venules during acute inflammatory response. Chemical mediators derived from cells and plasma proteins act on nearby vessels and cause increased permeability of microvasculature responsible for swelling (edema), tissue pain, and impaired function. Formation of exudate due to fluid exchange between blood and extracellular fluid in acute inflammation is shown in Fig. 3.4.

- Exudate is rich in plasma proteins (>3 g/dl), fibrinogen/fibrin, immunoglobulins, salts, water and neutrophils with specific gravity exceeding 1.020.
- Exudates formed in severe burns are life-threatening. Fibrin formation at the tissue injury site aids in localizing the spread of infectious microorganisms. Exudates dilute bacterial toxins and provide opsonins (IgG, C3b) to assist in phagocytosis. Comparison of exudate and transudate is given in Table 3.2.

LYMPHEDEMA

Lymphedema occurs due to obstruction of lymphatic channels by tumor emboli, surgical excision of lymph nodes, filariasis and congenital absence of lymphatic channels (Milroy's disease). Filariasis due to *Wuchereria bancrofti* causes lymphangitis and ultimately blockage of lymphatic channels. Scrotal and vulvar lymphedema occurs due to lymphogranuloma venereum. Breast lymphedema occurs due to blockage of subcutaneous lymphatic channels by cancer stem cells. Breast cancer undergoing modified radical mastectomy and lymph nodes dissection develops edema of upper arm due to obstruction of lymphatic drainage. Lymph fluid has high-protein content, which may induce

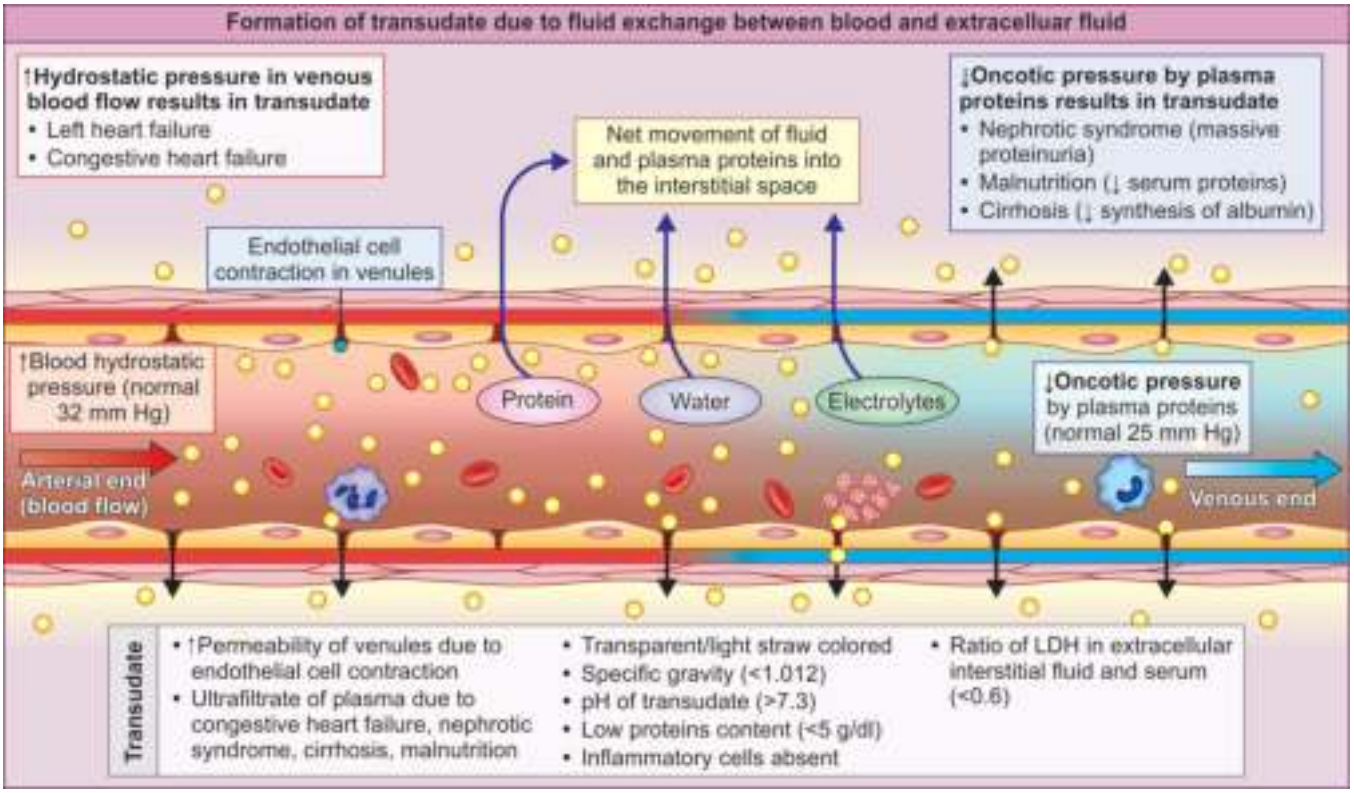


Fig. 3.3: Formation of transudate due to fluid exchange between blood and extracellular fluid. Transudate is formed as a result of increased hydrostatic pressure or decreased plasma oncotic pressure. Therefore, fluid leaves microvasculature along whole length with no reabsorption. There is low protein loss so this is a transudate.

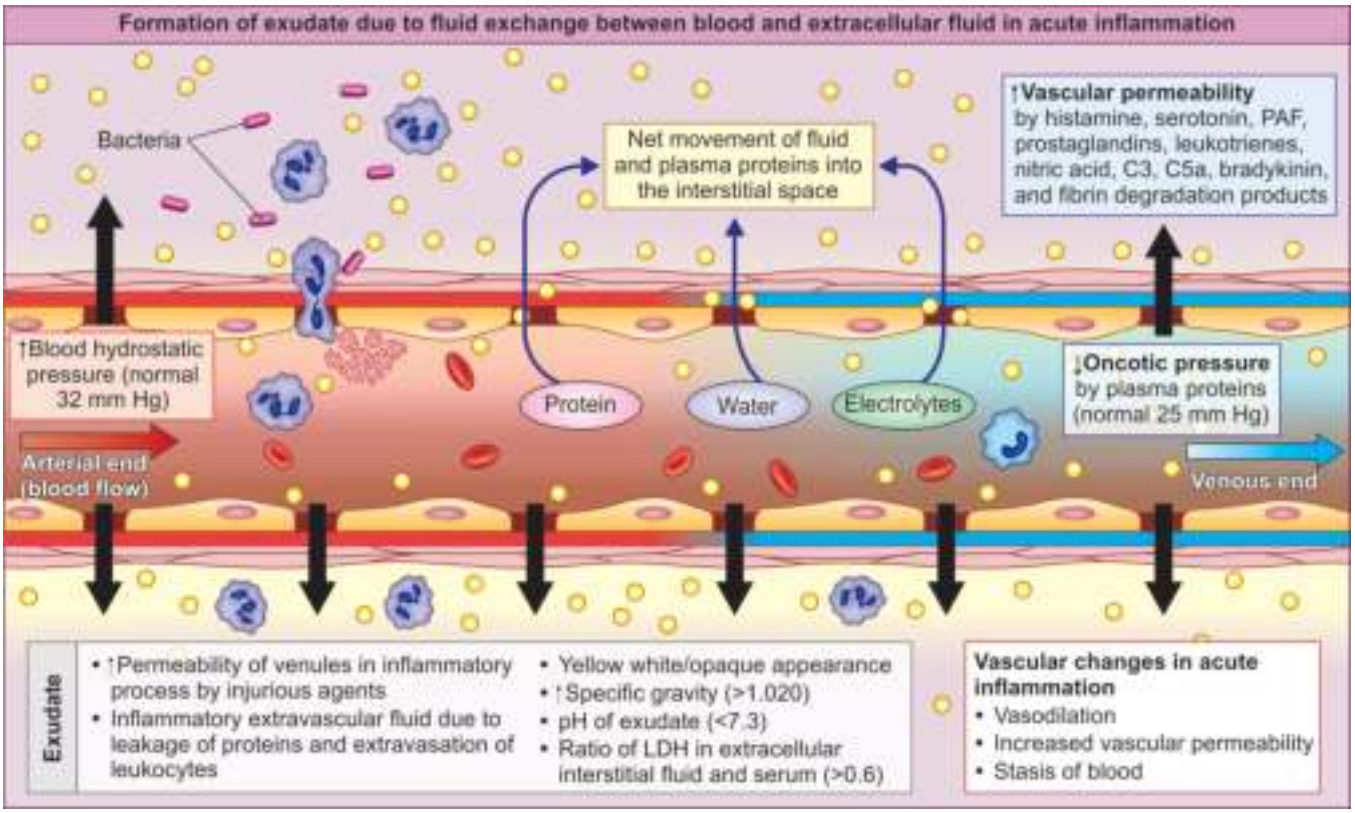


Fig. 3.4: Formation of exudate due to fluid exchange between blood and extracellular fluid in acute inflammation. An exudate is formed due to increased vascular permeability. Protein leaks out of microvasculature, so that there is no osmotic pressure difference between plasma and tissue (i.e. plasma oncotic pressure=0), therefore fluid leaves microvasculature. Fluid contains proteins, so this is an exudate.

Table 3.2 Comparison of exudate and transudate

Characteristics	Exudate	Transudate
Cause	Increased permeability of postcapillary venules in inflammation	Increased hydrostatic pressure or decreased oncotic pressure
Pathologic state	Acute inflammation	▪ Congestive heart failure ▪ Hypoproteinemia (malnutrition) ▪ Nephrotic syndrome (protein loss)
Endothelial permeability	Altered endothelial permeability	Normal endothelial permeability
Fluid nature	Inflammatory extravascular fluid	Ultrafiltrate of blood plasma
Color of fluid	Yellowish-white opaque appearance	Transparent/light straw colored
pH of fluid	Less alkaline (<7.3)	More alkaline (>7.3)
Specific gravity	>1.020 due to raised proteins content in exudate	<1.012
Composition of fluid	Mainly proteins and cellular debris	Mainly water and dissolved electrolytes
Proteins content in fluid	High-protein content, which raise specific gravity	Low protein fluid (<5 g/dl)
Glucose concentration	Low glucose in case of association with infections and cancer	Glucose level in fluid same as that of blood glucose
Ratio of LDH levels in fluids and serum	High ratio (>0.6)	Low ratio (<0.6)
Light microscopy	PMN cells in acute inflammation and later on macrophages and lymphocytes in chronic inflammation	Inflammatory cells absent

fibrogenic stimulus in the formation of dermal fibrosis. Lymphedema can be either pitting or non-pitting.

NON-PITTING EDEMA

Non-pitting edema occurs when excess fluid builds up in the body causing swelling that does not indent when pressure is applied. Non-pitting edema is caused by diseases related to thyroid gland and lymphatic system.

- **Lymphedema:** Lymphedema occurs due to blockage of lymphatic system in the settings of tumor emboli in lymphatic channels, modified radical mastectomy, radiation, and *Wuchereria bancrofti*. Lymphedema can be either pitting or non-pitting. Lymph fluid has high protein content, which may induce fibrogenic stimulus in the formation of dermal fibrosis resulting in non-pitting edema.
- **Myxedema:** Myxedema is severe form of hypothyroidism characterized by swelling of legs and feet including eyelids. Clinical examination of legs reveals non-pitting edema in peritibial region due to deposition of glycosaminoglycans such as hyaluronic acid, chondroitin sulfate and mucopolysaccharide, which binds water, producing non-pitting edema, in particular around feet, hands, eyes and supraclavicular fossae.
- **Graves' disease:** Graves' disease is characterized by nontender diffuse symmetric thyroid gland enlargement with features of thyrotoxicosis, exophthalmos in 60–90%, dermatopathy and constitutional symptoms. Retrobulbar ocular muscles are infiltrated by chronic inflammatory cells. There is deposition of hydrophilic mucopolysaccharides, fibrosis and contractures of extraocular muscles resulting in incoordination of eye movements. Patient presents with proptosis, lid lag, upper lid retraction, and muscle weakness.
- **Lipedema:** Lipedema is an abnormal fat build up in lower body that causes adipose tissue cells to grow and proliferate and increased fluid retention around adipocytes, which results in tender or painful non-pitting edema in legs and feet almost exclusively in women.

EDEMA: CLINICAL SIGNIFICANCE

Edema may be localized in body cavities, e.g. pleural effusion, pericardial effusion or ascites. Generalized edema in nephrotic syndrome is also known as anasarca. Clinically important type of edema is given in Table 3.3. Comparison of cardiogenic and noncardiogenic edema is given in Table 3.4.

Table 3.3 Clinically important type of edema

Type of Edema	Cause
Periorbital edema	Nephritic syndrome (post-streptococcal glomerulonephritis)
Generalized edema (anasarca)	Nephrotic syndrome
Peripheral edema (pitting leg edema)	Left heart failure
Brain edema	Head traumatic injury
Hydropericardium	Viral pericarditis
Hydrothorax	▪ Congestive heart failure ▪ Pleuritis
Ascites	Cirrhosis

Table 3.4 Comparison of cardiogenic and noncardiogenic edema

Characteristics	Cardiogenic Edema	Noncardiogenic Edema
Etiopathogenesis	▪ Left ventricular failure ▪ Myxoma in left atrium ▪ Mitral valve disease ▪ Acute respiratory distress syndrome ▪ Pulmonary vaso-occlusive disease	▪ Aspiration of noxious fluids ▪ Ingestion of drugs or poisons ▪ Inhalation of noxious fluids ▪ Pulmonary infection ▪ Systemic sepsis and trauma
Distribution of edema	Central region	More peripheral
Radiologic findings	▪ Septal lines common ▪ Peribronchial cuffing common ▪ Pleural effusion common ▪ Cardiomegaly present ▪ Pulmonary vasculature diversion in upper lobes	▪ Septal lines less common ▪ Peribronchial cuffing less common ▪ Pleural effusion less common ▪ Cardiomegaly absent ▪ No distribution of pulmonary vasculature

- **Subcutaneous edema:** Patient develops periorbital edema in nephritic syndrome, because proteinuria varies from mild to moderate <3.5 g/24 hours. Patient with nephrotic syndrome presents with generalized edema due to massive proteinuria ≥3.5 g/24 hours. Dependent edema is seen in congestive heart failure.
- **Pulmonary edema:** Congestive heart failure is most important cause of pulmonary edema, which may also occur in left ventricular failure, mitral valve disease, myxoma in left atrium, acute respiratory distress syndrome and pulmonary vaso-occlusive disease. Noncardiac edema causes include aspiration of noxious fluids, ingestion of drugs or poisons, inhalation of noxious fluids, pulmonary infection, systemic sepsis and trauma.
  - **Pathogenesis:** In patients with congestive heart failure, increased venous hydrostatic pressure leads to congestion of alveolar capillaries resulting in accumulation of a transudate in the alveoli. Passage of red blood cells into the alveoli are phagocytosed and degraded by macrophages. Hemosiderin-laden macrophages are known as 'heart failure cells'.
  - **Clinical features:** Patient presents with shortness of breath (dyspnea) on exertion and when recumbent (orthopnea). Patient may awaken from sleep due to sudden episodes of shortness of breath (paroxysmal nocturnal dyspnea). On auscultation, crackling breath sounds (rales) are heard caused due to expansion of fluid-filled alveoli. Right-sided heart failure reveals distended jugular veins, pitting edema of the lower extremities, and tender hepatomegally.
- **Ascites:** Excessive accumulation of serous fluid in the peritoneal cavity is known as **ascites**. Massive proteinuria (≥3.5 g/24 hours) in nephrotic syndrome and chronic liver disease lowers plasma albumin levels results in ascites. Ascites in liver cirrhosis occurs due to decreased synthesis of albumin by liver, the most significant contributor to reduced plasma oncotic pressure, is associated with increased sodium and water retention because of stimulation of the renin-angiotensin system. Portal hypertension in liver cirrhosis results in fluid transudation and increased secretion of hepatic lymph.
- **Pericardial effusion:** Pericardial effusion is collection of fluid in the pericardial sac beyond the 20–50 ml normally present. Pericardial effusion is caused by pericarditis (e.g. serous, fibrinous, suppurative, hemorrhagic or tubercular), metastatic deposits from lung cancer, breast cancer or lymphomas. Tubercular pericardial effusion contains straw/pale colored fluid formed by Mycobacterium tubercle bacilli.
- **Hydrothorax:** Hydrothorax refers to the accumulation of a significant volume of transudate about 200–400 ml within the pleural cavities to be detected by chest radiograph. Transudate is clear, straw colored fluid with low-protein content and low specific gravity. Cardiac failure is the most common cause of hydrothorax, which is usually bilateral.
- **Cerebral edema:** Increased intracranial pressure due to cerebral edema leads to herniation of the brainstem or cerebellar tonsils. Patient presents with projectile vomiting, headache and convulsive seizures.

HYPEREMIA AND CONGESTION

Hyperemia refers to increased blood flow (active hyperemia) or reduced drainage (passive hyperemia congestion) via capillaries and small vessels in a tissue or organ. Active hyperemia is usually a physiologic response to an increased functional demand associated with acute inflammation.

- Congestion refers to the engorgement of an organ with venous blood. Acute passive congestion occurs in shock or sudden right-sided heart failure due to obstruction to venous return or increased back flow from congestive heart failure.
- Deep vein thrombosis (DVT) of the leg veins causes edema of the lower extremity. Thrombosis of the hepatic veins in Budd-Chiari syndrome causes chronic passive congestion within liver. Differences between hyperemia and congestion are shown in Table 3.5.

HYPEREMIA

Hyperemia refers to active engorgement of vascular beds due to increased metabolic activity of tissue that results in localized increased concentrations of carbon dioxide and other metabolites. These metabolic changes act as local stimuli, which cause vasodilatation and increased blood flow to the tissue.

- Hyperemia can occur as a physiologic mechanism within the skin to dissipate heat. There is increased blood flow to gastrointestinal tract after a meal.
- Vascular changes in acute inflammation induces active hyperemia due to neurogenic reflexes and release of vasoactive substances such as histamine and prostaglandins, which promote the delivery of inflammatory metabolites to the injury site. Tissues

Table 3.5 Differences between hyperemia and congestion

Hyperemia	Congestion
Process	
Active process	Passive process
Blood flow	
Increased blood flow due to arteriolar dilatation in inflammation or skeletal muscles during exercise	Blood pooling due to impaired venous blood flow in congestive heart failure or isolated venous obstruction
Edema within organ	
Absent	Present
Gross morphology	
Organs with reddish discoloration due to engorgement with oxygenated blood	Organs blue-red color (cyanosis), due to accumulation of deoxygenated hemoglobin giving nutmeg appearance on gross examination
Light microscopy	
Acute pulmonary hyperemia with engorged capillaries, septal edema and minute intra-alveolar hemorrhage in lobar pneumonia	Chronic pulmonary congestion with alveolar spaces containing numerous hemosiderin-laden macrophages, heart failure cells and thickened septa
Acute liver hyperemia with distended sinusoids and fatty change	Chronic liver congestion with dilated central vein, distended sinusoids, fatty change progressing to cardiac cirrhosis

with hyperemic blood vessels are bright red and warm and there is engorgement of the arterioles and capillaries.

- Hyperemia is fundamental pathophysiologic process leading to overgrowth of epiphysis and early fusion in short stature persons with immature skeleton. Hyperemia with bone fracture healing may result in bone overgrowth.
- In the early acute stage of bacterial pneumonia, the upper lungs become red and microscopically blood vessels and alveolar capillaries are engorged with blood from hyperemia.

VENOUS CONGESTION

Venous congestion refers to the engorgement of an organ with venous blood. Acute passive congestion occurs in shock or sudden right-sided heart failure due to obstruction to venous return or increased backflow from congestive heart failure.

CHRONIC VENOUS CONGESTION IN LIVER

Diffuse venous congestion in the liver results from right-sided heart failure usually due to cardiomyopathy, tricuspid regurgitation, mitral insufficiency, constrictive pericarditis or cor pulmonale. In right-sided heart failure, liver is particularly vulnerable to chronic passive congestion, because the hepatic veins empty into the inferior vena cava. Liver is enlarged and tender.

- **Pathophysiology:** Moderate or severe right-sided heart failure increases central venous pressure, which is transmitted to the liver via the inferior vena cava and hepatic veins. Chronic venous congestion of liver leads to atrophy of hepatocytes, dilatation of sinusoids and centrizonal fibrosis. If severe chronic venous congestion of liver persists, it can progress to cardiac cirrhosis. The basis of hepatocytes cell death is probably sinusoidal thrombosis that propagates to the central veins and branches of the portal vein, causing ischemia. Pitting edema is seen in lower extremities in these patients.
- **Gross morphology:** Nutmeg liver is characteristic of chronic congestion of liver in patients with right-sided heart failure. Dark nutmeg-like spots reflect congestion and dilatation of sinusoids, venules and small hepatic veins in the centrilobular zones, which alternate with pale areas reflecting unaffected surrounding liver tissue in the periportal zones. Gross morphology of chronic venous congestion in liver is shown in Fig. 3.5.
- **Light microscopy:** The central veins and sinusoids are dilated and congested. Surrounding hepatocytes show brownish-yellow discoloration and fatty change. The increased venous pressure leads to dilation of the sinusoids and pressure atrophy of the centrilobular hepatocytes. Long-standing chronic passive congestion of liver leads to bridging fibrosis in extreme cases resulting in cardiac cirrhosis. Histology of chronic venous congestion in liver is shown in Fig. 3.6.
- **Clinical features:** Most patients of chronic venous congestion within liver remain asymptomatic. However, moderate chronic venous congestion causes discomfort in the right upper quadrant due to

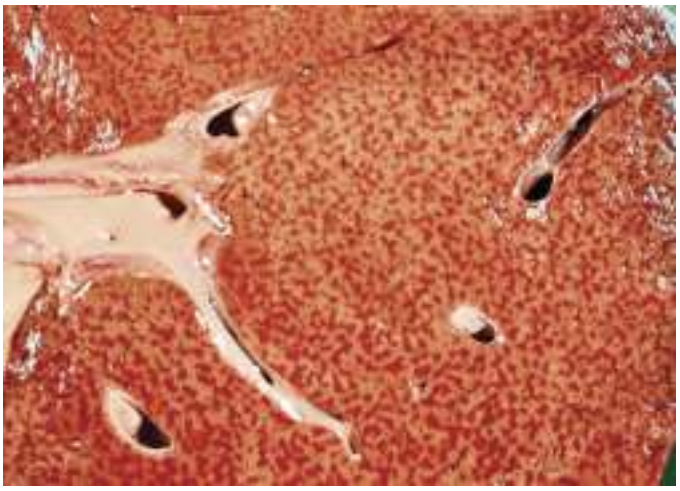


Fig. 3.5: Gross morphology of chronic venous congestion in liver. Nutmeg liver is characteristic of chronic congestion of liver in right-sided heart failure. Dark nutmeg-like spots reflect congestion and dilatation of sinusoids, venules and small hepatic veins in the centrilobular zones. These dark spots alternate with pale areas reflecting unaffected surrounding liver tissue in the periportal zones. Severe and long-standing hepatic congestion can lead to fibrosis, that is called cardiac cirrhosis.

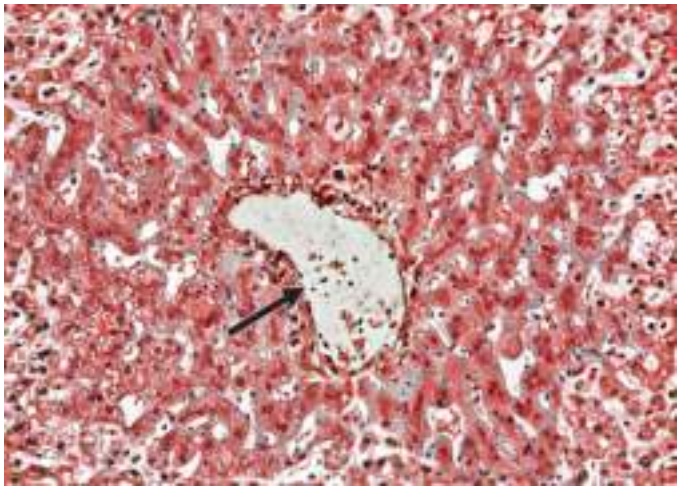


Fig. 3.6: Histology of chronic venous congestion in liver. Histologic findings suggestive of venous outflow impairment (i.e. chronic venous congestion in right-sided heart failure) in liver biopsies include dilatation and congestion of sinusoids and central veins, edema, hepatic cord atrophy and extravasation of red blood cells into the hepatocytes. Sinusoidal dilatation is a fairly common finding (arrow) (400X).

stretching of liver capsule, and tender hepatomegaly. Severe congestion leads to jaundice, massive hepatomegaly and ascites. Infrequently splenomegaly may result. Liver functions are impaired.

- **Diagnosis:** Laboratory test results are moderately abnormal in chronic venous congestion of liver, which include: unconjugated hyperbilirubinemia, elevated aminotransferases and prolonged prothrombin time/international normalized ratio (PT/INR). In addition, a high ascitic total protein content more than 2.5 g/dl plus ascitic albumin concentration more than 1.1 g/dl suggests congestive hepatopathy and differentiates portal hypertension from cirrhosis, in which ascitic total protein content <2.5 g/dl plus ascitic albumin concentration <1.1 g/dl.

BUDD-CHIARI SYNDROME

The cause of Budd-Chiari syndrome is thrombotic occlusion of the major hepatic veins, resulting in hepatomegaly. Thrombus in the hepatic veins cause obstruction to the blood flow to liver leading to chronic venous congestion in the liver. Liver shows severe centrilobular necrosis and hemorrhage as a result of localized obstruction to venous drainage. Zone 1 of liver shows a rim of viable hepatocyte. Budd-Chiari syndrome is shown in Fig. 3.7.

- Budd-Chiari syndrome occurs in the settings of hepatocellular carcinoma, polycythemia vera or other abdominal tumors and pregnant women.
- Less common causes of Budd-Chiari syndrome include paroxysmal nocturnal hemoglobinuria, promyelocytic leukemia, protein C deficiency, protein S deficiency, antithrombin III deficiency and antiphospholipid autoantibodies (lupus anticoagulant).
- Patient of Budd-Chiari syndrome presents with ascites, abdominal pain, hepatomegaly, mild jaundice, edema and eventual hepatic encephalopathy. Acute hepatic failure and death often occur rapidly.

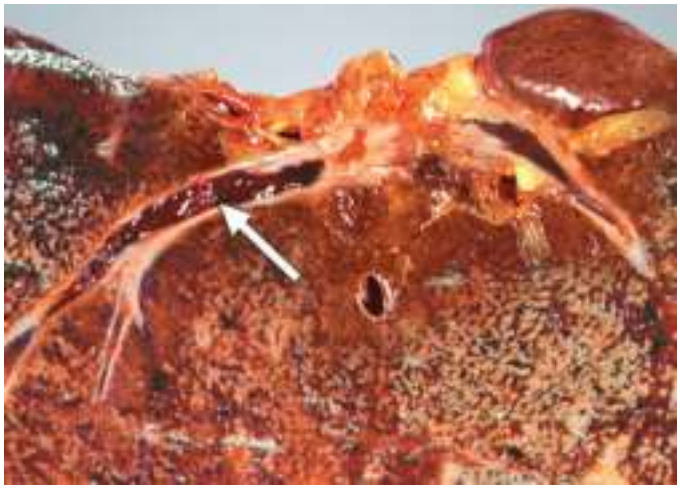


Fig. 3.7: Budd-Chiari syndrome. Budd-Chiari syndrome is characterized by narrowing and obstruction of the outflowing hepatic veins from either large region of the liver or entire liver. Blood clots or congenital webbing occur at the junction of these vessels with large veins that carries the blood from the inferior vena cava to the right atrium of the heart. Budd-Chiari syndrome may begin gradually or abruptly. Patient presents with pain in the upper part of the abdomen, jaundice, hepatomegaly and/or ascites. Thrombus can be seen in the hepatic veins (arrow).

CHRONIC VENOUS CONGESTION OF LUNGS

Chronic venous congestion of lungs is usually caused by left-sided heart failure in the settings of acute myocardial infarction, cardiomyopathies, hypertensive, or valvular heart disease (mitral stenosis, aortic stenosis in rheumatic heart disease), and left atrial myxoma. Pulmonary edema refers to intra-alveolar accumulation of fluid, which is a common complication of left heart failure.

- **Pathophysiology:** Left ventricular failure causes congestion of alveolar capillaries resulting to rupture, passage of red blood cells into the alveoli and accumulation of transudate in the alveoli. Alveolar macrophages degrade red blood cells and accumulate hemosiderin in macrophages. The hemosiderin-laden macrophages are called 'heart failure cells'. In long-standing chronic venous congestion, fibrosis of interstitial tissue and hemosiderin deposition results in brown induration of the lung. The alveoli in the lungs are filled with a smooth to slightly floccular pink material characteristic for pulmonary edema. Capillaries in the alveolar walls are congested with many red blood cells. Pulmonary edema is shown in Fig. 3.8. Heart failure cells in chronic venous congestion of lung are shown in Fig. 3.9.
- **Clinical features:** Patient presents with dyspnea due to pulmonary congestion, orthopnea (difficulty in breathing unless sitting up), frothy white pink sputum, pleural effusion with hydrothorax, weak pulse, cool moist skin as the peripheral vasoconstriction shunts blood to vital organs. Reduction in renal perfusion activates renin-angiotensin-aldosterone system and leading to retention of salt and water, which is less frequent. Cerebral anoxia is less frequent. On auscultation, crackles and wheeze breath sounds are heard.
- **Assay of B-type natriuretic peptide:** Assay of B-type natriuretic peptide is elevated in congestive heart failure, which may aid in the distinction of heart failure from acute coronary syndrome, bronchial asthma, chronic obstructive pulmonary disease, or pulmonary thromboembolism phenomenon, which may also present with dyspnea or pulmonary edema.

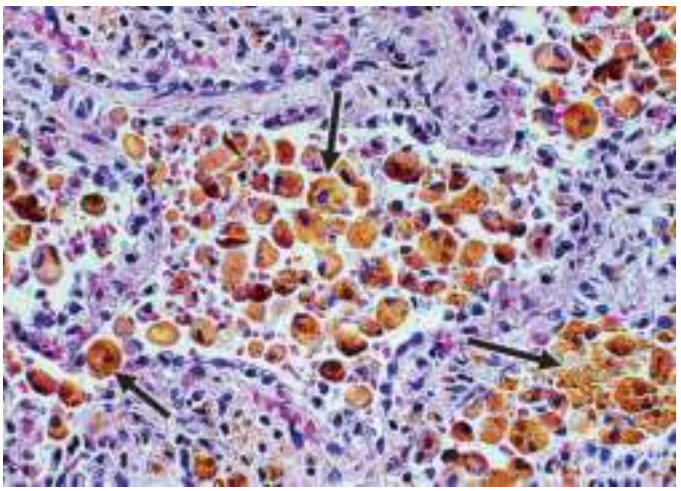


Fig. 3.9: Heart failure cells in chronic venous congestion of lung. Heart failure cells are basically macrophages laden with hemosiderin generated in the alveoli of the lungs of persons with left heart failure or chronic pulmonary edema, when the high pulmonary blood pressure causes breakdown of red blood cells to while passing through the vascular wall (arrows) (400X).

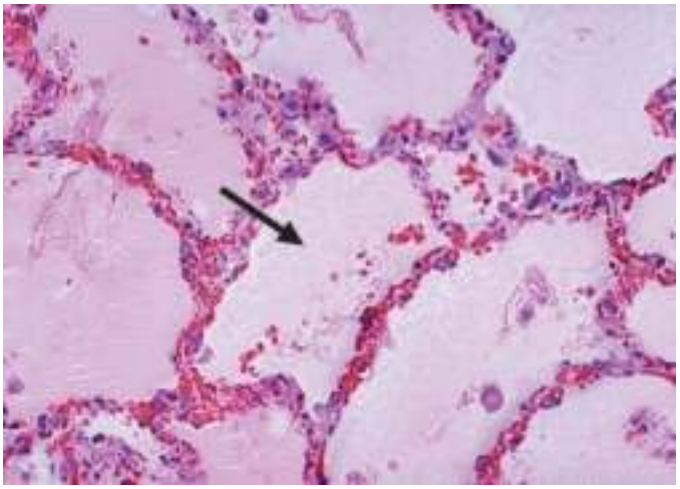


Fig. 3.8: Pulmonary edema. The alveoli in this lung are filled with a smooth to slightly floccular pink material characteristic of pulmonary edema. Alveolar capillaries are congested with many red blood cells. Congestion and edema of the lungs is common in patients with heart failure (arrow) (400X).

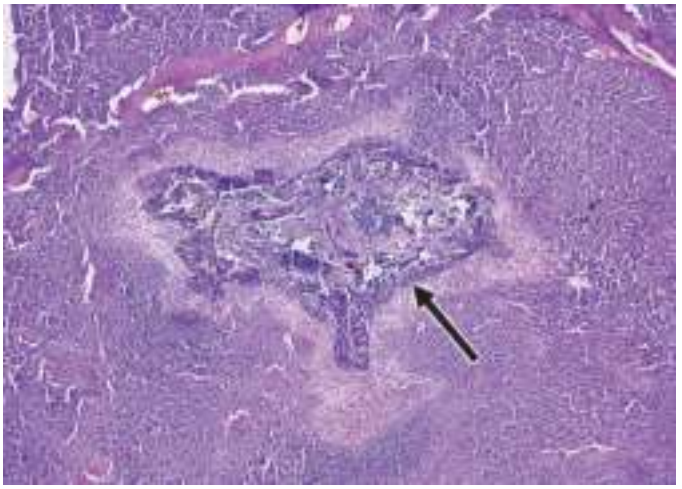


Fig. 3.10: Gamna-Gandy bodies in chronic venous congestion of spleen. Gamna-Gandy bodies or siderotic nodules are formed as a result of organization of hemorrhage with hemosiderin pigment and dystrophic calcification in spleen (arrow) (400X).

CONGESTIVE SPLENOMEGALY

Congestive splenomegaly most often occurs in portal hypertension due to cirrhosis and right-sided cardiac failure with cor pulmonale. Decreased portal venous drainage in these disorders leads to congestive splenomegaly. The increased portal venous pressure causes dilatation of sinusoids, with slowing of blood flow from the cords to the sinusoids that prolongs the exposure of the red blood cells to the macrophages, resulting in excessive trapping and destruction of red blood cells by macrophages in spleen (hypersplenism). Perivascular hemorrhages result in organization and formation of Gamna-Gandy bodies with hemosiderin pigment and dystrophic calcification.

- **Gross morphology**: Spleen shows irregular tan-white fibrous plaques over the purple capsular surface. Cut surface of fibrocongestive splenomegaly shows firm and brown fibrotic nodules termed 'Gamna-Gandy bodies'.
- **Light microscopy:** Gamna-Gandy body is an organized hemorrhage forming nodule with hemosiderin pigment and dystrophic calcification in spleen. Gamna-Gandy bodies in chronic venous congestion of spleen are shown in Fig. 3.10.

HEMOSTASIS

Hemostasis is a host-defense mechanism that protects the integrity of the vascular system after tissue injury, that causes free blood flow in the vascular system by inhibiting activation of platelets, coagulation system and fibrinolytic system.

- Hemostasis has integrating five major components: vascular endothelium, platelets, coagulation proteins, anticoagulant proteins (antithrombin III, protein C, protein S, and tissue factor pathway inhibitor), and fibrinolytic proteins (plasminogen-plasmin system).
- Hemostasis components are generally quiescent, but following tissue injury, these are rapidly activated to stop bleeding at the injured site by formation of a temporary 'platelet plug', activation of coagulation cascade and formation of stable 'fibrin polymer plug'. The clot seals the injured blood vessel and prevents further bleeding.
- Once the injured site starts to heal by regenerating process, the stable fibrin plug slowly undergoes remodeling and dissolution with restoration of normal tissue at the injured site, and maintenance of normal blood flow and vascular integrity.
- Thrombosis is opposite to hemostasis. Bleeding and coagulation disorders are the result of the failure of hemostatic plug formation mechanisms. Hemostasis and thrombosis are regulated by three components: blood vessel, platelets and coagulation system. All components of the hemostatic mechanism exist under resting conditions in an inactive form.

HEMOSTASIS: COMPONENTS

There are various cellular components in the process of coagulation, which include vascular endothelium, platelets and clotting factors.

- Endothelial cells synthesize clotting factors III and IX, while the clotting factor IV comes from the plasma. Factors III, IV and VIII undergo vitamin K-dependent γ-carboxylation of their glutamic acid residues, which allows their binding with calcium and other ions while in the coagulation cascade.
- Platelets are produced by megakaryocytes in the bone marrow, which have structural elements, which include plasma membrane, open canalicular system, spectrin and actin cytoskeleton, microtubules, mitochondria, lysosomes, α- and δ-granules, and peroxisomes. Platelets release proteins involved in formation of primary hemostatic plug to maintain hemostasis.
- The liver produces the majority of the clotting factors that function as procoagulants and anticoagulants.

VASCULAR ENDOTHELIUM

The blood vessel wall is the first-line of defense in maintaining hemostasis. Blood vessel is lined by endothelial cells and supported by subendothelial tissue, which maintains the vascular structural integrity. Subendothelial tissue, an extracellular matrix is composed of collagen, elastin, mucopolysaccharides (including heparan sulfate, chondroitin sulfate), laminin, fibronectin, von Willebrand factor (vWF), vitronectin and thrombospondin, synthesized by the endothelial cells.

- Vascular endothelium forms a barrier between platelets and plasma clotting factors and the subendothelial connective tissue.
- Vascular endothelium inhibits platelet aggregation and coagulation cascade. Disruption of normal laminar flow, imbalance between antithrombotic and prothrombotic molecules synthesized by endothelium result in thrombus formation.
- Vascular endothelium synthesizes antithrombotic (i.e. prostacyclin, ADPase, heparin-like molecules, thrombomodulin) and prothrombotic molecules

(i.e. von Willebrand factor, tissue factor, plasminogen activator inhibitors).

- Antithrombotic properties of vascular endothelium are shown in Fig. 3.11. Prothrombotic properties of vascular endothelium are shown in Fig. 3.12. Antithrombotic and prothrombotic properties of vascular endothelium are given in Table 3.6.

Maintenance of Laminar Blood Flow by Vascular Endothelium

Laminar blood flow dilutes clotting factors and inhibits endothelial cell activation, which increases the inflow of inhibitors of clotting factors. Endothelial cells influence vascular tone, blood pressure, and blood flow by induction of vasoconstriction and vasodilatation. Prostacyclin and nitric oxide cause vasodilation and inhibit platelet aggregation. Laminar blood flow is achieved by secretion of renin, endothelin, endothelial-derived relaxing factor (EDRF) or nitrous oxide (NO), adenosine diphosphatases (ADPase), prostacyclin, and surface enzymes that convert or inactivate other vasoactive peptides such as angiotensin and bradykinin.

Antiplatelet and Anticoagulant Properties of Vascular Endothelium

Intact vascular endothelium is nonthrombogenic that exerts a powerful inhibitory influence on hemostasis by a range of factors of endothelium either synthesizes or expresses on its surface.

Antiplatelet Properties of Vascular Endothelium

For example, platelets adhere to subendothelium tissue rather than vascular endothelium. It is because vascular endothelium synthesizes prostacyclin, endothelial-derived relaxing factor (EDRF) and adenosine diphosphatase (ADPase) enzyme, which inhibit platelet adherence.

- Adenosine diphosphate (ADP) released by platelets play important role in platelet activation and adhesion of platelet to each other.
- Vascular endothelium synthesizes adenosine diphosphatase enzyme (ADPase), which degrades ADP resulting in inhibition of platelets adhesion to subendothelial tissue.

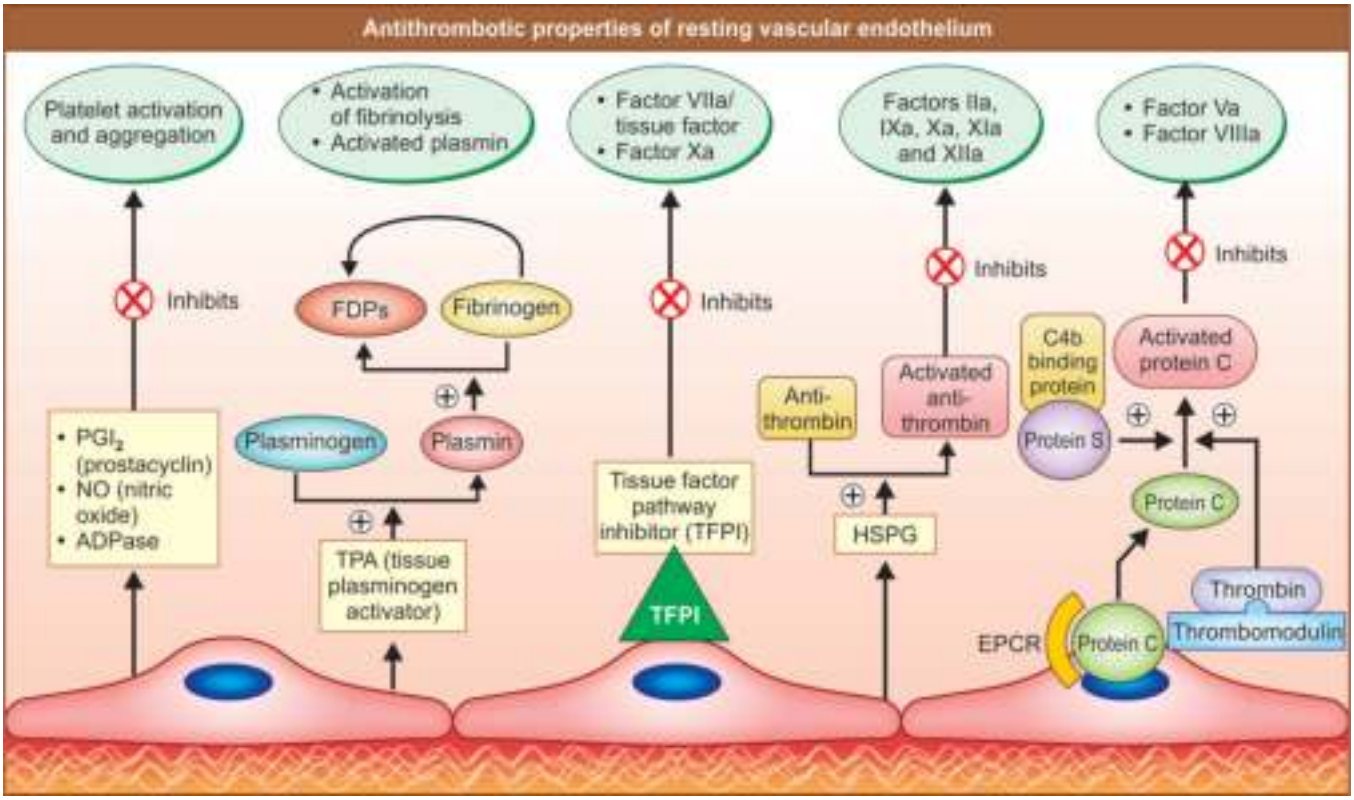


Fig. 3.11: Antithrombotic properties of vascular endothelium. Most important inhibitors of platelet activation generated by endothelial cells are nitric oxide (NO) and prostacyclin (PGI_2). Inhibitors of coagulation generated by endothelial cells are heparin-like molecules, tissue factor pathway inhibitor (TFPI) and thrombomodulin. Heparin-like molecules serve as a cofactor for antithrombin III inactivating several coagulation factors. Tissue factor pathway inhibitor (TFPI) limits the action of tissue factor (TF) and inhibits excessive tissue factor (TF)-mediated activation of coagulation factors VII and X. Thrombomodulin binds thrombin activating protein C and degrades factor Va and factor VIII.

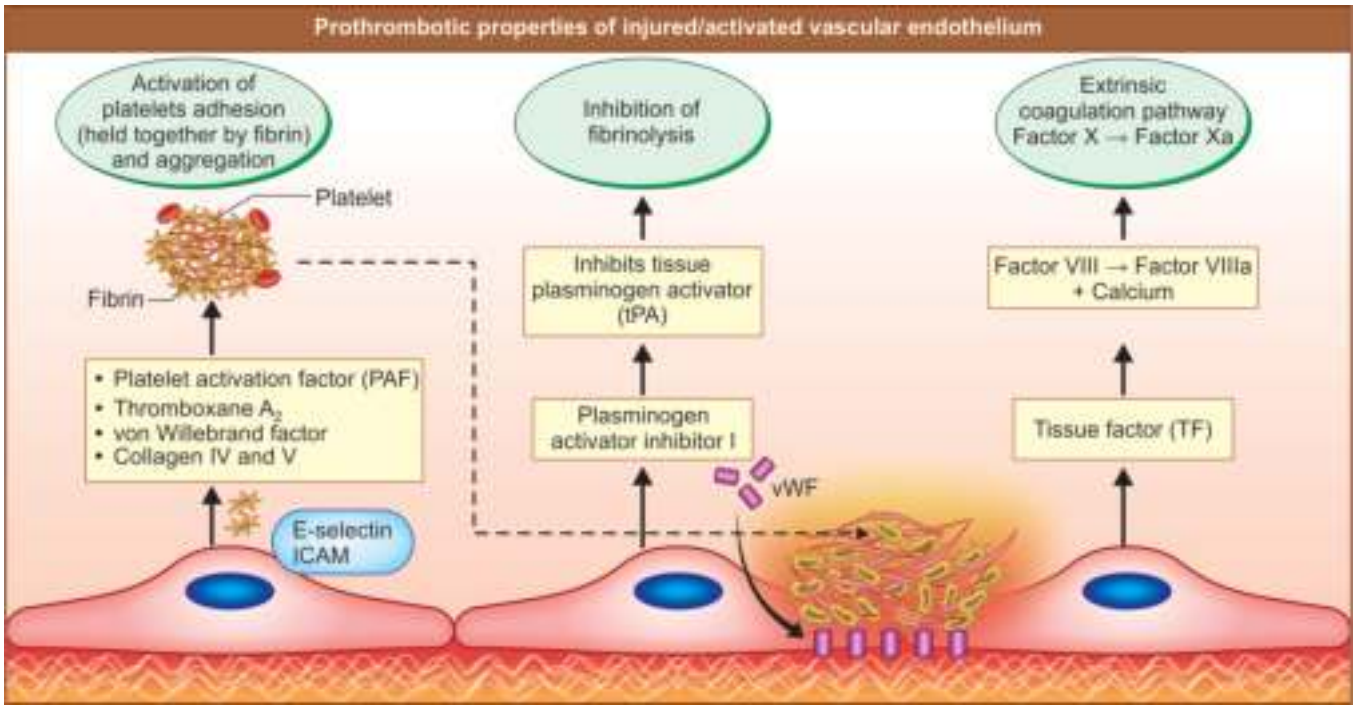


Fig. 3.12: Prothrombotic properties of vascular endothelium. At the site of vascular injury, von Willebrand factor (vWF) binds to the exposed subendothelial collagen. vWF then captures platelets from the bloodstream via the interaction of vWF with GpIb on the platelet surface. The binding of glycoprotein VI with subendothelial collagen leads to further platelet activation, which results in the integrin-mediated platelet adhesion and cellular activation releasing mediators. Thrombin binds to GpIb and activates protease-activated receptor 1 (PAR1) and protease-activated receptor 4 (PAR-4). Adenosine diphosphate (ADP) activates P2Y1 and P2Y2. Thromboxane A_2 (TXA_2) activates thromboxane receptor (TP). Agonists stimulation leads to aIIb-β_3 activation, which binds fibrinogen and mediates platelet aggregation.

Table 3.6 Antithrombotic and prothrombotic properties of vascular endothelium

Component Characteristics	Functions
Antithrombotic functions of vascular endothelium	
Negatively charged endothelial surface	Repels platelets and hemostatic proteins
Nitric oxide (NO)	Vasodilates; inhibits platelet recruitment and accumulation
ADPase (CD39)	▪ Antiplatelet property ▪ Degrades platelet ADP to AMP and adenosine; hence prevents platelet aggregation
Prostacyclin (PGI_2)	▪ Potent vasodilator ▪ Antiplatelet property by inhibiting platelet activation, adhesion and aggregation
Heparin-like molecules (heparan sulphate)	▪ Inhibitor of coagulation ▪ Inhibits fibrin formation (cofactor for antithrombin) and inactivates thrombin, factor X and other clotting factors
Thrombomodulin (TM)	▪ Inhibitor of coagulation ▪ Binds thrombin, enhances activation of protein C, that inhibits the activity of factor V and hence inhibits conversion of fibrinogen to fibrin
Endothelial protein C receptor (EPCR)	EPCR binds protein C; facilitates activation of protein C
Tissue factor pathway inhibitor (TFPI)	TFPI binds tissue factor/factor VIIa/factor Xa complex; inhibits extrinsic pathway of coagulation
Tissue plasminogen activator (tPA)	tPA activates fibrinolytic system
Annexin A2 (TPA receptor A2)	Annexin A2 binds tissue plasminogen activator (tPA) and plasminogen; activates fibrinolysis
Urinary-type plasminogen activator receptor (uPAR)	uPAR binds urinary plasminogen activator and plasminogen; activates fibrinolysis
Prothrombotic functions of vascular endothelium	
Endothelin (ET)	Vasoconstriction of blood vessel

Contd...

Table 3.6 Antithrombotic and prothrombotic properties of vascular endothelium (*Contd...*)

Component Characteristics	Functions
von Willebrand factor (vWF)	▪ Carrier of factor VIII in plasma; facilitates platelet adhesion ▪ Promotes platelet aggregation ▪ Stabilizes factor VIII procoagulant protein
Tissue factor (TF) induced by bacterial toxins, IL-1, TNF-α	▪ Procoagulant property ▪ Initiates fibrin formation, activates factor VII
Plasminogen activator inhibitor 1 (PAI-1)	▪ Procoagulant property ▪ Inhibits activation of fibrinolytic system
Nonhemostatic functions of vascular endothelium	
Selective blood/tissue barrier	Keeps blood cells and macromolecules in blood vessels; allows nutrient and gas exchange
Processing of blood-borne antigens	Contributes to cellular immunity
Basement membrane collagen synthesis	Provides back up protection for endothelial cells
Collagen of the matrix synthesis	Promotes platelet adhesion
Elastin synthesis	Vasodilates and vasoconstricts
Fibronectin synthesis	Binds one cell to another
Laminin	Contributes to platelet adhesion after injury
Vitronectin	Binds one cell to another, possibly promotes platelet adhesion
Thrombospondin (encoded by THBS1 gene)	Adhesive glycoproteins bind one cell to another, possibly promotes platelet adhesion

Normal blood flow dilutes clotting factors, inhibits activation of vascular endothelial cells and enhances inflow of inhibitors of coagulation.

Anticoagulant Properties of Vascular Endothelium

Vascular endothelium synthesizes membrane-associated molecules such as heparin-like molecules, thrombomodulin, tissue factor pathway inhibitor (TFPI) and protease nexin 1, which inhibit coagulation system. Major anticoagulant property of vascular endothelium is via the endothelial expression of thrombomodulin and tissue factor pathway inhibitor (TFPI).

- **Heparin-like molecules:** Heparin-like molecules synthesized by vascular endothelium binds to antithrombin III, which inactivates thrombin, factor Xa and other coagulation factors. Cell-surface heparan sulfate enhances the effect of antithrombin III in forming thrombin–antithrombin complexes.
- **Thrombomodulin:** Thrombomodulin is a natural anticoagulant synthesized by vascular endothelium that acts as a cofactor for thrombin-catalyzed activation of protein C, which inhibits the procoagulant functions of thrombin. Protein C inhibits the activity of factor Va and factor VIIIa, that can no longer convert fibrinogen to fibrin. Heparan sulfate proteoglycan potentiates the activation of antithrombin (AT) 15-fold. Thrombomodulin stimulates the activation of protein C by thrombin 30-fold.
- **Tissue factor pathway inhibitor (TFPI):** Vascular endothelium synthesizes tissue factor pathway inhibitor (TFPI). TFPI rapidly inactivates VIIa and factor Xa in circulation, so limiting conversion of prothrombin to thrombin.
- **Protease nexin 1:** Endothelium also synthesizes protease nexin 1 enhanced by heparan sulfate that inactivates thrombin by covalent binding to the thrombin active sites.

Procoagulant Properties of Vascular Endothelium

Normal vascular endothelium inhibits platelet adherence and coagulation cascade. In contrast to antiplatelet and anticoagulant properties of normal vascular endothelium, injured endothelium induces synthesis of prothrombotic molecules such as tissue factor, plasminogen activator inhibitor and von Willebrand factor (vWF).

Tissue Factor

Tissue factor (TF) triggers coagulation cascade by binding to factor VIIa, and the resulting tissue factor—factor VIIa complex activates the coagulation factors IX and X, ultimately leading to formation of fibrin and blood clot. Vascular endothelium synthesizes endothelin 1 (ET-1), which is a potent vasoconstrictor.

von Willebrand Factor

The von Willebrand factor (vWF) is a carrier of factor VIII in plasma, that stabilizes factor VIII procoagulant protein and facilitates platelet adhesion and aggregation to subendothelial collagen fibers.

Plasminogen Activator Inhibitors

Plasminogen activator inhibitors have procoagulant property, which inhibit activation of fibrinolytic system leading to thrombus formation.

Fibrinolytic Properties of Vascular Endothelium

Vascular endothelium secretes several components involved in fibrinolysis, which include tissue plasminogen activator (tPA) and plasminogen activator inhibitors bound to the endothelial cell surface, which enable assembly of active complexes.

Repair Properties of Vascular Endothelium

After simple minor injury to vascular endothelium, vascular repair process involves migration of adjacent resident proliferation and subsequent proliferation of these cells leading to restoration of functional endothelial monolayer and reestablishment of junctions to reform semipermeable membrane. More severe blood vessel wall injuries require migration and proliferation of smooth muscle cells and fibroblasts. Vascular endothelium secretes platelet-derived growth factor (PDGF), fibroblast growth factor-β and vascular permeability factor. Vascular endothelium is also responsive to PDGF and fibroblast growth factor-β.

Interactive Properties of Vascular Endothelium with Leukocytes

Cell adhesion molecules (CAMs) present on both endothelial cells and leukocytes mediate interaction of vascular endothelium and leukocytes. CAMs play key role in the inflammatory response. Selectins, integrins and immunoglobulin superfamily of receptors mediate rolling, adhesion, emigration and migration of leukocytes from the bloodstream to the site of tissue injury.

PLATELETS

Platelets play a central role in normal hemostasis by forming 'primary hemostatic plug'. Platelets activate coagulation system through the platelet phospholipid complex, which maintain the physical integrity of the vascular endothelium. During platelet activation, platelets release a number of cytokines and chemical mediators via degranulation, which include adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin, von Willebrand factor, thromboxane A_2, from dense granules of platelets, which can activate neighboring platelets via ADP and ATP sensitive receptors. Platelet-derived growth factor (PDGF) repairs injured endothelium. Platelet structure is shown in Fig. 3.13.

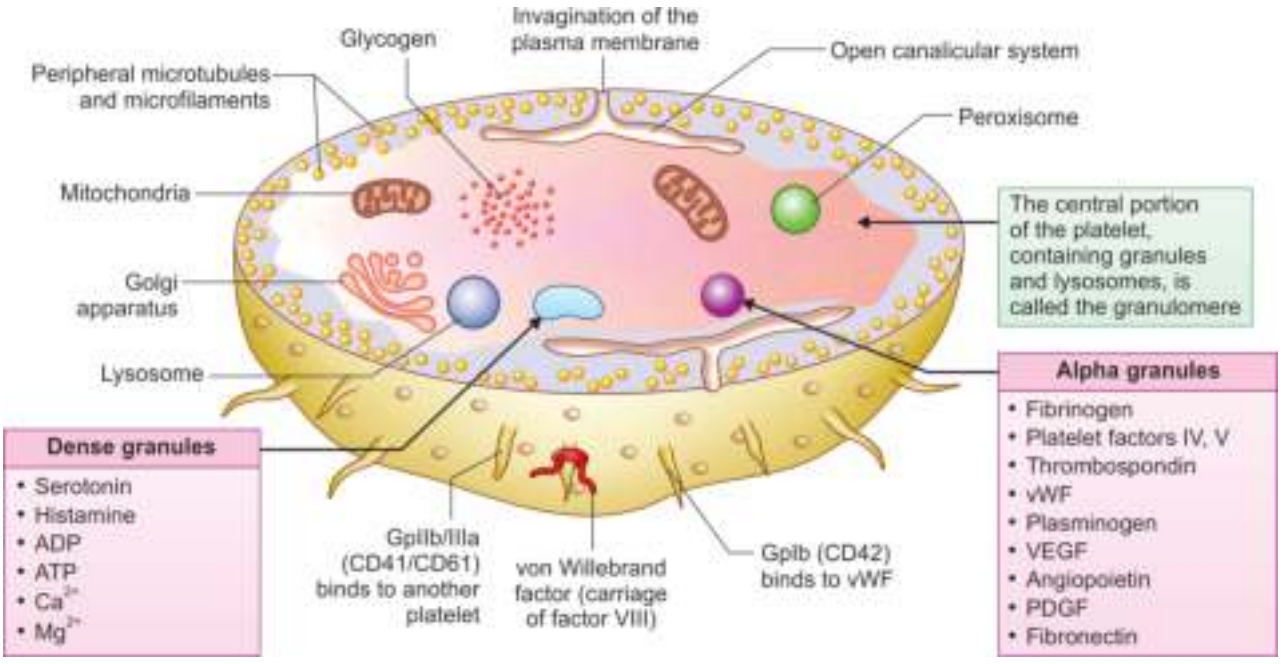


Fig. 3.13: Platelet structure. Platelets are fragments of cytoplasm derived from the megakaryocytes of the bone marrow and then enter the bloodstream. Circulating inactivated platelets are biconvex discoid structures. Platelets regulate hemostasis by forming primary hemostatic platelet plug. Platelets contain two types of granules, i.e. α-granules and dense bodies (dense granules). α-Granules contain fibrinogen, factors V and VIII; platelet-derived growth factor, transforming growth factor β, fibronectin and platelet factor IV. Dense bodies contain adenosine diphosphate (ADP), adenosine triphosphate (ATP), ionized calcium, serotonin and epinephrine.

Thrombopoiesis

Thrombopoietin, vitamin B_{12} and folic acid participate in platelet production in bone marrow. Platelets are formed due to cytoplasmic fragments of megakaryocytes. Thrombopoiesis is shown in Fig. 3.14.

- Megakaryocytes undergo endomitosis to increase ploidy. Megakaryocytes produce platelets by remodeling of cytoplasm into long projections called proplatelets. The lamellipodial and filopodial formation that accompanies platelet activation is driven by the actin cytoskeleton.
- Nearly one trillion, platelets circulate in an adult human. Platelets range in size from less than 5 femtoliters to more than 12 femtoliters; however, the average platelet is about 7.3 femtoliters.
  - Two-thirds of platelets remain in circulation, while one-third is stored in spleen. Recent studies revealed that platelet life span in 3–7 days. Platelet normal count for all ages is 150–450 × 10^9/L.
  - The discoid shape of resting platelets is maintained by a cytoskeleton composed of microtubules, actin filaments, and spectrin-based membrane skeleton.
- Platelets possess several glycoproteins on their surface, which act as adhesive receptors and antigens involved in autoimmune and immune reactions.
  - The main glycoproteins of platelets are the GpIb/IX/V complex, the aIIbβ3 integrin, CD36 (GpIV or GpIIIb), and GpVI. For each of these glycoproteins, several polymorphisms have been described, influencing both platelet function and platelet immunogenicity. The identification and characterization of the gene encoding these glycoproteins by crystallographic studies have contributed to analyze the role of platelet surface glycoproteins.

Platelet Structure

Platelets are membrane bound porous disc-like structures, which express glycoprotein receptors of the integrin family on their surface.

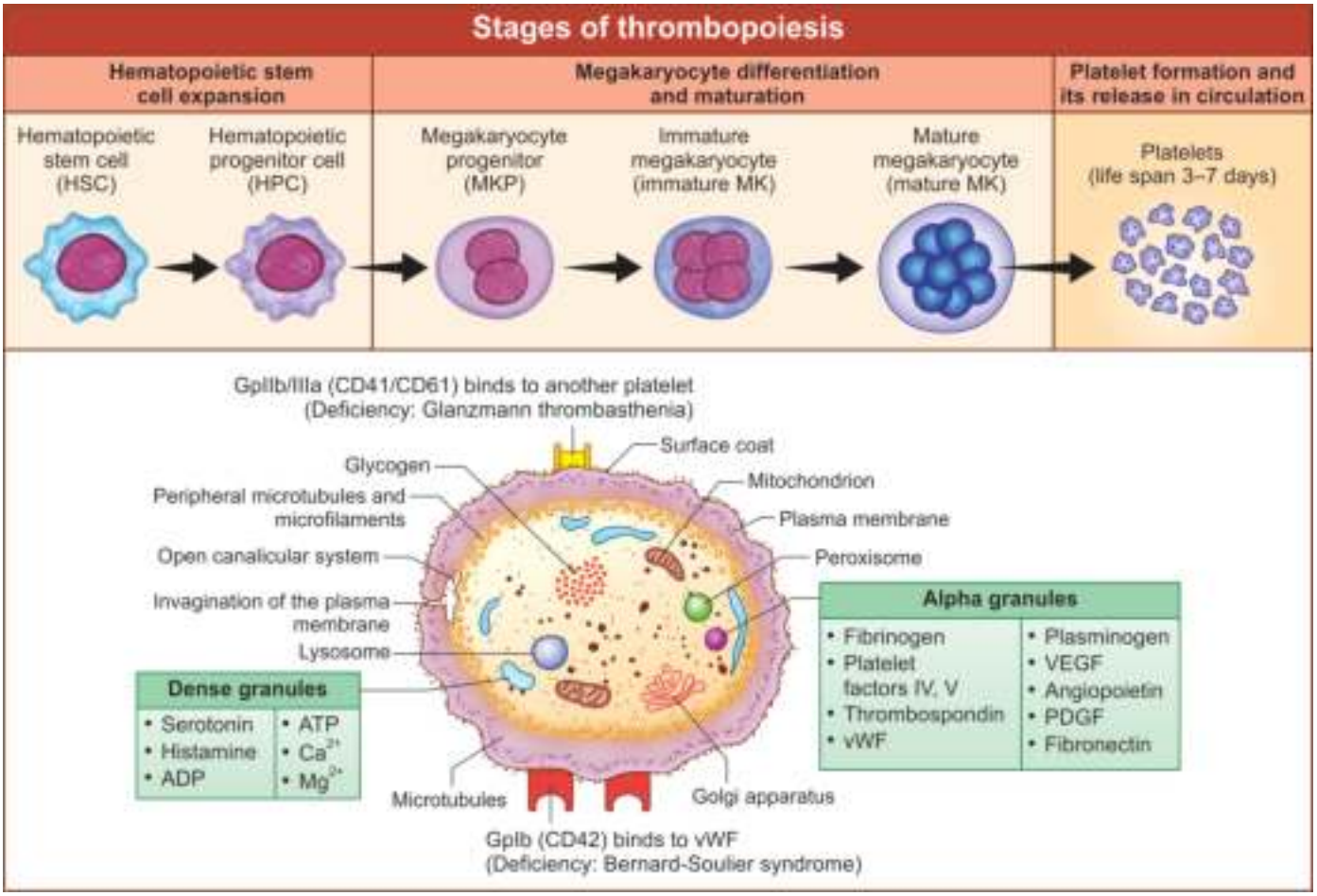


Fig. 3.14: Thrombopoiesis. Platelets are produced by process of thrombopoiesis in the bone marrow. Thrombopoietin produced by liver, which regulates production of platelets in the bone marrow, which stimulates differentiation of common myeloid progenitor cells into promegakaryocytes and then into megakaryocytes that buds off numerous platelets and their release into the circulation. Life span of platelets is 3–7 days. Platelets play important role in formation of primary hemostatic plug.

Table 3.7 Important platelet plasma membrane glycoproteins

Glycoproteins	Copies per Platelet	Receptors
Glycoprotein Ia	$2–4 \times 10^3$	Collagen
Glycoprotein IIa	$5–10 \times 10^3$	Fibronectin, laminin
Glycoprotein Ic	$3–6 \times 10^3$	Fibronectin, laminin
Glycoprotein Ib/IX	$25–30 \times 10^3$	vWF, thrombin essential in the platelet vessel wall interaction
Glycoprotein IIb/IIIa complex	$40–50 \times 10^3$	Fibrinogen, vWF, fibronectin and vitronectin
Glycoprotein IV	Unknown	Collagen, thrombospondin
Glycoprotein V	Unknown	Thrombin

Platelet Plasma Membrane

Plasma membrane of platelets allows adherence of platelets with the help of fibrinogen. Integrin α-IIb-β3 (GpIIb/IIIa) is most abundant glycoprotein on the platelet surface with a number of 60,000–80,000 copies per cell and an additional intracellular pool that is exposed on the surface on activation.

- Platelet plasma membrane glycoproteins function as receptors for different agonists, adhesive proteins, coagulation factors and adjacent other platelets.
- The platelet plasma membrane is a source of phospholipid (prostaglandins synthesis), site of calcium mobilization and localization of coagulant activity to the platelet surface. Important platelet plasma membrane glycoproteins are given in Table 3.7.

Platelet Antigens

Platelets also have many surface specific antigens associated with platelet membrane glycoproteins: HLA class I antigen and ABO group antigen.

Platelet Cytoskeleton

Beneath plasma membrane, platelets contain actin and myosin filaments that cause the platelets to contract. Microtubules maintain the disc-like shape in the resting platelets, but disappear temporarily on platelet aggregation.

Platelet Surface Connecting Canalicular System

The surface-connected canalicular system is formed by invaginations of platelet plasma membrane, that increases the surface area across membrane transport through which platelet granules discharge their contents during the secretory phase of platelet aggregation. The dense tubular system represents the smooth endoplasmic reticulum, which is thought to be site of prostaglandin synthesis and release of calcium ions.

Platelet Organelles and Platelet-specific Storage Granules

Platelet contains many cell organelles (mitochondria, lysosomes, peroxisomes and glycogen granules) and two types of platelet-specific storage dense granules and α-granules. Platelet aggregation results in release of platelet-specific granules.

- **Dense granules:** Dense granules of platelets contain 60% of platelet storage pool of adenosine diphosphate (ADP), adenosine triphosphate (ATP), ionized calcium, bioactive amines (serotonin and histamine) and epinephrine. ADP and ATP play important role in recruitment of platelets and activation of neighboring platelets resulting in platelet aggregation.
- **α-Granules:** α-Granules of platelets contain fibrinogen, von Willebrand factor (vWF), adhesive proteins, platelet factor IV, factor V, β-thrombospondin, plasminogen, vascular endothelial growth factor (VEGF), angiopoietin, platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and fibronectin. Fibrinogen, von Willebrand factor (vWF), adhesive proteins mediate platelet–platelet and platelet–endothelial interactions.

Platelet Function

Platelets play important role in hemostasis, thrombosis and wound healing. Following vascular endothelial injury, platelets become activated in the blood resulting in platelet adhesion to the exposed subendothelial extracellular matrix, platelet hemostatic plug formation, and finally formation of a thrombus consisting of both core and shell. Platelet structure components and their functions are given in Table 3.8. Lysosomal enzymes in platelets are given in Table 3.9.

COAGULATION SYSTEM CASCADE

Platelets initially arrest bleeding by forming temporary primary hemostatic plug in severed blood vessels. Formation of clot by activation of coagulation system is necessary to secure the repair of the damaged blood vessel. Coagulation system pathways are shown in Fig. 3.15.

- On activation, platelets undergo conformational change in shape resulting in synthesis of platelet factor 3 derived from plasma membrane phospholipid of platelets, which serve as catalytic sites for calcium

Table 3.8 Platelet structure components and their functions

Platelet Structure Components	Functions
Platelet membrane	
Glycocalyx	▪ Outermost coat comprising glycolipids, glycoproteins and mucopolysaccharide ▪ Negative charge due to sialic acid residue of proteins and lipids
Plasma membrane	▪ Plasma membrane is composed of glycolipids, cholesterol and glycoproteins ▪ Lipoprotein layer containing platelet factor 3 involved in blood coagulation
Membrane glycoproteins (acting as receptors for cell–cell and ligand–cell interaction)	
Glycoprotein IIb/IIIa	Cross-linking of GpIIb/IIIa to vWF and fibrinogen leading to platelets aggregation. Deficiency of GpIIb/IIIa results in Glanzmann's disease
Glycoprotein Ib–IX	In Bernard-Soulier syndrome, deficiency of GpIb–IX results in bleeding diathesis
Cytoskeleton proteins	
Short actin filament	Present under plasma membrane involved in maintaining discoid shape
Actin microfilament network	Present in cytoplasm
Microtubules	Present in peripheral part of cytoplasm involved in maintaining discoid shape
Dense granule products	
Serotonin (bioactive amine)	Vasoconstriction
Histamine (bioactive amine)	Allergic and inflammatory response
Adenosine diphosphate (ADP)	Recruitment of platelets and activation of neighboring platelets resulting in platelet aggregation
Adenosine triphosphate (ATP)	Agonist for cells other than platelets
Calcium (Ca^{++})	Extracellular source for hemostatic reactions
Magnesium (Mg^{++})	Essential for the movement of sodium, potassium and calcium into and out of cells
α-Granule products	
Fibrinogen	▪ Fibrinogen mediates platelet–platelet and platelet–endothelial interactions ▪ Platelets aggregation and fibrinogen conversion to fibrin
Platelet factor 4	Platelets aggregation
Thrombospondin	Platelets aggregation
Factor V	Platelets adhesion
von Willebrand factor (vWF)	vWF mediates platelet–platelet and platelet–endothelial interactions
Plasminogen	Plasminogen conversion to plasmin, that causes fibrinolysis
VEGF, angiopoietin, PDGF	Angiogenesis
Fibronectin	Plasma form of fibronectin circulates in the blood, and upon tissue injury. Fibronectin is incorporated into fibrin clots to exert effects on platelet function and mediate hemostasis
Osteonectin	Osteonectin is a regulatory protein involved in the adhesion of osteoblasts and platelets to their extracellular matrix and in early stromal mineralization

Table 3.9 Lysosomal enzymes in platelets

Cathepsin D
Cathepsin E
Acid hydrolases
Carboxypeptidase A
Carboxypeptidase B
Proline carboxypeptidase
Beta-N-acetyl-o-hexosaminidase
Beta-o-glucuronidase
Beta-o-galactosidase

and coagulation factors in the intrinsic coagulation system pathway.

- Coagulation system comprises extrinsic and intrinsic pathways, which reflect how clotting occurs in the test tube during tests. Clotting in the body is initiated differently in both coagulation system pathways.
  - **Extrinsic pathway of blood coagulation system:** Tissue factor binds and activates factor VII. Activated factor VII (VIIa) further activates factors X and IX via proteolysis. Activated factor IX (IXa) binds to its cofactor-activated factor VIII (factor VIIIa), which results in activation of factor X (factor Xa). Factor Xa binds to its cofactor-activated factor V (factor Va) and calcium and generates

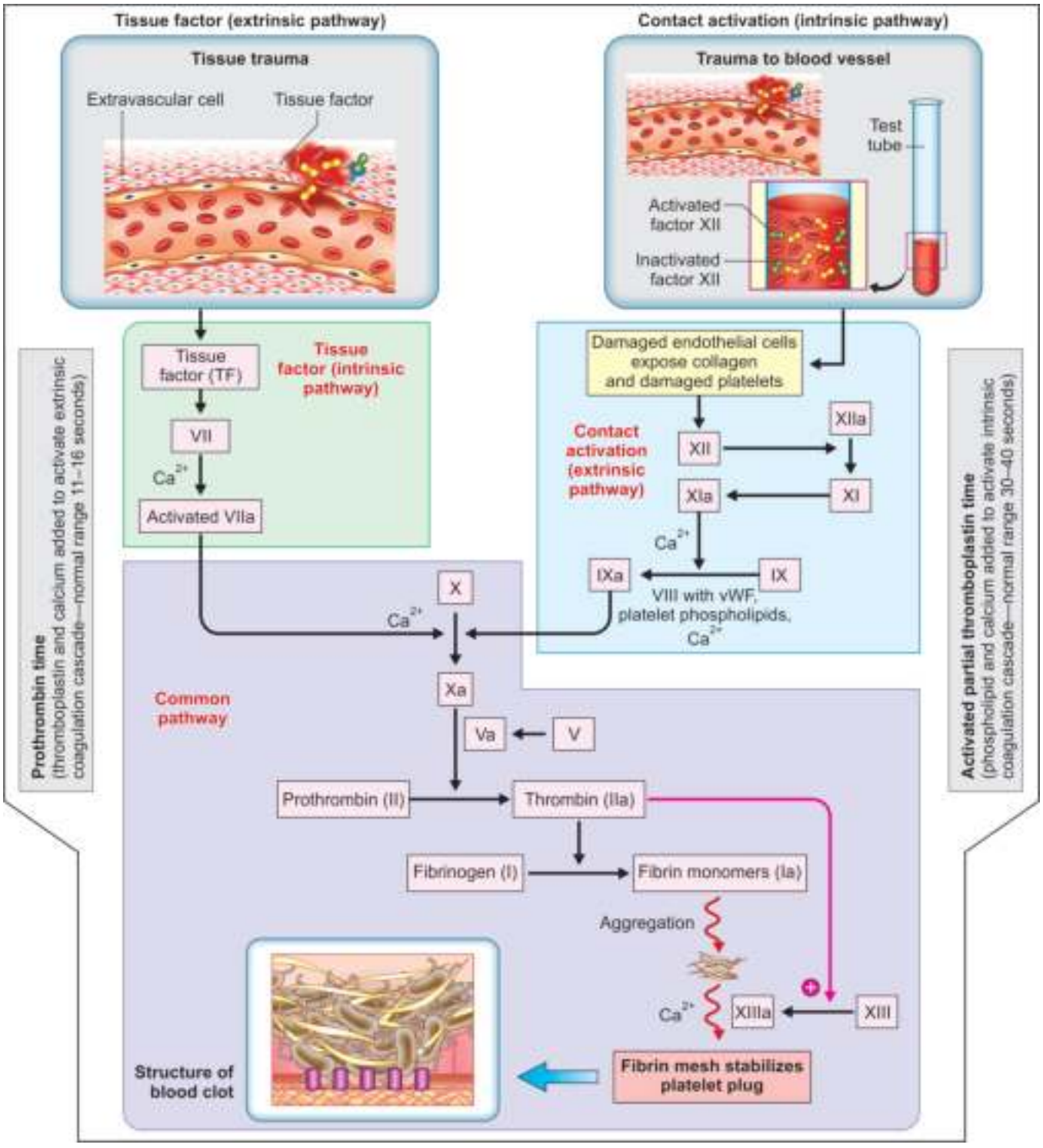


Fig. 3.15: Coagulation system pathways. Coagulation system consists of extrinsic, intrinsic and common pathways, that interact together to form a stable blood clot. Both extrinsic and intrinsic pathways converge to common pathway by independently activating factor X. Extrinsic pathway involves interaction by factor III (i.e. tissue factor) and its interaction with factor VII, whereas factors XII, XI, IX and VIII are utilized in the intrinsic pathway. Then the common pathway uses factors X, V, II, I and XIII leading to formation of a protein web made up of polymerized cross-linked fibrin.

a prothrombinase complex that cleaves the prothrombin into thrombin.

- **Intrinsic pathway of blood coagulation system:** With production of thrombin, factor XI is converted to activated factor XI (factor XIa). Activated factor XI (factor XIa) binds to activated factor VII and tissue factor, and converts factor IX to activated factor IX (factor IXa). Factor IXa activates factor X. Activated factor X (factor Xa) converts factor VIII to activated factor VIII (VIIIa). Factor VIIIa activates

factor X. Activated factor X (factor Xa) binds with activated factor V (factor Va) and converts prothrombin to thrombin. Thrombin acts a cofactor and catalyzes and enhances bioactivity of many of the aforesaid proteolytic pathways.

- **Fibrin clot formation:** Both extrinsic and intrinsic pathways of coagulation cascade converge and convert fibrinogen to fibrin monomers, which polymerizes and forms fibrin polymer mesh leading to cross-linked fibrin clot. This reaction is catalyzed by activated factor XIII (factor XIIIa) that stimulates the lysine and glutamic acid side chains causing cross-linking of the fibrin molecules and formation of stabilized fibrin clot.
- **Blood clot dissolution (tertiary hemostasis):** Activated platelets contract their internal actin and myosin fibrils in their cytoskeleton leading to shrinkage of the blood clot volume. Plasminogen then activates to plasmin, which promotes dissolution of the fibrin blood clot; and restores the blood flow in the blood vessels.

Coagulation Factors

Liver is actively involved in synthesis of coagulation factors, which include factors I, II, V, VII, VIII, IX, X, XI, XIII, and protein C and protein S. Ionized calcium (Ca^{++}) is present in the blood, that is also derived from intracellular source. Phospholipid component is present on surface of the cells including platelets, which provide a surface upon which the chemical reactions of coagulation system can take place.

Thrombin

Thrombin is formed from its precursor prothrombin following tissue injury. Thrombin converts fibrinogen to fibrin in the final stage of blood coagulation cascade. Thrombin is multifunctional serine protease with both procoagulant and anticoagulant properties. Thrombin plays a central regulator of hemostasis and thrombosis.

- Thrombin having procoagulant properties participates in activation of fibrinogen to fibrin, activation of factors V and VIII and platelets.
- Thrombin having anticoagulant properties participates in activation of protein C via thrombomodulin and activation of thrombin activatable fibrinolysis inhibitor (TAFI).
- Thrombin activates platelets causing shape change, generation of thromboxane A_2, ADP release and ultimately platelet aggregation.
- Thrombin also activates the cofactors of coagulation factors V, VIII and XIII. Thrombin bound to thrombomodulin activates protein C. In addition to its procoagulant and anticoagulant activities, thrombin also plays key role in cell growth, activation migration.
- Thrombin plays key role in endothelial cell contraction and acquisition of prothrombin properties, and promotion of cell division.

Tissue Factor

Tissue factor is an integral transmembrane protein found on the surface of vascular cells and many avascular tissues.

- Inflammatory cytokines or endotoxin can upregulate tissue factor on vascular endothelium and monocytes. After blood vessel injury, tissue factor (TF) plays a central role in hemostasis and thrombosis.
- Tissue factor binds to factor VII via calcium ions. Tissue factor (thromboplastin)–factor VIIa complex activates extrinsic pathway of coagulation cascade, which results in fibrin deposition and activation of platelets.
- Tissue factor acts as a cofactor enhancing the proteolytic activity of factor VIIa towards factors IX and factor X.

Factor V

Factor V is plasma procoagulant glycoprotein, that circulates in inactive form. Plasma factor V is predominantly synthesized in the liver. Platelet factor V is synthesized in the megakaryocytes and stored in α-granules of platelets. Factor V interacts with other coagulation proteins including factor X and prothrombin to facilitate the conversion of prothrombin to thrombin to many folds. Factor V is inactivated by activated protein C and its cofactor protein C. Plasma factor V has half-life of approximately 12 hours.

Factor VII

Factor VII is a vitamin K-dependent plasma glycoprotein and serine protease synthesized in the liver. Its main role is to initiate the blood coagulation cascade in conjunction with tissue factor to generate enzyme complex that activates factors X and IX. Activated factor VII has no catalytic activity until bound to tissue factor. Factor VII has half-life of 4–6 hours.

Factor VIII

Factor VIII (antihemophilic factor) is a glycoprotein synthesized in the liver. It is one of the longest and least stable coagulation factors with complex polypeptide composition. Factor VIII circulates in the plasma in a noncovalent complex with von Willebrand factor (vWF), which functions to protect factor VIII from premature proteolytic degradation and concentrate factor VIII at the sites of vascular injury. Factor VIII functions as a cofactor in the blood coagulation cascade for the proteolytic activation of factor X by factor IX, facilitate the conversion of factor X to factor Xa by 200,000-fold. Inactivation of factor VIII also occurs via activated

protein C and its cofactor protein C. Factor VIII has half-life of about 12 hours.

Factor IX

Factor IX (Christmas factor) is a vitamin K-dependent plasma glycoprotein and serine protease, which cleaves factor X in blood coagulation cascade leading to conversion of fibrinogen to fibrin. Calcium binding properties of factor IX are crucial to its normal function and biological activity. Factor VIII together with factor IX proceed to activate factor X. In addition, factor IXa may also activate factor VII. Factor IX has a half-life of 24 hours.

Factor X

Factor X (Stuart-Prower factor) is a vitamin K-dependent plasma glycoprotein and serine protease synthesized in the liver. It plays a central role in the blood coagulation cascade at the point of convergence of the intrinsic and extrinsic pathways of coagulation system. Calcium binding properties of factor X are crucial to its normal function and biological activity. Activated factor X, in conjunction forms a complex on phospholipid surfaces with factor V to form the prothrombinase complex, which converts prothrombin to thrombin. Factor X is inhibited by antithrombin and α_2-macroglobulin. Factor X has half-life of about 36 hours.

Factor XI

Factor XI is a plasma glycoprotein and serine protease, that circulates bound to kininogen. Factor XI is cleaved to active factor XIa by activated factor XIa in the presence of kininogen. Factor XIa activates factor IX in the presence of calcium. Both factor XI and factor XIa bind to platelets. Factor XI has a half-life of about 72 hours.

Factor XII

Factor XII (Hageman factor) is plasma glycoprotein and serine protease synthesized in the liver that circulates in blood as a zymogen. Factor XII may undergo auto-activation to factor XIIa after binding to negatively charged surfaces. Factor XIIa activates prekallikrein to kallikrein that in turn further autoactivates factor XII. Factor XIIa can activate factor XI to promote downstream activation of the blood coagulation cascade.

Factor XIII

Factor XIII (fibrin stabilizing factor) is a **transglutaminase** that forms γ-glutamyl-lysyl amide cross-linking of fibrin, stabilizing the insoluble blood clot against shear stress. As a result, it achieves further protection from degradation of clot in the coagulation cascade. Factor XIII also works on α_2-antiplasmin to cross-link it to fibrin, as a part of its another antifibrinolytic property.

von Willebrand Factor

von Willebrand factor is a glycoprotein synthesized in both endothelium and megakaryocytes involved in primary hemostasis plug formation. Vascular endothelium secretes vWF into the plasma constitutively, but is stored in Weibel-Palade bodies of **α**-granules of platelets. The vWF functions as **carrier protein** for coagulation factor VIII. The vWF functions as adhesive protein involved in vascular endothelial–platelet interaction, via platelet membrane glycoprotein Ib and IIb/IIIa complex. Its function as a adhesive protein is important in setting of high shear stress.

Coagulation System Cascade

Coagulation cascade refers to series of steps that occur during formation of a blood clot after vascular injury by activating clotting factors. There are three pathways of coagulation cascade, which include extrinsic, intrinsic and common pathways. Factor Xa proceeds and forms a link between both extrinsic and intrinsic pathways. Prothrombin time and international normalized ratio (PT–INR) assesses the extrinsic and common pathways of coagulation. Activated partial thromboplastin time (APTT) analyzes factors XII, IX, X, VII, V, II or prothrombin, kininogen and prekallikrein.

Extrinsic Pathway of Coagulation System

Binding of tissue thromboplastin and calcium ions activates factor VII, which further activates factors X and IX via proteolysis. Activated factor IX (IXa) binds with its cofactor-activated factor VIII (factor VIIIa), which results in activation of factor X (factor Xa).

- Activated factor X (factor Xa) binds to its cofactor-activated factor V (factor Va) and calcium and generates a prothrombinase complex that cleaves the prothrombin into thrombin.
- Thrombin first splits off fibrinopeptides A and B from fibrinogen, and then form fibrin monomers, which undergo spontaneous polymerization by hydrogen bonding to form fibrin network. It precedes stabilization of fibrin network by factor XIII (fibrin stabilizing factor) resulting in cross-linking of fibrin network. This product is insoluble and resistant to digestion by plasmin.
- Absence of clotting factors VII, X, V, II, or fibrinogen or presence of inhibitors interfere with conversion of fibrinogen to fibrin, which leads to abnormal prothrombin time. Similarly, abnormalities of fibrinogen and inhibitors of the conversion of fibrinogen to fibrin result in an abnormal thrombin time.

Intrinsic Pathway of Coagulation System

With production of thrombin, factor XI is converted to activated factor XI (factor XIa). Activated factor XI

(factor XIa) binds with activated factor VII and tissue factor, and converts factor IX to activated factor IX (factor IXa), which activates factor X (factor Xa). Platelets provide phospholipid in this process.

- Factor Xa proceeds and forms a link between both extrinsic and intrinsic pathways. Factor Xa binds with factor V and prothrombin. Factor Xa cleaves prothrombin to form thrombin. Thrombin converts fibrinogen into fibrin, which is stabilized by fibrin-stabilizing factor XIII.
- Activated partial thromboplastin time (APTT) analyzes factors XII, IX, X, VII, V, II or prothrombin, kininogen and prekallikrein. Factor XII, kininogen and prekallikrein are essential for normal clotting in the test tube. Activation of factor XII begins, when it is exposed to glass surface in presence of kininogen and prekallikrein. In human, many substances such as collagen, fatty acids, or joint cartilage can activate factor XII (factor XIIa).
- Abnormalities in any one of the following coagulation factors produce an abnormal activated partial thromboplastin time (APTT) such as factors XII, IX, X, VII, V, II or prothrombin, kininogen and prekallikrein. Persons deficient in kininogen and prekallikrein have no significant bleeding disorders.

Stabilized Fibrin Clot Formation

Both extrinsic and intrinsic pathways of coagulation cascade converge and convert fibrinogen to fibrin monomers, which polymerize and form fibrin polymer mesh resulting in cross-linked fibrin clot. This reaction is catalyzed by activated factor XIII (factor XIIIa) that stimulates the lysine and glutamic acid side chains causing cross-linking of the fibrin molecules and formation of stabilized clot.

Anticoagulant Pathways

Natural, physiologic anticoagulants fall into two broad categories: (a) serine protease inhibitors and (b) protein C system that neutralizes specific activated coagulation factors. These anticoagulant systems have physiologic significance. These are active from the initiation of coagulation process until fibrin deposition has occurred.

Serine Protease Inhibitors (SERPINs)

SERPINs are natural **ser**ine **p**roteases **in**hibitor**s**. Serine proteases are controlled by serpins. Serine proteases drive important physiologic processes such as blood coagulation, fibrinolysis inflammation and angiogenesis. Serine protease inhibitors include antithrombin III, heparan cofactor II, protein C inhibitor, plasminogen inactivators and α_2-antiplasmin.

- Out of these, antithrombin III is most important inhibitor of coagulation that inhibits all the coagulation serine proteases (II, VII, IX, X, XI and XII).
- Antithrombin III antagonizes thrombin activity. Heparin-like molecules synthesized by endothelium activates antithrombin III. Hence in clinical practice, heparin is administered to minimize thrombosis.
- Antithrombin III (ATIII) that has anti-Xa activity. ATIII activity/inhibition is enhanced in the presence of heparin and other sulfated glycosaminoglycans.
- Heparin is not normally present in circulation and normally, antithrombin III binds to heparin sulfate on the vascular endothelial cells.

Anticoagulant Protein C System

Protein C is a vitamin K-dependent natural anticoagulant synthesized in the liver and present in circulation. Protein C is the key enzyme that inactivates both coagulation factors Va (cofactor in the activation factor in the prothrombin) and VIIIa (cofactor in the activation factor X). Protein C is activated on the surface of vascular endothelium by thrombin-thrombomodulin endothelial protein C receptor (EPCR). Thrombin thrombomodulin complex is a potent activator of protein C.

Anticoagulant Protein S System

Protein S is a vitamin K-dependent natural anticoagulant present in circulation. It is synthesized in the liver, endothelial cells and megakaryocytes. About 60% of protein S is complexed to C4b binding protein. Only unbound or free protein S is physiologically active. Protein S serves as a cofactor for protein C and enhances its activity of protein C resulting in inhibition of factors V and VIII. Protein S does not require activation.

Fibrinolytic System

The fibrinolytic system is a highly regulated enzymatic process that functions to remove the fibrin clot after the vasculature is repaired, as well as to degrade fibrin clots that form in the blood circulation. The final step in fibrinolytic pathway is the plasminogen-plasmin-mediated cleavage of fibrin creating fibrin degradation products (FDPs).

- Plasminogen is normally present in blood circulation in an inactive form. Plasminogen is activated by tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA).
- Urokinase-type plasminogen activator (uPA) is a serine protease that binds to its receptor (uPAR) and catalyzes the conversion of plasminogen into plasmin and activates signaling pathways leading to promote cell migration, proliferation, and survival.
- Tissue-type plasminogen activator (tPA) is synthesized by vascular endothelium in response to thrombin, shear stress and venous occlusion. Tissue-type plasminogen activator is considered the major intravascular activator of plasminogen.

- Tissue-type plasminogen activator converts plasminogen (inactive form) to plasmin (active form) that binds to specific receptors on fibrin strands network and begins degrading the fibrin strands resulting in dissolution of blood clot. Plasmin also degrades fibrinogen, factor V, factor VIII, prothrombin, and factor XII.
- Circulating plasmin is rapidly inactivated by plasmin inhibitor, which limits the fibrinolytic process in the blood clot and prevents it from occurring in the entire circulation.
- Many tissue-type plasminogen activators (alteplase, reteplase, tenecteplase), prepared by recombinant DNA technology, are now available for use in treatment of acute myocardial infarction, acute ischemic stroke, and pulmonary embolism.

HEMOSTASIS: STAGES

The mechanism of hemostasis can be divided into various stages: vascular constriction, formation of a temporary 'platelet plug', activation of the coagulation cascade, thrombin generation and formation of 'fibrin plug'.

- Binding of thrombin to platelet surface receptors along with adenosine diphosphate (ADP) and thromboxane A_2 cause further platelet aggregation. ADP produces conformational changes of the platelets surface GpIIb–IIIa receptors, to which noncleaved fibrinogen binds. Fibrinogen bridges by binding to aggregated platelets together resulting in formation of secondary hemostatic plug. The platelet hemostatic plug is stabilized by polymerized fibrin mesh.
- Hemostasis facilitates formation of blood clot with platelets and fibrin polymer. The blood clot seals the injured blood vessels in the area and prevents further bleeding so that tissue regeneration process takes place. Once the tissue injury starts to heal, the platelets and fibrin polymer plug remodel and get dissolved resulting in restoration of normal tissue at the site of the tissue damage.

PRIMARY HEMOSTASIS (PLATELET HEMOSTATIC PLUG FORMATION)

Hemostasis is a stepwise process for stoppage of bleeding by sequentially vasoconstriction of the blood vessel, formation of a primary hemostatic platelet plug and development of a blood clot.

- Blood clotting process requires the presence of platelets produced by bone marrow, von Willebrand factor generated by the vascular endothelium, and clotting factors synthesized in the liver, using vitamin K.
- Within seconds of vascular injury, von Willebrand factor (vWF) is released by endothelium that binds to platelet receptors. This phenomenon is responsible for adhesion of platelets to the exposed subendothelial collagen fibers.
- Platelets undergo morphologic changes (disc shape to spherical shape) and activation leading to release of adenosine diphosphate (ADP), thromboxane A_2 (TXA_2), serotonin, and epinephrine. ADP and TXA_2 attract additional platelets leading to platelet aggregation. Platelet factor 3, a surface phospholipid accelerates clotting by formation of thrombin.
- Blood clot retraction and its dissolution also play significant role in hemostasis. These processes involve the interaction of substrates, enzymes, protein cofactors released from platelets and vascular endothelium, and calcium ions in the blood.

Transient Vasoconstriction of Microvasculature

Following vascular endothelial injury, there is immediate transient vasoconstriction of adjacent microvasculature via neural stimulation reflex and endothelin chemical mediator released from damaged vascular endothelial cells and release of thromboxane A_2 by platelets. This reduces the blood flow into the damaged area that allows contact activation of platelets. Neural reflex and thromboxane A_2-mediated vasoconstriction in an injured blood vessel is given in Fig. 3.16.

- Vasoconstriction effect is temporary and needs to be supplemented by aggregation of platelets and blood coagulation factors to maintain permanent hemostasis of the injured blood vessels.

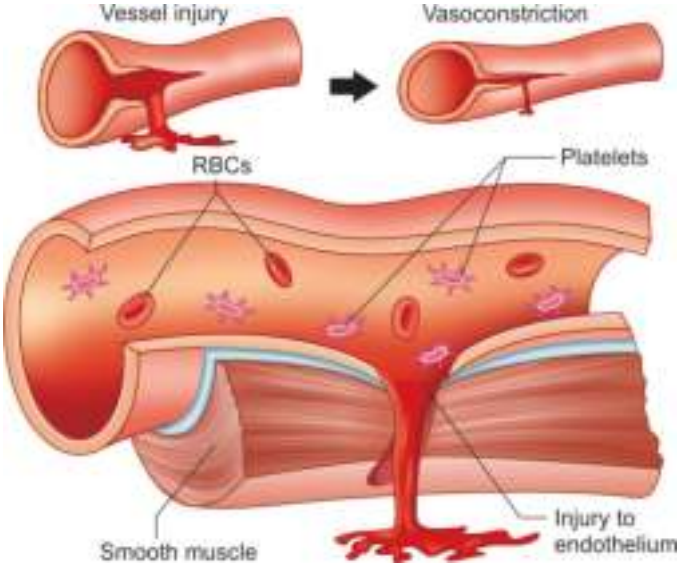


Fig. 3.16: Neural reflex and thromboxane A_2-mediated vasoconstriction in an injured blood vessel. Vasoconstriction of blood vessel occurs due to neural reflex and release of thromboxane A_2 by platelets resulting in reduction of blood flow.

- Patient with genetic deficiency of von Willebrand factor (vWF) or platelet-surface glycoprotein receptors (GpIb) results in defective platelet adhesion and bleeding tendencies, known as **Bernard-Soulier syndrome**.

Platelet Adhesion to Exposed Subendothelial Collagen

Within seconds of tissue injury, endothelial injury exposes subendothelial collagen or noncollagenous microfibrils resulting in platelets adhesion to the subendothelial surface. The von Willebrand factor (vWF) released from Weibel-Palade bodies of the endothelial cells and α-granules of platelets, binds to the platelet-surface GpIb glycoprotein receptors resulting in platelets adhesion to the exposed subendothelial collagen fibers of damaged blood vessels.

Platelets Release Reaction (Secretion)

Subendothelial collagen exposure and local thrombin generation results in release of platelet α-granule contents such as adenosine diphosphate (ADP), serotonin, calcium (Ca^{++}) and fibrinogen, further enhances platelet activation, chemotaxis of additional circulating platelets, platelet aggregation and platelet plug formation.

- Activated platelets stick to each other via bridges formed by the binding of fibrinogen, fibrin or vWF to activated aIIbβ3 integrin.
- The platelet hemostatic plug is extended as additional platelets are activated via the release (secretion) of thromboxane A_2 (TXA_2) generated from arachidonic acid through cyclooxygenase pathway.

Platelet Agonists

Agonist is a substance, which initiates a physiologic response when combined with a receptor. Agonists promoting platelet aggregation, include ADP, thromboxane A_2, epinephrine, collagen, platelet activating factor (PAF) derived from mast cells and basophils. Platelet-derived agonists and their receptors are given in Table 3.10.

- Platelet agonist thromboxane A_2 causes vasoconstriction and promotes platelet aggregation by activating guanyl cyclase, which increases intracellular levels of cGMP. The inhibition of cyclooxygenase pathway by low-dose aspirin is the basis of aspirin therapy for prevention of thrombotic disease.
- Platelet agonists bind to **P2Y12 receptor** (G protein-coupled receptor) on the platelet surface induce GpIIb/GpIIIa expression at platelet surface and induction of signal and conformational and morphologic changes of platelet from a disc shape to a spherical shape.
- Conformational and morphological changes are followed by constriction of the microtubules and releasing contents of the granules (primarily ADP, catecholamine, serotonin and PDGF) into the open canalicular system.
- Pseudopodia develop on the surface of platelet, which contain a network of actin and myosin. The microtubule circumferentially contracts resulting in activation of phospholipids and glycoprotein IIb/IIIa receptors. Internal biochemical changes occur in the platelets resulting in granule secretion.

Table 3.10 Platelet-derived agonists and their receptors

Platelet-derived Agonist	Agonist Receptors
Adenosine diphosphate (ADP)	▪ P2Y1 receptor ▪ P2Y2 receptor
Serotonin	5-HT2 receptor
Platelet activating factor (PAF)	PAF receptor
Thromboxane A_2	TP receptor

Agonist is a substance, which initiates a physiological response when combined with a receptor.

Platelet Aggregation Inhibitors

Prostacyclin (PGI_2) synthesized in the vascular endothelium is a potent vasodilator, which prevents platelet aggregation. TXA_2 causes vasoconstriction and promotes platelet aggregation. Both prostacyclin and TXA_2 perform balanced function for modulating platelet function. In hemostasis, fibrinolysis and fibrin degradation products (FDPs), compete with fibrinogen for binding to platelet membrane and interfere with platelet aggregation. Fibrin degradation products attract leukocytes (neutrophils and monocytes).

Pathology Pearls: Platelet Activation

- Platelet activation *in vivo* can be part of the hemostatic response to vascular injury or a pathologic response to drugs or disease.
- Collagen, thrombin, and ADP are critical platelet agonists, causing granule exocytosis, activation of the aIIbβ3 integrin, and platelet aggregation.
- Once platelet aggregation begins, the close proximity of the adjacent platelets allows contact-dependent and contact facilitated interactions that promote growth and stability of the platelet mass.
- Achieving a platelet hemostatic plug that is large enough to be stable but small enough to avoid vascular occlusion is the result of tight regulation of initial intracellular signaling events and the presence of molecules on the platelet surface that help limit the extent of platelet activation.
- Platelet receptors and signaling events have provided targets for the development of antiplatelet agents, which are in widespread clinical use. It is reasonable to expect that new discoveries in the basic science of platelets will lead to the identification of new targets and new approaches to the manipulation of platelet behavior *in vivo*.

Hematology Pearls: Platelet Plug Formation (Primary Hemostasis)

Resting platelets maintain low levels of cytoplasmic calcium via uptake by dense tubular system (DTH) and active extrusion, probably via a calcium pump. These mechanisms oppose a passive diffusion of intracellular calcium. With platelet activation, platelets increase intracellular cytoplasmic calcium due to release of calcium from the dense tubular system and increase in calcium influx. Formation of primary platelet plug is shown in Figs 3.17 and 3.18.

- Platelet agonists such as adenosine diphosphate (ADP) and thromboxane A_2 released from platelets, play important role in platelet aggregation and initial hemostatic platelet plug formation, that stops bleeding.
- Platelet agonist in the presence of calcium stimulates high-affinity fibrinogen receptors to bind activated glycoprotein IIb/IIIa on the surface of platelet and linked platelets. Fibrinogen binds horizontally to two platelets by peptide sequences at the terminal end of its γ-chain and α-chain in the D domains, one γ-chain to GpIIb receptors of each platelet.
- Fibrinogen, thus becomes a bridge between the two platelets. Initial platelet hemostatic plug is reversible, unstable and easily dislodged, when ADP release is minimal. It is worth mentioning that PGI_2 and nitric oxide (NO) released from vascular endothelial cells inhibit platelet aggregation.
- Leukocytes and RBCs are also present in hemostatic plug. Leukocytes adhere to vascular endothelium and produce inflammatory response.

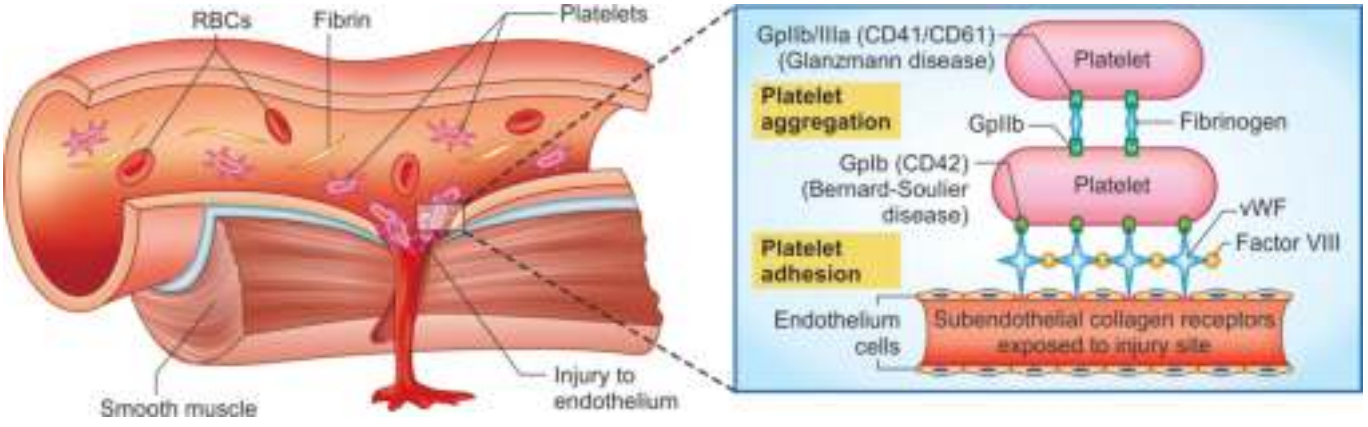


Fig. 3.17: Formation of primary platelet plug. Platelets adhere to be exposed collagen fibers due to binding of von Willebrand factor to platelet receptors. Activated platelets release ADP and TXA_2 resulting in attraction of additional platelets and aggregation.

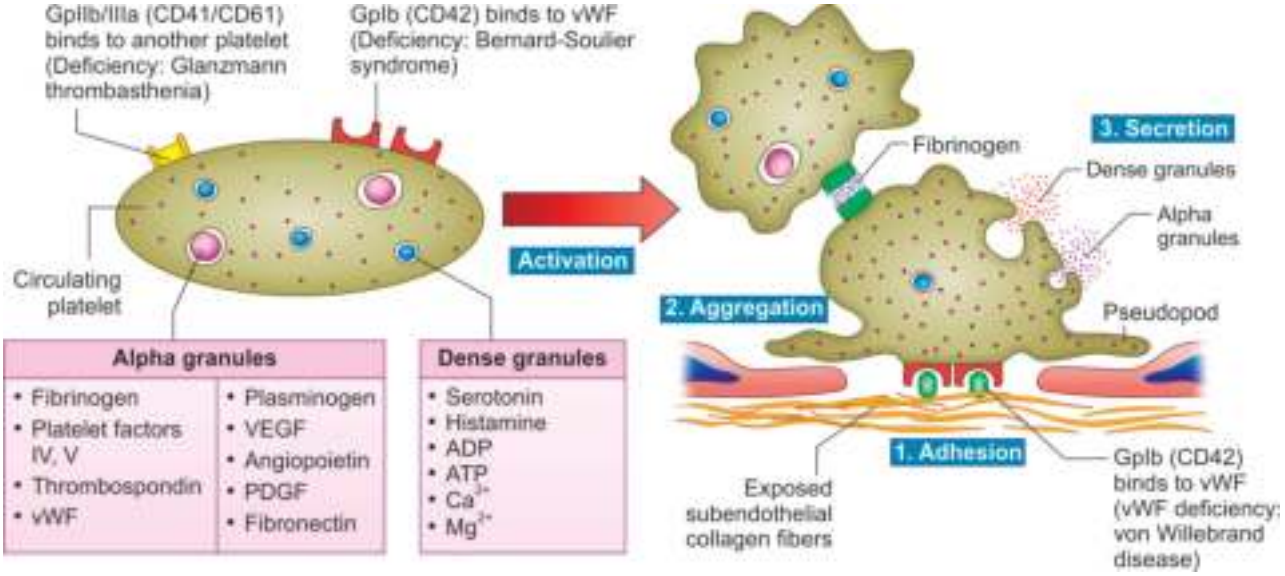


Fig. 3.18: Formation of platelet hemostatic plug. Platelet activation involves three overlapping mechanisms. (a) Adhesion of platelets to the exposed subendothelium is mediated by the binding of von Willebrand factor (vWF) to glycoprotein, i.e. GpIb/IX (CD42) resulting in initiation of signal for activation of platelets, (b) Exposure of GpIIb/IIIa (CD41/61) to the fibrinogen receptor on the platelet surface permits for platelet aggregation, (c) At the same time, platelets synthesize their granule contents, which facilitate further activation.

SECONDARY HEMOSTASIS (STABLE HEMOSTATIC PLUG FORMATION)

Finally, close contact between platelets in the growing platelet plug, along with a fibrin meshwork help to perpetuate and stabilize the platelet plug. Formation of stable hemostatic plug is shown in Figs 3.19 and 3.20.

- Coagulation system cascade results in formation of insoluble polymerized fibrin mesh. Secondary hemostasis refers to the coagulation system cascade of enzymatic reactions that ultimately leads to the conversion of fibrinogen to fibrin monomers. Fibrin monomers are then cross-linked into insoluble polymerized fibrin strands that serve to stabilize the loose platelet hemostatic plug formed in primary hemostasis. Vascular injury activates intrinsic and extrinsic pathways of coagulation system. Intrinsic pathway of coagulation begins in blood circulation by activation of circulating factor XII. Extrinsic pathway of coagulation system is activated by cellular lipoprotein known as tissue factor including platelet phospholipids.

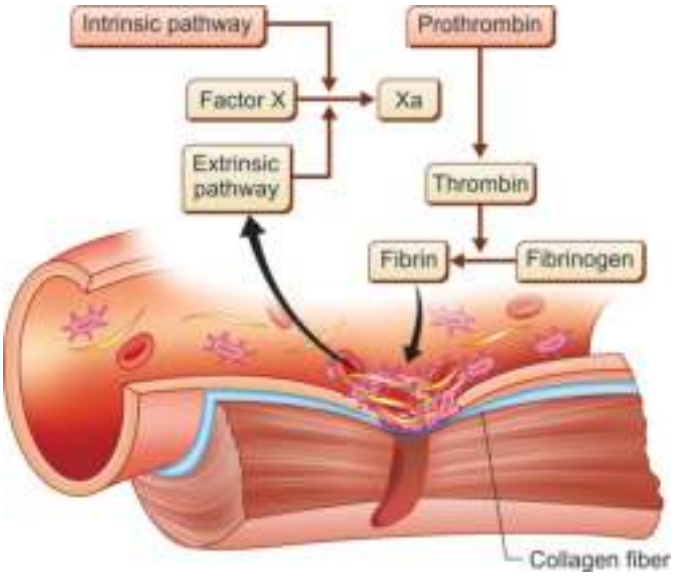


Fig. 3.19: Formation of stable hemostatic fibrin plug. Blood coagulation occurs by intrinsic and extrinsic pathways as a result of vascular injury. Both these coagulation pathways lead to activation of factor X, conversion of prothrombin to thrombin. Thrombin converts fibrinogen to fibrin. Fibrin stabilizing factor stabilizes fibrin leading to holding of clot by insoluble fibrin strands.

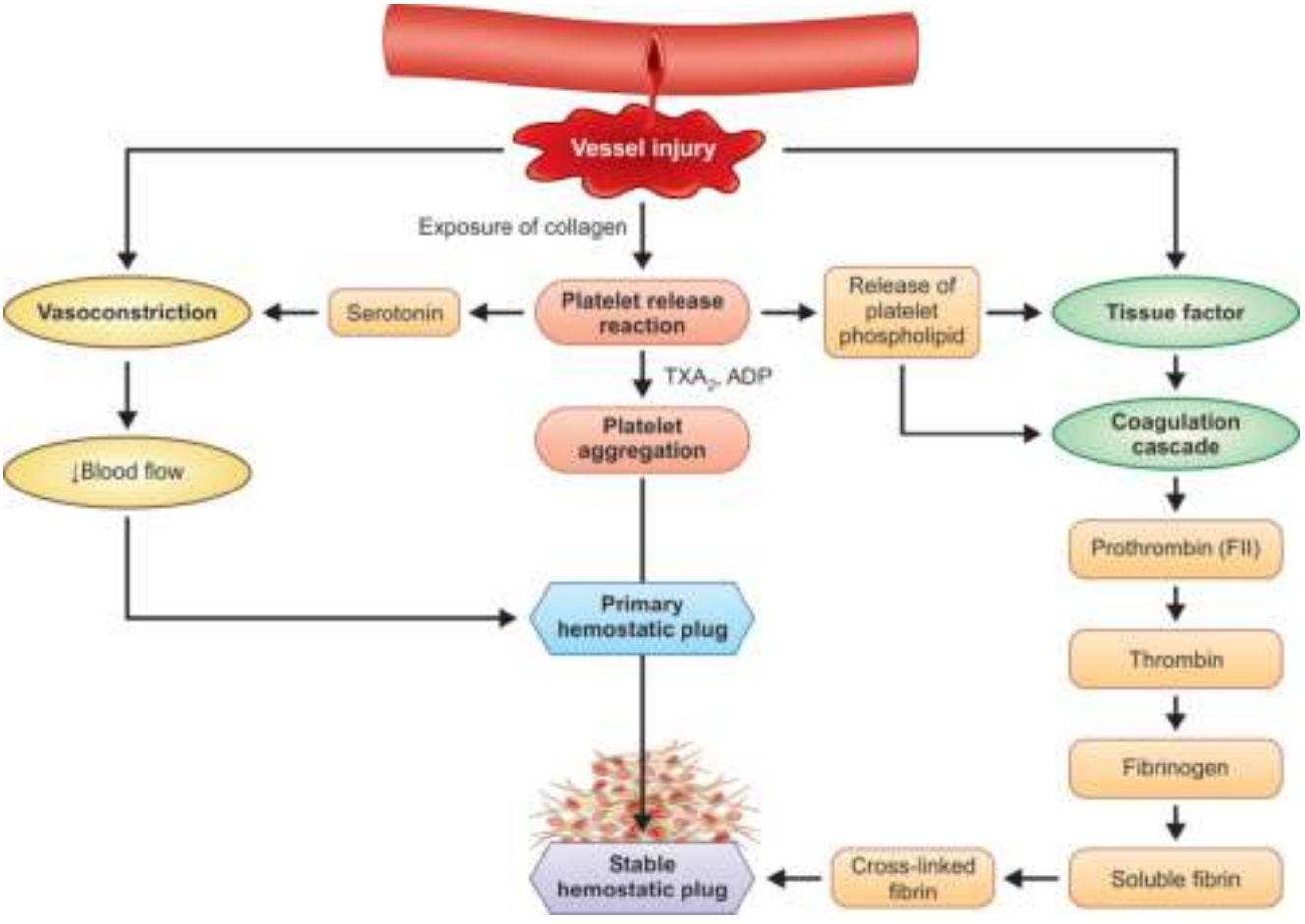


Fig. 3.20: Formation of stable hemostatic fibrin plug. During primary hemostasis, after vasoconstriction, platelets adhere and aggregate to form a platelet hemostatic plug around the site of injury. Then in the secondary hemostasis, platelet hemostatic is reinforced by a protein mesh made up of polymerized cross-linked fibrin.

- Calcium, factor X, factor V, platelet phospholipids combine to form prothrombin activator, which then converts prothrombin to thrombin. The terminal steps in both intrinsic and extrinsic pathways of coagulation system are same.
- Thrombin converts fibrinogen to fibrin strands stabilized by fibrin stabilizing factor (factor XIII) that create the secondary hemostatic stable plug of insoluble polymerized fibrin strands, blood clot, with platelets and red blood cells resulting in stoppage of bleeding.
- Binding of thrombin to platelet surface receptors along with adenosine diphosphate (ADP) and thromboxane A_2 (TXA_2) cause further platelet aggregation resulting in formation of stable hemostatic plug.
  - ADP produces conformational changes of the platelets surface GpIIb–IIIa receptors, to which noncleaved fibrinogen binds.
  - Cross-linking of fibrin by fibrin stabilizing factor XIII holds the blood clot and results in activation of platelets and fibrinolytic pathways. Plasmin dissolves fibrin strands in the course of wound healing.
  - Additionally, thrombin interacts with nonhemostatic systems to promote cellular chemotaxis, fibroblast growth factor and wound healing and tissue repair.
- Vascular endothelium also undergoes a series of changes moving from its resting phase with predominantly anticoagulant properties to a more active procoagulant properties.

BLOOD CLOT RETRACTION

Blood clot retraction occurs within 20–60 minutes after a blood clot is formed, which contributes to hemostasis. Activated platelets contract their internal actin and myosin fibrils in their cytoskeleton leading to shrinkage of the blood clot volume. Actin and myosin in the cytoskeleton of activated platelets that are trapped in the blood clot, contract and pull the blood clot towards platelets. This phenomenon causes squeezing serum from the blood clot leading to shrinkage of blood clot. Blood clot retraction requires large numbers of platelets, and failure of blood clot retraction is indicative of a low-platelet count. Blood clot retraction is shown in Fig. 3.21.

BLOOD CLOT DISSOLUTION (THROMBOLYSIS)

Blood clot dissolution (thrombolysis) begins immediately after blood clot formation. This allows blood flow to be reestablished and permanent blood vessel repair to take place. The process by which a blood clot dissolves is known as **fibrinolysis**. Blood clot dissolution requires a sequence of steps controlled by plasminogen activators and inhibitors. Blood clot dissolution or lysis is shown in Fig. 3.22.

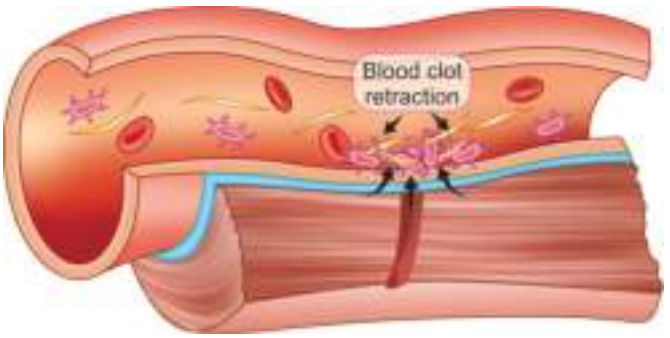


Fig. 3.21: Blood clot retraction. Within a few minutes after a blood clot is formed at injured site, actin and myosin in the platelets that are trapped in the blood clot begins to contract. As a result, fibrin strands of the blood clot are pulled towards the platelets, thereby squeezing serum (plasma without fibrinogen) from the blood clot and causing it to shrink to become smaller.

- Plasminogen activators such as tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) circulate in the plasma as a reversible complex with plasminogen activator inhibitor 1 (PAI-1).
  - When the fibrin clot is formed, tPA and uPA bind to the fibrin clot and convert plasminogen to plasmin.
  - Then elastase, cathepsin G and uPA derived from neutrophils, monocytes/macrophages, and high shear stress including plasmin result in lysis of the cross-linked fibrin into fibrin degradation products (FDPs).

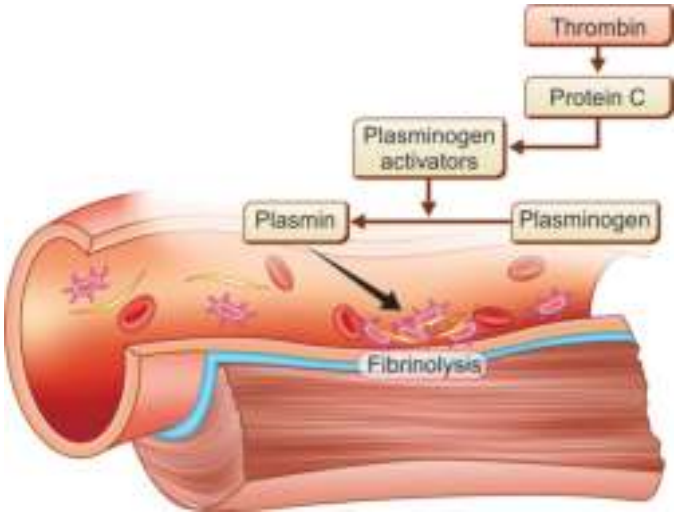


Fig. 3.22: Blood clot dissolution (thrombolysis). Blood clot dissolution begins immediately after a blood clot is formed. This process begins with activation of plasminogen, an inactive precursor of the proteolytic enzyme, and plasmin. After the formation of blood clot, large amounts of plasminogen are trapped in the blood clot. tPA released from injured tissues and vascular endothelium converts plasminogen to plasmin. Plasmin digests the fibrin strands, causing the clot to dissolve.

- To binding of plasminogen activator inhibitor 1 (PAI-1) fibrin can irreversibly inhibit plasminogen activators.

- Blood clot dissolution (thrombolysis) is inhibited by release of plasminogen activator inhibitor 1 (PAI-1) by platelets; and elastase and cathepsin G from white blood cells that become trapped on the thrombus, which directly breakdown fibrin; the plasminogen activation by tissue plasminogen activator (tPA) and urinary plasminogen activator (uPA). The thickness and porosity of fibrin fibers also determines structural stability and susceptibility to thrombolysis. Lp(a), a homologue of plasminogen that can inhibit tPA-mediated plasminogen activation.

Clinical Pearls: Recombinant Tissue Plasminogen Activator (tPA) used in Clinical Practice

- Tissue plasminogen activator functions by catalyzing the conversion of plasminogen to plasmin, the primary enzyme involved in dissolving blood clots.
- Recombinant biotechnology has allowed to manufacture tissue plasminogen activator such as alteplase, reteplase and teneplase in the laboratories.
- Recombinant tissue plasminogen activators are administered in ischemic cerebral stroke within 3 hours of onset, and myocardial infarction. If there is delay of more than 1 to 2 hours before percutaneous transluminal coronary angioplasty, severe pulmonary thromboembolism causing severe instability due to pressure in heart, and deep vein thrombosis (DVT).

HEMORRHAGIC DISORDERS

Spontaneous bleeding can be caused by primary or secondary structural defects in the blood vessel wall, platelet disorders, coagulation disorders. Three most common hereditary bleeding disorders are hemophilia A (factor VIII) deficiency, hemophilia B (factor IX deficiency) and von Willebrand disease. Causes of inherited and acquired abnormalities of hemostasis are given in Table 3.11.

Table 3.11 Causes of inherited and acquired abnormalities of hemostasis

Inherited Abnormalities of Secondary Hemostasis		
Hemophilia A	▪ X-linked recessive disorder ▪ Decreased or depletion of coagulation factor VIII deficiency ▪ Activated partial thromboplastin time (APTT) prolonged	▪ Severity and risk of bleeding depend on severity of deficiency of factor VIII ▪ About 5–20% of patients develop inhibitors of factor VIII due to treatment
Hemophilia B	▪ X-linked recessive disorder ▪ Decreased or depletion of coagulation factor IX	▪ An activated partial thromboplastin time (APTT) prolonged ▪ About 1–3% of patients develop inhibitors to factor IX due to treatment
Hemophilia C	▪ Decreased or depletion of coagulation factor XI ▪ Autosomal recessive disorder	▪ Common in Ashkenazi Jews ▪ 1:8 Heterozygotes ▪ 1:90 Homozygotes
Afibrinogenemia	▪ Complete absence of fibrinogen–homozygous gene mutation ▪ Prothrombin time and international normalized ratio (PT–INR) prolonged	▪ Activated partial thromboplastin time (APTT) prolonged ▪ Thrombin time (TT) prolonged ▪ Some patients have severe bleeding with minor challenges than hemophiliacs
Hypofibrinogenemia	▪ Patient has fibrinogen level 50% of normal level–heterozygous gene mutation with mild bleeding ▪ Thrombin time (TT) prolonged	▪ Activated partial thromboplastin time (APTT) prolonged ▪ Prothrombin time and international normalized ratio (PT–INR) prolonged
Dysfibrinogenemia—structural abnormality	▪ Structural abnormality of fibrinogen–usually heterozygous state ▪ About 50% of patients have no clinical bleeding, 25% of cases with clinical bleeding; and 25% of cases develop thrombosis	▪ Prothrombin time normal ▪ Activated partial thromboplastin time (APTT) normal ▪ Thrombin time (TT) prolonged ▪ Fibrinogen levels variable
von Willebrand disease (some cases)	Decreased vWF leads to decreased stability and function of factor VIII	

Contd...

Table 3.11 Causes of inherited and acquired abnormalities of hemostasis (*Contd...*)

Acquired Abnormalities of Secondary Hemostasis	
Vitamin K deficiency	Impaired synthesis of coagulation factors II, VII, IX and X; and natural anticoagulant protein C and protein S
Liver disease	Impaired synthesis of coagulation factors I, II, V, VIII, IX, X, XI; and natural anticoagulant protein C, protein S and antithrombin C
Disseminated intravascular coagulation (DIC)	Multiple coagulation factors deficiencies especially fibrinogen depletion; as well as thrombocytopenia
Nephrotic syndrome	Loss of antithrombin III and antiplasmin in urine along with massive proteinuria
Multiple myeloma and amyloidosis	Consumption of coagulation factor X
Direct oral administration of anticoagulants for therapeutic purpose	Oral anticoagulants antagonize thrombin

Table 3.12 Common clinical manifestations of hemorrhagic disorders

Organs	Clinical Manifestations	
Skin, mucosal or serosal surfaces	▪ Petechiae (pin-point sized lesions) ▪ Ecchymoses (bruises)	▪ Purpura (systemic disease characterized by numerous petechiae and ecchymoses)
Gastrointestinal tract	▪ Hematemesis (blood in vomiting) ▪ Hematochezia (fresh blood in stool)	▪ Melena (blackened blood in the stool, caused by exposure of blood to hydrochloric acid in the gastric region)
Respiratory tract	▪ Epistaxis (nose bleeding)	▪ Hemoptysis (expectoration of blood)
Genitourinary tract	▪ Metrorrhagia (excessive uterine bleeding)	▪ Hematuria (blood in the urine)

- Patients suffering from hemorrhagic disorders often present with only mild bleeding due to traumatic injuries or surgical procedures. However, some patients have life-threatening bleeding. Blood can be released from the circulation to the exterior of the body or into surrounding tissues, hollow organs or body cavities. Common clinical manifestations of hemorrhagic disorders are given in Table 3.12.
- Simple screening tests can be performed to find out the cause of the bleeding by taking family history that provides information whether the bleeding tendency is hereditary or acquired. Clinical examination can reveal the type of bleeding. Platelet count can be done to exclude thrombocytopenia. Coagulation tests, such as prothrombin time and international normalized ratio (PT–INR) and activated partial thromboplastin time (APTT) can supplement initial information concerning deficiency states of coagulation disorders. Bleeding time is often prolonged in patients suffering from von Willebrand disease, thrombocytopenia or thrombocytopathy. Further testing of platelets and coagulation factors is required in tertiary hospitals.

DEFECTS IN HEMOSTASIS

Defects in hemostasis lead to susceptibility to bleeding (also known as hemorrhagic diathesis). Bleeding may result from abnormalities in platelets, coagulation factors and blood vessels.

- Bleeding diathesis is classified into disorders of primary hemostasis caused by platelet abnormality, and secondary hemostasis caused by defects in the extrinsic and/or intrinsic and common pathways of coagulation system cascade, and hyperfibrinolysis (when there is increased clot degradation).
- Although clinical features may overlap. Mucocutaneous bleeding (e.g. petechiae, epistaxis and gastrointestinal bleeding) is associated with disorders of primary hemostasis. Bleeding into potential spaces (e.g. hemarthrosis and bleeding in skeletal muscles) is characteristic of disorders of secondary hemostasis.
- Diagnostic workup of a hemorrhagic diathesis begins with a detailed clinical history, family history, clinical examination, complete blood count, and a coagulation panel, which typically allows the bleeding disorder to be classified as one of primary or secondary hemostasis. Specialized studies are performed to determine the specific etiology of disorder so that treatment can be initiated.
- Treatment of hemorrhagic diathesis may include transfusion of blood products and replacement of specific coagulation factors or administration of adjuvant medications (e.g. desmopressin or tranexamic acid).

Pathology Pearls: Clinical Findings in Hemorrhagic Disorders

- **Erythema:** Erythema is an inflammatory redness of the skin.
- **Purpura:** Purpura is a diffuse superficial hemorrhage in the skin up to 1 cm in diameter.
- **Petechial hemorrhages:** Petechial hemorrhages are pinpoint hemorrhages occur in the skin, mucous membranes (conjunctiva), which represent the rupture of capillaries or arterioles and occur in conjunction with coagulopathies or vasculitis. In bacterial endocarditis, microemboli from infected cardiac valves may cause rupture of capillaries and arterioles results in pinpoint hemorrhages under nails known as Janeway's lesions.
- **Ecchymosis:** Ecchymosis is a larger diffuse superficial hemorrhage in the skin and subcutaneous tissue (a 'black and blue' mark). Following hemorrhage, skin shows purple discoloration, turning green and then yellow before resolution. These changes reflect progressive oxidation of bilirubin released from the hemoglobin of degraded erythrocytes. A 'black eye' is a good example of an ecchymosis.
- **Hematoma:** Hematoma is localized extravascular accumulation of clotted blood within a tissue or organ. Hemorrhage into skeletal muscles can be merely painful or fatal, if hematoma is located in the brain.
- **Hemorrhage in body cavities:** Hemorrhage refers to discharge of blood from the vascular compartment into the extravascular body spaces or to the exterior. Hemorrhage may occur in the pleural cavity, pericardial sac, peritoneal cavity or a synovial space. Cardiac tamponade occurs when the pressure in the pericardial sac rises to exceed the filling pressure of the heart.
- **Hemopericardium:** Pericardial fluid may accumulate rapidly, particularly with hemorrhage caused by a ruptured myocardial infarct, dissecting aortic aneurysm due to intimal tear, or trauma. Dissecting aortic aneurysm occurs due to weakening of the aortic media (cystic medial necrosis) or hypertension.
- **Hemarthrosis:** Bleeding into the joint cavity is known as hemarthrosis. It is most often caused by hemophilia. Repeated bleeding in joint cavity may cause deformities and may limit the mobility of the joints.
- **Hematemesis:** Hematemesis is presence of blood in vomitus. Massive hematemesis is a frequent cause of death in patients with esophageal varices in alcoholic cirrhosis, who has portal hypertension. Bleeding occurs due to increased intravascular hydrostatic pressure.
- **Hemoptysis:** Hemoptysis is coughing up blood.
- **Hematochezia:** Hematochezia is passage of bloody stools caused by lower gastrointestinal hemorrhage.
- **Hematobilia:** Hematobilia is bleeding into the biliary passages, a complication of trauma or neoplasia.
- **Hematocephalus:** Hematocephalus is an intracranial infusion of blood.
- **Melena:** Melena (black stool) is a symptom of upper gastrointestinal bleeding. Blood from ruptured esophageal varices or a peptic ulcer is partially digested by hydrochloric acid. Hemoglobin is transformed into a black pigment (hematin), which imparts a typical "coffee-grounds" color to the stool.

DEFECTS OF PRIMARY HEMOSTASIS (PLATELET DEFECTS AND VON WILLEBRAND DISEASE)

Hemostasis refers to the response to vascular injury that produces a platelet clot at the injured site. Primary hemostasis serves to immediately limit bleeding through the formation of a loose primary hemostatic platelet plug.

- Platelets play key role in primary hemostasis. Initial platelet adhesion, activation and aggregation upon tissue injury, stimulates coagulation factors and other potent mediators to achieve hemostasis. Defects in primary hemostasis are generally associated with mucocutaneous bleeding, characterized by epistaxis, ecchymosis, genitourinary bleeding or gingival bleeding. Platelet count and platelet function tests are performed to assess primary hemostasis.
- The von Willebrand factor (vWF) is an adhesive plasma glycoprotein, which performs its primary hemostatic functions through binding to factor VIII, platelets surface glycoproteins, and constituents of connective tissue. The vWF acts as a stabilizer of factor VIII in the blood circulation.
  - Defects in vWF concentration or activity are very common, affecting about 1% of the population. The von Willebrand disease is an inherited bleeding disorder in which the blood does not clot properly.
  - Patient presents with excessive bleeding from tissue injury or after surgery or dental procedure, epistaxis, hematuria, easy bruising, heavy menstrual bleeding and heavy bleeding during delivery and postpartum period.

DEFECTS OF SECONDARY HEMOSTASIS (COAGULATION FACTOR DEFECTS)

Secondary hemostasis is defined as the formation of insoluble, cross-linked fibrin by activated coagulation factors in coagulation system cascade, specifically thrombin. Fibrin stabilizes the primary platelet plug at the injury site, where the primary hemostatic platelet plug is insufficient alone to limit hemorrhage.

- The most common inherited defect of secondary hemostasis is hemophilia A (factor VIII deficiency). Failure to stabilize primary platelet plug results in hemorrhagic diathesis. Patient presents with hemarthrosis soft tissue bleeding, hematomas, or retroperitoneal bleeding in hemophilia A.

- Two screening tests are most often done in the initial assessment of secondary hemostasis. Both prothrombin time and international normalized ratio (PT–INR) and activated partial thromboplastin time (APTT) are performed on plasma separated from whole blood collected in test tube containing sodium citrate anticoagulant.

DEFECTS IN BLOOD VESSELS

Vascular bleeding disorders can result from primary or secondary defects in blood vessels, typically causing petechiae, purpura and bruising except hereditary hemorrhagic telangiectasia, which can cause serious blood loss. Vascular bleeding may occur in the setting of defects in vascular and perivascular collagen in Ehlers-Danlos syndrome, and hereditary connective tissue disorders (Marfan's syndrome, pseudoxanthoma and osteogenesis imperfecta). Vascular bleeding may be a prominent feature of scurvy, immunoglobulin A associated vasculitis during childhood. Tests of hemostasis are usually normal in vascular bleeding disorders.

Atherosclerotic Aneurysm Rupture

Atherosclerosis means hardening (sclerosis) or loss of elasticity of large elastic arteries and medium-sized arteries due to atheromatous plaque formation. Severe atherosclerosis may so weaken the wall of the abdominal aorta that it balloons to form an aneurysm, which may rupture and bleed into the retroperitoneal space. The atherosclerotic aneurysm may be demonstrated by ultrasonography and MRI.

Mycotic Aneurysm Rupture

Mycotic aneurysm occurs in the root of aortic arch and its descending branches, due to direct extension from aortic valve endocarditis. Emboli from infective endocarditis may involve cerebral blood vessels result in hemorrhage into the basal ganglia, extending into the subarachnoid space.

Berry Aneurysm Rupture

The most common site of berry aneurysm formation is between the anterior communicating and the anterior cerebral arteries in the circle of Willis.

- Berry aneurysm develops as saccular lesions (1.0–1.5) at site of congenital defects in smooth muscle of the arterial wall, which occurs in 10–15% of patients with adult polycystic kidney disease.
- Rupture of berry aneurysm causes subarachnoid hemorrhage. Patient presents with history of severe headache, nuchal rigidity from irritation of the meninges.

Aortic Dissection

Aortic dissection can occur due to systemic hypertension, atheromatous plaque, trauma to chest, pregnancy, Marfan's syndrome (missense mutations of the fibrillin gene 1 located on chromosome 15 causing defective cross linkage), Ehlers-Danlos syndrome (defective synthesis of procollagen fibers) and copper deficiency (cofactor in lysyl oxidase participating in collagen fibers). Aortic dissection rupture causes massive bleeding associated with fatal outcome.

Infections and Cancers

Certain infections (e.g. pulmonary tuberculosis) and invasive cancers may erode blood vessels and allow hemorrhage to occur. Malignant tumors that arise in organs (e.g. ovaries, gastrointestinal tract, or lung) adjacent to body cavities may shed cancer stem cells into these spaces, which invariably results in malignant effusion.

Capillary Fragility in Vitamin C Deficiency

Vitamin C deficiency is associated with capillary fragility and bleeding, owing to a defect in the supporting connective tissue structures. Vitamin C is essential for cross linkage of collagen fibrils gives structural stability to tropocollagen. Patient presents with bleeding diathesis (e.g. bleeding into skin and joints) and poor wound healing.

Traumatic Injury to Blood Vessels

Hemorrhage occurs depending on the severity of traumatic injury. Damage to the large blood elastic vessels or heart causes massive hemorrhage associated with fatal outcome. Blunt trauma to capillaries causes ecchymosis.

Bleeding and Coagulation Disorders

Severe decrease in the number of platelets (thrombocytopenia) or a deficiency of a coagulation factor (e.g. factor VIII in hemophilia A) is associated with spontaneous hemorrhage into various parts of the body without apparent injury.

Gastrointestinal Tract Disorders

A person may exsanguinate into an internal cavity, as in gastrointestinal hemorrhage from a peptic ulcer disease (arterial hemorrhage) or esophageal varices (venous hemorrhage). In such cases, fresh blood may fill the entire gastrointestinal tract. Bleeding into a serous cavity can also result in the accumulation of a large amount of blood, even to the point of exsanguination.

THROMBOSIS

Thrombosis is intravascular coagulation of blood, often causing significant interruption of blood flow. A thrombus is blood clot formed from constituents of blood (coagulation factors, platelets, red blood cells) in the circulatory system (blood vessels, cardiac chambers and valvular cusps) during life. Most important factor in thrombus formation is vascular endothelial injury. Alterations of blood flow (turbulence blood flow) and hypercoagulability of blood also play a role in thrombus formation. Size and shape of thrombus depends on its site of origin in veins or an artery. Virchow's triad contributing to thrombosis is shown in Fig. 3.23.

- Thrombus is attached to the site of its formation and remains there, and interrupting blood flow to organs due to partial or complete occlusion of blood vessels.
- Hemostasis is a physiologic phenomenon, whereas thrombosis is usually a pathologic phenomenon that precedes embolism with serious complications. However, embolism may have antecedent causes other than thrombosis.
- Thromboembolism remains a leading cause of mortality and disability. Thrombi may partially or completely obstruct arteries or veins leading to local ischemic complications.
  - Thromboembolic phenomenon can be life-threatening by causing myocardial infarction, cerebrovascular infarction and pulmonary embolism.
  - Embolus is intravascular solid, liquid or gaseous mass carried in the blood from its origin and lodged in another site.
  - Infarct is localized area of ischemic tissue necrosis generally caused by an impaired blood supply.

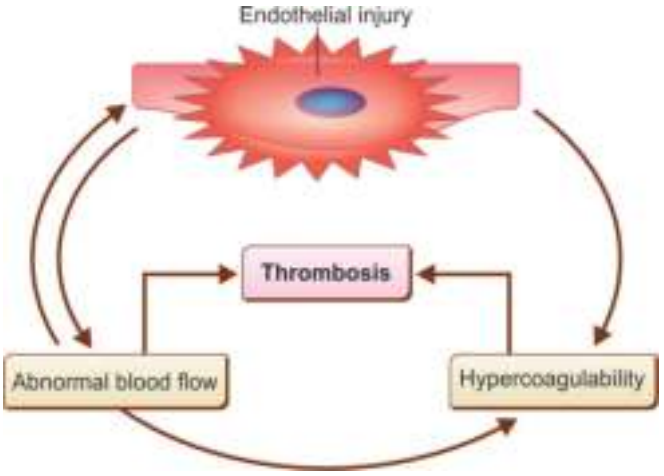


Fig. 3.23: Virchow's triad contributing to thrombosis. Three factors of Virchow's triad include endothelial injury, stasis of blood flow, the presence of hypercoagulable state. It is named after the renowned German physician Rudolf Virchow (1821–1902). Thrombus is a solid mass of blood formed within the cardiovascular system involving the interaction of endothelial cells, platelets and the coagulation cascade.

- A thrombus is the final product of the blood coagulation step in hemostasis, which occurs in cardiovascular system leading to obstruction of blood flow. A blood clot is formation of semi-solid mass in blood vessels that helps control bleeding.

THROMBOGENESIS

Thrombosis occurs when there is a breakdown in the balance between thrombogenic factors and protective antithrombogenic factors. Thrombogenesis results from the interaction of platelets, damaged vascular endothelial cells, and the vascular coagulation system cascade. Intravascular thrombogenesis is influenced by a complex interplay of procoagulant, anticoagulant and fibrinolytic factors.

VASCULAR ENDOTHELIAL INJURY

Vascular endothelial cells line the entire circulatory system, from the heart to the smallest capillaries. Vascular endothelium forms a barrier between platelets and plasma clotting factors and the subendothelial connective tissue. In normal health, vascular endothelium inhibits platelet aggregation and coagulation cascade including protein C and protein S pathway, thus plays a crucial role in providing the proper hemostatic balance.

- Vascular endothelium controls coagulation system by regulating the expression of binding sites for anticoagulant factors (i.e. prostacyclin, ADPase, heparin-like molecules, thrombomodulin) and procoagulant factors (i.e. von Willebrand factor, tissue factor, plasminogen activator inhibitors including platelet adhesion molecules inducing platelet adhesion) on the endothelial cell surface.
- Vascular endothelial damage can be caused by hemodynamic forces, hyperlipidemia, cigarette smoke, immune complex, bacterial toxins, irradiation, direct trauma and various mutagens.
- Vascular endothelial injury favors thrombus formation through disruption of normal laminar flow and coordinated induction of procoagulant and suppression of anticoagulant mechanisms.

Increased Synthesis of Procoagulant Molecules

Vascular endothelial injury upregulates procoagulant molecules (i.e. von Willebrand factor, tissue factor, plasminogen activator inhibitors including platelet adhesion molecules). Increased expression of platelet adhesion molecules enhances adhesion of platelets on injured vascular endothelium. Tissue factor activates coagulation system. Plasminogen activator inhibitors inhibit fibrinolytic system. Tumor necrosis factor

suppresses synthesis of thrombomodulin, vascular endothelial anticoagulant cofactor, and induced the expression of tissue factor, which is a procoagulant cofactor.

Decreased Synthesis of Anticoagulant Molecules

Vascular endothelial injury downregulates anticoagulant molecules such prostacyclin (PGI_2), nitric oxide, adenosine diphosphatase enzyme, heparin-like molecules and thrombomodulin. Prostacyclin and nitric oxide (NO), potent inhibitors of platelets. Loss of prostacyclin and nitric oxide promotes platelets aggregation. Loss of adenosine diphosphatase enzyme is not able to degrade adenosine diphosphate (ADP) that results in platelets aggregation. Loss of heparin-like molecules and thrombomodulin activate coagulation system.

ALTERATIONS OF BLOOD FLOW

Blood flow within cardiovascular system can be either laminar (physiologic state) or turbulent (pathologic state). Laminar blood flow is smooth constant, and streamlined, whereas turbulent blood flow is irregular and chaotic. In laminar blood flow, platelets circulate in central column separated from subendothelial collagen by plasma. Laminar blood flow dilutes clotting factors by fresh blood. Laminar blood flow increases the inflow of inhibitors of clotting factors, and inhibits vascular endothelial cell activation.

- Blood vessel wall damage due to atherosclerosis, hypertension or vascular anomalies is a major predisposing factor for arterial thrombosis, by inducing turbulent blood flow, which allows platelets adhesion to injured vascular endothelium. Consequently, hyperactivity of platelets also plays a role in the pathogenesis of **thrombus formation** within arteries, veins and cardiac chambers. Both arterial and venous thrombosis can reduce or totally impede blood flow.
- Turbulent blood flow disrupts laminar blood flow bringing platelets in contact with vascular endothelium. Turbulent blood flow prevents dilution of clotting factors by fresh flowing blood and retards inflow of clotting factors inhibitors resulting in buildup thrombi. Turbulent blood flow promotes vascular endothelial cell activation resulting in thrombus formation and leukocytic adhesion.

ROLE OF PLATELETS

Platelets are essential in the initial stages of thrombus formation, because they adhere and aggregate at the site of exposed subendothelial extracellular matrix in injured blood vessel and then serve as a surface for coagulation system cascade, the overall rate which determined the final structure of fibrin.

- Following severe injury to vascular endothelium, platelets adhere to exposed subendothelial extracellular matrix, which is mediated by von Willebrand factor (vWF). Soon after platelet adhesion; platelets release adenosine diphosphate (ADP), catecholamine, serotonin and platelet-derived growth factor (PDGF) into the open canalicular system.
- Platelet aggregation is promoted by ADP, thromboxane A_2, epinephrine, collagen, and platelet activating factor (PAF) derived from mast cells and basophils. Then integrin, glycoprotein IIb/IIIa becomes receptor for fibrinogen, which forms bridges between adjacent platelets leading to formation of initial reversible hemostatic platelet plug, when ADP release is minimal.
- On the surface of stimulated platelets, coagulation system cascade is accelerated that stabilize primary hemostatic platelet plug by polymerized fibrin leading to generation of thrombus.

Clinical Pearls: Agonists, Promoters and Inhibitors of Platelets Aggregation

Agonists Promoting Platelets Aggregation

- **Platelets aggregation:** Platelets aggregation is promoted by ADP, thromboxane A_2, epinephrine, collagen, platelet activating factor derived from mast cells and basophils.
- **Thromboxane A_2:** Thromboxane A_2 is generated from arachidonic acid through cyclooxygenase pathway. Thromboxane A_2 causes vasoconstriction and promotes aggregation of platelets by activating guanyl cyclase, which increases intracellular levels of cGMP. The inhibition of cyclooxygenase by low-dose aspirin is the basis of aspirin therapy for prevention of thrombotic disease.

Inhibitors of Platelets Aggregation

- **Prostacyclin:** Prostacyclin (PGI_2) synthesized by endothelium is a potent vasodilator and prevents platelet aggregation. TXA_2 causes vasoconstriction and promotes platelets aggregation. Both prostacyclin and TXA_2 perform balanced function for modulating platelets function.
- **Fibrin degradation products:** Under physiologic state, intravascular platelets aggregation is also prevented by fibrin degradation products, but after vascular endothelial injury, hemostatic plug is formed. Leukocytes and RBCs are also present in hemostatic plug. Leukocytes adhere to endothelium and produce inflammatory response.
- **Thrombin:** Thrombin stimulates leukocytic adhesion and cleaves fibrinogen into fibrin. Fibrin degradation products attract leukocytes (neutrophils and monocytes).

HYPERCOAGULABLE STATE

Hypercoagulable state or thrombophilia is the increased tendency of blood to form thrombus. It is less frequent cause of thrombus formation that may occur in settings of hereditary or acquired disorders. Hereditary causes

of hypercoagulable state are given in Table 3.13. Acquired causes of hypercoagulable state are given in Table 3.14.

Inherited Hypercoagulable State

Inherited hypercoagulable state occurs due to factor V Leiden mutation (most common), prothrombin gene mutation, deficiencies of natural anticoagulant proteins (i.e. antithrombin III, protein C and protein S), elevated levels of fibrinogen or dysfunctional fibrinogen (dysfibrinogenemia, abnormal fibrinolytic system and elevation of plasminogen activator inhibitor 1 (PAI-1).

Acquired Hypercoagulable State

Acquired hypercoagulable state occurs due to prolonged immobilization, hormonal replacement therapy, oral contraceptive pills, pregnancy, obesity, recent trauma or surgery, central venous catheter placement, mucin secreting tumors, some medications used to treat cancer (i.e. tamoxifen, bevacizumab, thalidomide and lenalidomide), heparin-induced thrombocytopenia, antiphospholipid antibody syndrome, myeloproliferative disorders (i.e. polycythemia vera or essential thrombocythemia), previous history of deep vein thrombosis (DVT) or pulmonary embolism, paroxysmal nocturnal hemoglobinuria, nephrotic syndrome and HIV/AIDS.

Table 3.13 Hereditary causes of hypercoagulable state

Common Mutations
Factor V Leiden mutation (abnormal factor V resists degradation by protein C)
Prothrombin G20210A (factor II) mutation
Methyl tetrahydrofolate reductase (MTHFRC677T) mutation
Rare Mutations
Antithrombin III mutation
Protein C mutation
Protein S mutation
Very Rare Mutations
Dysfibrinolysis

Table 3.14 Acquired causes of hypercoagulable state

High-risk Disorders
Myocardial infarction
Atrial fibrillation
Prosthetic valve
Trauma (surgery, fracture, burns)
Disseminated intravascular coagulation (DIC)
Malignant tumors (mucin secreting tumors)
Heparin-induced thrombocytopenia (HIT)
Antiphospholipid antibody syndrome (lupus anticoagulant syndrome)
Low-risk Disorders
Nephrotic syndrome (urinary loss of antithrombin III)
Hyperestrogenic state (pregnancy)
Oral contraceptive use
Tobacco smoking
Sickle cell anemia

THROMBOTIC DISORDERS

In normal health, hemostatic balance exists between procoagulant, anticoagulant and fibrinolytic systems. Numerous inherited and acquired thrombotic disorders can tip the balance in favor of coagulation leading to thrombus formation in arteries and veins. Thrombi can obstruct blood flow and cause dysfunction of organs. Comparison between inherited and acquired thrombophilia is given in Table 3.15. Laboratory diagnosis of thrombophilia is given in Table 3.16.

- Common manifestations of thrombotic disorders include deep vein thrombosis (DVT) and life-threatening pulmonary thromboembolism. Coronary artery disease can cause myocardial infarction. Occlusion of mesenteric arteries lead to gangrene gut. Occlusion of arteries supplying brain can lead to cerebral stroke.
- Most of the inherited thrombotic disorders do not begin to cause an increased risk of thrombosis until young adulthood, although thrombus can form in blood vessels at any age. Women may have a history of multiple spontaneous abortions.
- Hereditary thrombophilia is a prothrombotic familial syndrome caused by deficiency of a number of anti-thrombotic proteins, including antithrombin III, protein C, and protein S. Hereditary thrombophilia is characterized by recurrent venous thrombosis and thromboembolism, which most often occurs in adolescents or young women.
- Patients should be investigated in the settings of history of thrombosis at young age, unexplained recurrent episodes of thromboembolic phenomenon, strong family history of thrombosis in the first-degree relatives, recurrent abortions and pregnancy associated thrombi in women.

INHERITED THROMBOTIC DISORDERS

Significant advances in identification of etiologies of inherited thrombotic disorders associated with recurrent arterial or venous thrombosis have been recently

Table 3.15 Comparison between inherited and acquired thrombophilia

Feature	Inherited Thrombophilia	Acquired Thrombophilia
Common causes	▪ Factor V Leiden mutation ▪ Prothrombin G20210A gene mutation ▪ Antithrombin III deficiency ▪ Protein C (PC) deficiency ▪ Protein S (PS) deficiency	▪ Autoimmune disorders ▪ Antiphospholipid syndrome (lupus anticoagulant causing pregnancy loss)
Family history of thrombophilia	Present (family member with known thrombophilia)	Absent
Venous thromboembolism	Unprovoked venous thromboembolism or with minor risk factor	Unprovoked venous thromboembolism
Site of thrombosis	Recurrent venous thromboembolism in <50 years	Thrombosis at unusual sites especially in patient >50 years (e.g. splanchnic region)
Clinical manifestations	Skin necrosis associated with vitamin K antagonists	Late or recurrent pregnancy loss

Table 3.16 Laboratory diagnosis of thrombophilia

Laboratory Test	First Diagnostic Step	Comments
Activated protein C resistance (APCR)/factor V Leiden (FVL)	Deficient factor V Leiden (FVL) coagulation based functional assay	DNA analysis for factor V Leiden mutation
Prothrombin G20210A mutation (FII G20210A)	DNA analysis	Measurement of protohemin (FII) activity in plasma should not be used to screen thrombophilic patients for this mutation due to its inability to clearly distinguish carriers from noncarriers of the mutation
Antithrombin (AT) deficiency	Functional chromogenic assay	Measurement of antithrombin antigen by an immunoassay in order to classify the type of deficiency as type 1 or 2
Protein C deficiency	Functional chromogenic assay	Measurement of protein C antigen by an immunoassay in order to classify the type of deficiency as type 1 or 2 or 3
Protein S (PS) deficiency	Functional coagulation-based assay	Measurement of protein S antigen by an immunoassay in order to classify the type of deficiency as type 1 or 2 or 3
Antiphospholipid antibodies (aPLAs): LA, ACL and anti-β2-Gp1	▪ Lupus anticoagulant: a panel of screening two or more assays and at least one confirmatory assay ▪ ACL and anti-β2-Gp1: enzyme immunosorbent assays for both IgM and IgG isotopes	Repeat testing for a positive test result with at least 12 weeks apart in order to confirm a positive test result
Increased factor VIII level (FVIII >159%)	Coagulation or chromogenic functional assay	Repeat testing 3 to 6 months after initial testing
Homocysteinemia	Plasma level of homocysteine	Repeat testing in case of questionable or borderline test result or to confirm a positive test result
Dysfibrinogenemia	Screening assays: thrombin time (TT) and replitase time (RT), functional fibrinogen level correlated with von Clauss fibrinogen level	Parallel analysis of functional and immunoreactive fibrinogen as confirmatory assays

reported. A point mutation in coagulation factor (factor V Leiden) results in resistance to activated protein C probably represents the most common genetic risk factor for venous thrombosis. Homocysteinemia is a metabolic disorder linked to both arterial and venous thrombosis. Many patients with recurrent thrombosis will have more than one genetic risk factor. Thrombophilias in approximate descending order of probability are given in Table 3.17.

Factor V Leiden Mutation

Mutation of factor V Leiden has been named after the city in Netherlands, which is the most common cause of hereditary thrombophilia. Risk of hereditary thrombophilia is greatly increased during pregnancy and following oral contraceptive intake.

- **Molecular genetics:** Abnormal factor V protein is formed due to specific point mutation by substitution of glutamine for normal arginine at position 506,

Table 3.17 Thrombophilias in approximate descending order of probability

Blood protein/platelet defects leading to thrombosis
Antiphospholipid syndrome
Factor V Leiden mutation (abnormal factor V resists degradation by protein C)
Other factor V mutations
Sticky platelet syndrome
Plasminogen activator inhibitor 1 (PAI-1) mutation
Prothrombin G20210 mutation
Methylene tetrahydrofolate reductase (MTHFRC677T) mutation
Antithrombin III mutation
Protein C mutation
Protein S mutation
Heparin cofactor II mutation
Plasminogen mutation
Factor XII mutation
Dysfibrinogenemia
Homocysteinemia
Lipoprotein (a)
Tissue plasminogen activator mutation
Tissue factor pathway inhibitor mutation

which alters the cleavage site targeted by activated protein C. Abnormal factor V protein becomes resistant to cleavage by protein C. As a result, an important antithrombotic counter-regulatory mechanism is lost. There is increased generation of prothrombinase complex and thrombin generation.

- **Clinical features:** Patient presents with recurrent deep vein thrombosis (DVT). Factor V Leiden mutaion predisposes to unchecked thrombosis especially in deep veins of leg.
- **Laboratory tests:** Laboratory tests for factor V Leiden mutation include activated protein C resistance assay and genetic analysis by polymerase chain reaction.

Prothrombin G20210A Mutation

Prothrombin is the precursor of thrombin. Normally, prothrombin protein is produced to assist hemostasis. The prothrombin G20210A mutation is the second most common cause of inherited risk factor for thrombosis that leads to elevated plasma levels of prothrombin, which demonstrates a higher risk for arterial and venous thrombotic events.

- **Single point mutation** in prothrombin gene occurs due to a single nucleotide change (G to A) in the 3′-untranslated regions at position 20210. It is more commonly seen in Caucasians and individuals of African Americans.
- Prothrombin testing is done by taking a blood sample and using a genetic test to look at the prothrombin gene. DNA is isolated from blood cellular components and the prothrombin gene is analyzed to see if there is a mutation in the DNA code.

Methylene Tetrahydrofolate Reductase (MTHFRC677T) Mutation

Methylene tetrahydrofolate reductase (MTHFRC677T) gene mutation results in mild elevation of homocysteine levels in 5–15% of White and East Asia populations. Elevated levels of homocysteine inhibit antithrombin III and thrombomodulin. There is increased risk of thrombosis. Increased levels of homocysteinemia are also associated with an increased risk of neural tube defects and possibly a number of diverse neoplasms. Increased homocysteine can be reduced by dietary supplementation with folic acid and vitamins B_6 (pyridoxine) and B_{12} (cobalamin).

Increased Concentration of Factor VIII, Factor IX, Factor XI, or Factor I

Increased levels of factor VIII (antihemophilic globulin A), factor IX (antihemophilic globulin B), factor XI (antihemophilic globulin C), or factor I (fibrinogen) are also associated with increased venous thrombosis.

Increased Concentration of Factor VIII

Higher levels of factor VIII increase the risk of thrombosis especially in African-Americans, which also correlate with acute phase reactant synthesis, estrogen usage, pregnancy and after aerobic exercise. Elevated factor VIII level may cause activated protein C resistance. In contrast, low levels of factor VIII correlate with bleeding in hemophilia patients.

Increased Concentration of Factor IX

Factor IX is a vitamin K-dependent glycoprotein that plays a key role in hemostasis. Factor IX is activated through intrinsic pathway as well as extrinsic pathway of coagulation system.

- Factor XI, when activated by factor XIa or factor VIIa tissue factor, converts factor X into Xa and accelerated by factor VIIIa, calcium ions and a phospholipid membrane, which eventually results in formation of fibrin clot.
- Elevated factor IX levels increase risk of deep venous thrombosis (DVT) after adjustment for factors VIII, XI and other known risk factors.
- Various studies revealed that enhanced level of factor IX occur with advancing age and in women on oral contraceptive pills. In contrast, deficiency of factor IX leads to hemophilia B.

Increased Concentration of Factor XI

Factor XI is a vitamin K-dependent protein that plays a role in hemostasis. Factor XI is activated through intrinsic pathway of coagulation system cascade involved in the creation of a stable fibrin clot. Elevated factor IX levels increase the risk of venous thrombosis after adjustment with factor VIII, factor IX and other known factors.

Increased Concentration of Factor I

Factor I (fibrinogen) is a protein synthesized in the liver. Major physiologic function of fibrinogen is the formation of polymerized fibrin that binds to platelets and some plasma proteins in a hemostatic plug. Elevated fibrinogen levels increase risk of blood clots associated with higher risk of heart disease, blood vessel dysfunction and cerebral stroke.

Antithrombin III, Protein C and Protein S Deficiency

Vitamin K-dependent clotting factors (II, VII, IX and X), protein C and protein S are synthesized by liver. Antithrombin III synthesis is not vitamin K-dependent. In normal health, antithrombin III, protein C and protein S prevent thrombus formation.

- Hereditary deficiency of antithrombin III and protein C have an autosomal dominant pattern of inheritance with identical clinical manifestations of thrombosis.
- Approximately, 50% reduction in the plasma concentration of protein C results in venous thrombosis and recurrent thromboembolic pulmonary phenomenon in adolescents or early life. Diagnostic tests of hemostasis used for detection of congenital thrombotic disorders are given in Table 3.18.

Antithrombin III Deficiency

Antithrombin III is a protein in the blood synthesized in the liver that inhibits thrombus formation by binding to heparin on endothelial cells and forming a complex with thrombin (thrombin-antithrombin) thus inhibiting coagulation cascade.

- Qualitative defects of antithrombin III describe mutations, which either affect heparin binding site, reactive site or has pleomorphic effects.
- Homozygous antithrombin III deficiency is incompatible with life unless affecting the heparin-binding site.
- Persons with antithrombin III deficiency present with venous thrombosis and less likely with arterial thrombosis.

Protein C Deficiency

Protein C interacts with thrombomodulin to become activated protein C. Activated protein C inactivates coagulation factors V and VIII leading to inhibit coagulation system cascade. Protein C deficiency can cause thrombosis in teenagers.

- Protein C and protein S deficiency can be inherited or acquired in the stetting of liver dysfunction, vitamin K antagonists, renal failure, disseminated intravascular coagulation (DIC) and active thrombosis.
- Protein C is rapidly consumed in disseminated intravascular coagulation. A total lack of protein C is usually associated with death *in utero*.
- Newborns with total absence of protein C suffer from purpura fulminans neonatalis. Repeated infusions of prothrombin complex help in keeping these children alive.
- The half-life of protein C is shorter than half-life of other vitamin K-dependent coagulation factors.

Protein S Deficiency

Protein S enhances the effect of activated protein C. Protein S deficiency is linked to develop thrombus formation. Protein S deficiency can be classified as type 1 (reduced quantity of protein C), type 2 (low activated protein C activity) and type 3 (low free protein S to increased binding to the complement factor C4b). Interaction of protein S with C4b is an active phase reactant linked to coagulation, inflammation and autoimmunity.

Dysfibrinolysis

Dysfibrinolysis occurs due to dysfibrinogenemia, plasminogen deficiency, tissue plasminogen activator (tPA) deficiency, elevated plasminogen activator inhibitor 1 (PAI-1) and factor XII deficiency.

Table 3.18 Diagnostic tests of hemostasis used for detection of congenital thrombotic disorders

Diagnostic Test	Type of Assay	Application
Protein C (PC)	Functional clotting and/or chromogenic assays	Congenital deficiency of protein C
Protein S (PS)	Functional clotting and/or antigenic assays	Congenital deficiency of protein S
Antithrombin III (AT-III)	Chromogenic or antigenic assays	Congenital deficiency of antithrombin III
Activated protein C resistance (APCR)	Activated partial thromboplastin time (APTT) or Russell's viper venom time (RVVT)	Congenital or acquired thrombophilia (APCR, FVL—factor V Leiden)

- Plasminogen deficiency clinically appears similar to protein C deficiency associated with thrombosis during teen-age years.
- Plasminogen activator inhibitor I (PAI-1) and deficient tissue plasminogen activator are linked to diabetes mellitus, inflammatory bowel syndrome and coronary arteriosclerosis.
- In patients with functional changes in fibrinogen (dysfibrinogenemia) are linked to thrombosis or bleeding.

Sticky Platelet Syndrome

Sticky platelet syndrome is an autosomal dominant thrombophilic disorder characterized by increased *in vitro* platelet aggregation after low concentrations of ADP and/or epinephrine. Antiphospholipid antibodies bind to phospholipid molecules in the blood and induce autoimmune reaction resulting in platelets sticking together. Clumping of platelets causes the build-up of blood clots in blood vessels.

ACQUIRED THROMBOTIC DISORDERS

Patients with acquired hypercoagulable states are more likely to develop venous and arterial thrombosis than healthy individuals. Venous thrombosis and pulmonary embolism are associated with significant morbidity and mortality. Acquired hypercoagulable states associated with increased risk of thrombosis are given in Table 3.19.

Table 3.19 Acquired hypercoagulable states associated with increased risk of thrombosis

Acquired Hypercoagulable States Leading to Abnormal Platelet Functions
Atheromatous plaque
Diabetes mellitus
Hyperlipidemia
Tobacco smoking
Thrombocytosis
Acquired Hypercoagulable States Leading to Abnormal Coagulation System Cascade
Congestive heart failure
Postsurgical state
Immobilization
Use of oral contraceptives
Pregnancy
Heparin-induced thrombocytopenia (HIT)
Antiphospholipid syndrome
Thrombotic thrombocytopenic purpura (TTP)
Nephrotic syndrome (loss of antithrombin III and antiplasmin in urine)
Myeloproliferative disorders

Antiphospholipid Syndrome

Antiphospholipid syndrome (APS) is most common acquired thrombophilia associated with high titers of circulating antibodies (IgG) directed against cell membranes, and phospholipids, which may cause arterial or venous thrombosis and recurrent miscarriages. Proposed mechanism of thrombosis in antiphospholipid antibody syndrome is given in Table 3.20. Common sites of arterial and venous thrombosis in antiphospholipid syndrome are given in Table 3.21.

- *In vitro* antiphospholipid antibodies interfere with the assembly of phospholipid complexes and thus

Table 3.20 Proposed mechanism of thrombosis in antiphospholipid antibody syndrome

Interference with endothelial phospholipids and thus prostacyclin release
Inhibition of prekallikrein, thus inhibition of fibrinolysis
Inhibition of thrombomodulin, thus protein C and protein S activity
Activated protein C resistance (nonmolecular)
Interaction with platelet membrane phospholipids
Direct inhibition of protein S
Inhibition of endothelial cell tissue plasminogen activator release
Inhibition of annexin-V, a cell surface protein that inhibits tissue factor—also referred to as 'placental anticoagulant protein (serine only)
Induction of monocyte release of tissue factor

Table 3.21 Common sites of arterial and venous thrombosis in antiphospholipid syndrome

Arterial Thrombi
Coronary arteries
Cerebral arteries
Retinal arteries
Brachial artery
Mesenteric arteries
Peripheral (extremity) arteries
Aorta
Placental arteries (recurrent miscarriage)
Venous Thrombi
Deep vein thrombosis (DVT)/pulmonary embolism (PE)
Intracranial veins
Retinal vein
Inferior vena cava
Renal vein
Hepatic vein (Budd-Chiari syndrome)
Portal vein
Placental veins (recurrent miscarriage)

inhibit coagulation. However, *in vivo*, the antibodies induce a hypercoagulable state.

- Antiphospholipid syndrome antibodies are being tested, which include lupus anticoagulant, anti-cardiolipin and anti-β_2 glycoprotein. Lupus anticoagulant leads to prolongation of coagulation analyzed by activated partial thromboplastin time (APTT) *in vitro*.
- Antiphospholipid antibodies may also occur secondary to systemic lupus erythematosus, rheumatoid arthritis, and drugs (e.g. phenytoin and cocaine). The most common thrombotic event is deep vein thrombosis (DVT). Any person with cerebral stroke and rheumatological disorder should be screened for antiphospholipid antibody syndrome.
- Patient with antiphospholipid syndrome presents with recurrent venous especially in deep veins of legs or arterial thrombi. But renal, hepatic, and retinal veins are also susceptible. There is history of repeated miscarriages, cardiac valvular vegetations, or thrombocytopenia.

Heparin-induced Thrombocytopenia Syndrome

Heparin is widely used for treatment and prophylaxis of thromboembolism in clinical practice. Heparin-induced thrombocytopenia (HIT) is a serious complication of administration of low-molecular weight heparin products. A fall in platelet counts and hypercoagulable state characterize heparin-induced thrombocytopenia. Patients are prone to thromboembolic complications linked to morbidity and mortality. Two types of heparin-induced thrombocytopenia syndrome include: type 1 HIT and type 2 HIT syndrome.

Type 1 Heparin-induced Thrombocytopenia Syndrome

Type 1 heparin-induced thrombocytopenia syndrome is a nonimmune-mediated reaction, which is more common than type 2 HIT, which can occur within a day of heparin therapy. This is mild reaction that results in mild to moderate drop of platelets. Type 1 HIT syndrome is not associated with any complications and platelet counts will spontaneously normalize even if heparin is continued, which is not a contraindication to future heparin use.

Type 2 Heparin-induced Thrombocytopenia Syndrome

Type 2 heparin-induced thrombocytopenia is an immune-antibody mediated reaction in patients that results in severe drop of platelet counts (often <50% of baseline) within 5–10 days of heparin administration.

- In normal person, platelet factor 4 is positively charged and stored in α-granules of the platelets, which is released upon platelet activation. Platelet factor 4 binds to the negatively charged heparin-like substance present on vascular endothelium. Platelet factor 4 can also bind to exogenous heparin with a much higher affinity than endogenous heparan.
- Platelet factor 4 binding to exogenous heparin may trigger the formation of IgG, IgA or IgM antibodies specific to the heparin–PF4 complex. IgG attached to the heparin–platelet factor 4 (PF4) complex binds to the Fc receptor on the platelet surface and leads to platelet activation. Repeated cycles of platelet activation leads to release prothrombotic substances such as thrombin and platelet factor 4 leading to severe hypercoagulable state. Continuous chain can be broken on discontinuation of heparin and initiation of treatment.
- Most characteristic clinical finding in type 2 HIT is thrombocytopenia that occurs due to destruction of IgG-coated platelets by reticuloendothelial cells. Simultaneously, activated platelets lead to platelets aggregation leading to arterial and venous thrombosis and simultaneous low-platelet count.
- The most common complications of type 2 HIT are deep vein thrombosis (DVT) and pulmonary thromboembolism within the first 10 days. However, prothrombotic state persists up to 30 days after withdrawal of heparin.
- However, if a patient has been exposed to heparin within last 100 days, antibodies may remain in the body, causing an immune reaction to manifest as soon as day one of re-exposure to heparin.

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation (DIC) is characterized by widespread clotting with resultant consumption of platelets and coagulation factors, especially factors II, V, and VIII, and fibrinogen. Pathophysiology of DIC is shown in Fig. 3.24. Causes of DIC are given in Table 3.22.

- Disseminated intravascular coagulation (DIC) occurs as a result of release of tissue thromboplastin (tissue factor) or activation of the intrinsic pathway of coagulation, as well as secondary activation of the fibrinolytic system.
- Clinical manifestations of DIC are thrombotic phenomena in small blood vessels of multiple organs and hemorrhage.
- Hematologic findings in disseminated intravascular coagulation include microangiopathic hemolytic anemia with fragmented red blood cells

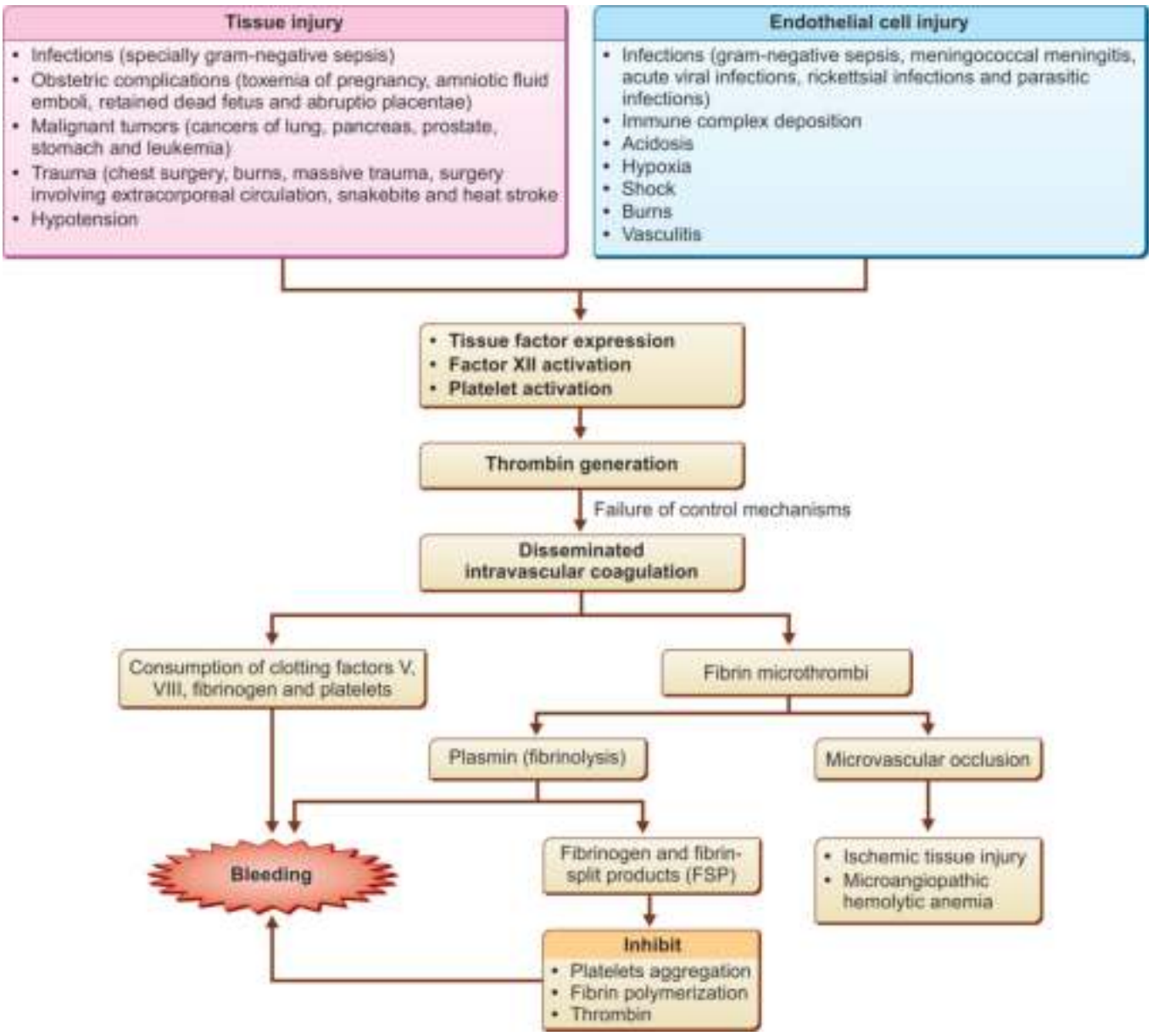


Fig. 3.24: Pathophysiology of disseminated intravascular coagulation (DIC). DIC is triggered by tissue injury, endothelial injury or combination of both these processes, and characterized by widespread activation of coagulation leading to formation of blood clots throughout the bloodstream, occlusion of small vasculature, organ dysfunction, consumption of coagulation factors and platelets, and life-threatening hemorrhage.

(schistocytes), increased fibrin and fibrinogen degradation (split) products, thrombocytopenia and prolonged bleeding time, prothrombin time and international normalized ratio (PT–INR), activated partial thromboplastin time (APTT), and thrombin time (TT).

Procoagulant Substance Secreting Malignant Tumors

Malignancy is the second most common acquired hypercoagulable state that leads to a prothrombotic state through the production of procoagulant factors (tissue factor and cancer procoagulant factor) and the interaction of cancer stem cells with blood and vascular endothelium.

- Blood stasis from malignant tumor compression, paraproteinemia, and cytokine release pose an additional risk for thrombus formation.
- Cancer procoagulant factor is elevated in 85% of cancer patients. Procoagulant factor activates factor X thus causing hypercoagulable state in cancer patients. Polycythemia vera poses a thrombotic risk in addition to hyperviscosity. Migratory thrombophlebitis as a consequence of visceral malignancy is known as Trousseau syndrome.

Table 3.22 Causes of disseminated intravascular coagulation

Cause	Mechanism
Obstetric complications	▪ Toxemia of pregnancy ▪ Amniotic fluid emboli ▪ Retained dead fetus ▪ Abruptio placentae (premature separation of placenta)
Metastatic cancers	▪ Lung carcinoma ▪ Pancreatic carcinoma ▪ Prostate carcinoma ▪ Gastric carcinoma ▪ Leukemia
Infectious agents	▪ Gram-negative sepsis ▪ Meningococcal meningitis ▪ Acute viral infections ▪ Rickettsia infections (e.g. Rocky Mountain spotted fever) ▪ Parasitic infections (e.g. malaria)
Trauma or surgery	▪ Chest surgery ▪ Burns ▪ Massive trauma ▪ Snake bite ▪ Surgery involving extracorporeal circulation ▪ Heat stroke
Shock	▪ Septic shock ▪ Severe hypovolemic shock
Immunological mechanism	▪ Immune complex disease ▪ Hemolytic transfusion reactions

FATE OF THROMBUS

Thrombosis is process of formation of mass in circulation from the constituents of flowing blood, mass is called as thrombus.

- Thrombosis involves activation of platelets and coagulation system cascade leading to conversion of soluble fibrinogen to fibrin, which is polymerized and converted to insoluble fibrin.
- Thrombus formation can have one of five outcomes: propagation, dissolution, organization, recanalization and embolization to distant organs.
- Occasionally, instead of organization of thrombus, the central portion of thrombus undergoes enzymatic digestion as a result of the release of lysosomal enzymes from trapped leukocytes and platelets, which occurs in large thrombi formed in cardiac chambers or abdominal aortic aneurysm. If the bacterial seeding occurs, it is called mycotic aneurysm. Fate of thrombus is shown in Fig. 3.25A to D.

PROPAGATION OF THROMBUS

Platelet activation at the site of disrupted atheromatous plaque is an initial step in the thrombus formation. Activated platelets release adenosine diphosphate (ADP), serotonin and thromboxane A_2, which promote platelets activation further. This self-amplifying process results in thrombus propagation, in which increase in size of thrombus occurs due to accumulation of additional platelets and fibrin. Propagation of thrombus results in occlusion of the blood vessel.

DISSOLUTION OF THROMBUS

Fibrinolysis refers to the process of dissolution of the blood clot formed by activation of hemostatic pathways either in hemostasis in physiologic response to vascular injury or dissolution of thrombosis in pathologic response to vascular injury. Dissolution of thrombus occurs when the fibrinolytic mechanisms break up the thrombus and restoration of blood flow in the blood vessel. Tissue plasminogen activator (tPA), synthesized by vascular endothelium, converts plasminogen to plasmin in the vicinity of the thrombus.

- Plasmin is a fibrinolytic enzyme, which splits fibrin strands (fibrinolysis) and dissolves relatively small thrombus resulting in restoration of blood flow.
- Streptokinase enzyme can directly activate plasmin, which can dissolve occlusive thrombi. Activation of Hageman factor links the fibrinolytic system, coagulation system, complement system, and kinin system.

ORGANIZATION OF THROMBUS

Older thrombi undergo organization, due to ingrowth of vascular endothelial cells, fibroblasts, smooth muscle cells into the fibrin-rich thrombus. Contraction of these cells leads to incorporation of smaller thrombus in the subendothelial region of the blood vessel wall. Organized thrombus is firm and grayish white in color resulting in obliteration of lumen of blood vessel.

RECANALIZATION OF THROMBUS

The process of recanalization of the deep vein of the lower limbs after an episode of deep vein thrombosis (DVT) is a part of the natural evolution of the remodeling of the venous thrombosis in patients on anticoagulant therapy with heparin and vitamin K inhibitors. Recanalization is a process that initially involves adhesion of the thrombus to the wall of deep vein of lower extremities and inflammatory response in the blood vessel wall resulting in organization and subsequent contraction of the thrombus, neovascularization, spontaneous lysis of areas within the thrombus and restoration of partial blood flow.

THROMBUS EMBOLIZATION

Thrombus embolism occurs when a blood clot or thrombus formed in a blood vessel breaks loose and circulate in the bloodstream and blocks another blood vessel leading to infarction of organs at a distant

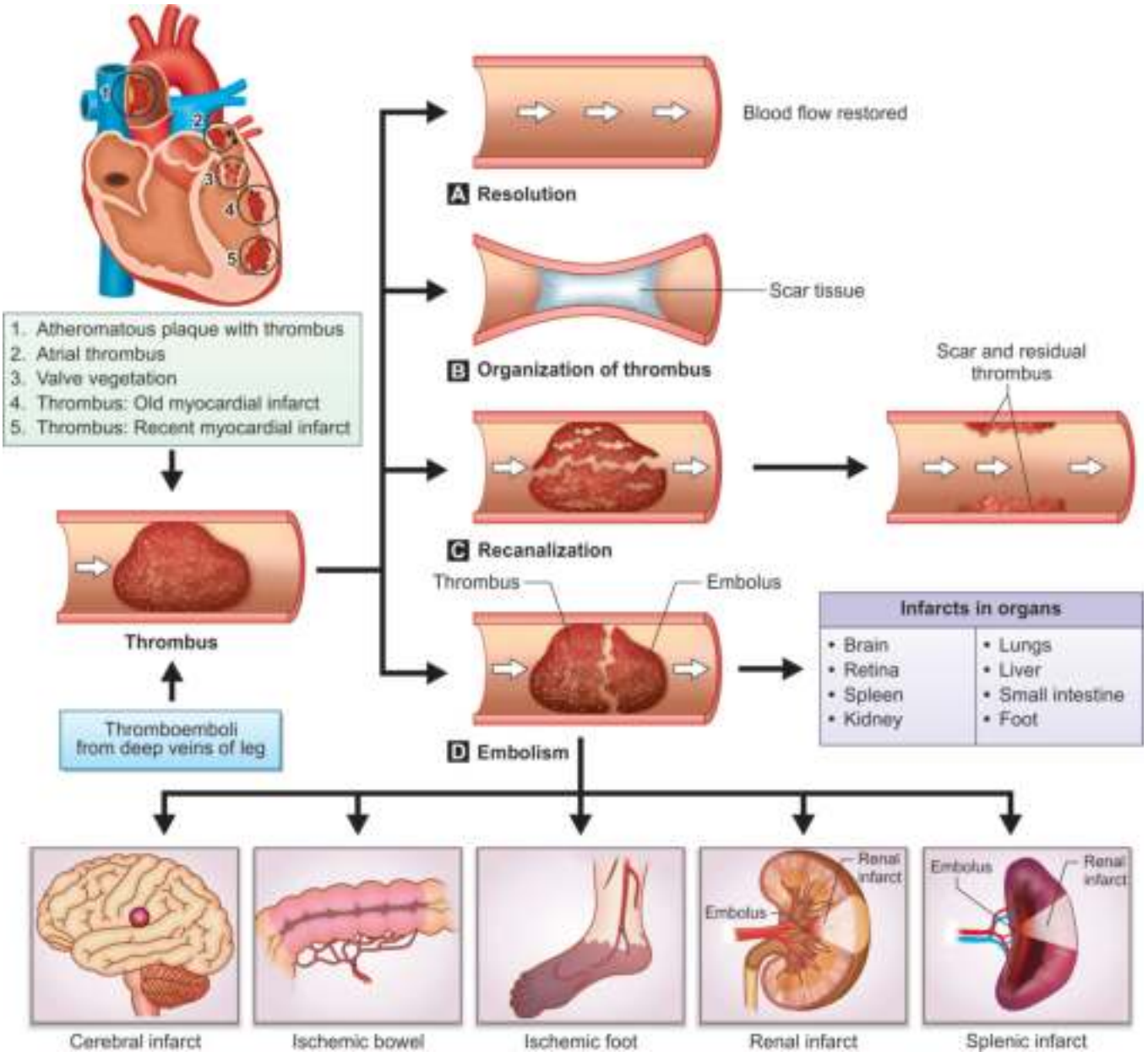


Fig. 3.25A to D: Fate of thrombus. The main outcomes of thrombosis are resolution, organization, recanalization, occlusion, embolism and propagation. Thromboembolism can cause cerebral stroke, ischemic bowel disease, peripheral vascular disease, renal infarct and splenic infarct.

site such as brain, kidney, intestine and lower limbs and even death. The thromboemboli may enter coronary arteries resulting in myocardial infarction. Venous thrombi may cause pulmonary embolism with fatal outcome.

THROMBOEMBOLISM

Thromboembolism refers to formation of a blood clot or thrombus in a blood vessel that breaks loose and is carried by the blood stream to obstruct another blood vessel.

- The blood clot or thrombus may obstruct a blood vessel in the lungs (pulmonary embolism), heart (myocardial infarction), brain (cerebral stroke), legs (deep vein thrombosis), and less common in gastrointestinal tract (gangrene gut) and kidneys (renal infarct). Thromboembolism is important cause of morbidity and mortality especially in adults.
- There are two main categories of thromboembolism depending on type of blood vessel affected: venous and arterial thromboembolism. Patients are treated with anticoagulants, aspirin and vasodilators.
  - Venous thromboembolism occurs in veins, which includes deep vein thrombosis (DVT) and pulmonary embolism (PE). Risk factors of venous thromboembolism include bed confinement and sitting for a long time without movement.

- Arterial thromboembolism occurs in arteries, that causes restriction of blood flow and oxygen leading to ischemia of organ. Risk factors of arterial thromboembolism are generally different.

Pathology Pearls: Arterial and Venous Thrombi

- Thrombi may be formed in arteries and veins during life. There are two main types of thrombi: arterial thrombus and venous thrombus. The risk factors of arterial thrombus and venous thrombus are generally different.
- The composition, physical properties, and evolution of arterial and venous thrombi are likely to differ mainly due to various local conditions and time since formation.
- Outcome of thrombi includes lysis of thrombus, propagation, organization, recanalization and embolization. Differences between arterial and venous thrombi are given in Table 3.23.

Arterial Thrombi

- Risk factors of arterial thrombi include cigarette smoking, diabetes mellitus, hypertension, hyperlipidemia, family history of arterial thrombosis and older age.
- Platelets play key role in the development of arterial thrombi formed at relatively high wall shear rates, generating what are often termed 'white thrombi'.
- Arterial thrombus is most often superimposed on an ulcerated atheromatous plaque in large elastic and medium-sized arteries (i.e. abdominal aorta and coronary arteries).
- Arterial thrombi commonly arise from the rupture of atherosclerotic plaque and exposure of procoagulant components such as collagen and lipid-rich activated macrophages being tissue factor in coronary, cerebral and other arteries supplying organs.
- Arterial thrombus grows in the direction of the blood flow (propagation) resulting in tissue infarction and ischemia either directly or indirectly through embolization.

Venous Thrombi

- Venous thrombi are attributed to a combination of hypercoagulable state together with injured or activated endothelium of veins, turbulent blood flow, blood stasis during surgery and immobilization, fracture, obesity, history of deep vein thrombosis (DVT), inherited disorders, autoimmune disorders (e.g. Crohn's disease), central venous catheter, pregnancy, and oral contraceptive pills.
- Most common sites of venous thrombi are deep veins of lower leg (90%), dural sinuses of skull, pelvic veins and portal tributaries.
- In contrast, venous thrombi are formed under low shear rate and mainly composed of red blood cells (RBCs) and fibrin (i.e. red thrombi).

Pathology Pearls: Postmortem Blood Clots

- Thrombosis is the formation of a solid mass by the constituents of blood within vascular system. Thrombus should not be confused with a blood clot, which occurs in nonflowing blood. A postmortem blood clot is defined as coagulation of blood after death. Postmortem blood clots may be confused with venous thrombi.
- Postmortem blood clots are gelatinous with dark red due to settling down of red blood cells by gravity giving 'red currant jelly' appearance.
- Yellow chicken fat cell-free supernatant resembles melted and clotted chicken. In contrast to thrombi; postmortem clots are not attached to the blood vessel wall. Differences between antemortem thrombus and postmortem blood clot are given in Table 3.24.

VENOUS THROMBOEMBOLISM

Venous thromboembolism occurs when a blood clot or thrombus breaks off and blocks a vein—a blood vessel that brings blood in need of oxygen back to heart.

- Venous thrombi are formed in areas of less active blood flow due to stasis of venous blood especially in immobilized patients, which extend in the direction of blood flow (i.e. toward heart). Propagating tail of venous thrombi may not be well attached to vascular endothelium resulting in fragmentation creating an embolus.
- Venous thrombi are occlusive, which often create a long cast of the lumen of vein. The venous thrombi are dark red with a higher concentration of red blood cells (RBCs) along with fair number of platelets and leukocytes than arterial thrombi. Lines of Zahn are not prominent in venous thrombi. Anticoagulant therapy with heparin and warfarin prevents formation of venous thrombi.
- Venous thromboembolism often starts in the lower extremities with a condition known as deep vein thrombosis (DVT) below knee joint (most common in 90%). Less common sites of venous thromboembolism include superior saphenous, periprostatic, ovarian or uterine veins, hepatic veins, renal veins, and dural sinuses. If the venous thrombus breaks off and lodges in the lung, it causes life-threatening condition known as pulmonary thromboembolism, which can be fatal depending on the location of the embolus and the degree of blood flow obstruction in lungs.
- The broader term venous thromboembolism denotes deep vein thrombosis (DVT), pulmonary embolism (PE) or a combination of the two conditions (called DVT/PE). Venous thromboembolism can involve

Table 3.23 Differences between arterial and venous thrombi

Parameters	Arterial Thrombi	Venous Thrombi
Blood flow	Formed in rapid blood flow in heart chambers or arteries	Formed in slow blood flow in veins (deep veins of leg)
Location of thrombus	Coronary, cerebral, iliac, femoral arteries and heart chambers in active blood flow	Superficial saphenous veins, deep veins of legs, popliteal veins, femoral veins, hepatic veins, renal veins and dural sinuses in less active blood flow
Pathogenesis	Vascular endothelial injury (atheromatous plaque), turbulent blood flow (e.g. bifurcation of vessel and aortic aneurysm) and hypercoagulable states	Stasis of venous blood especially in immobilized patients, disseminated cancers, pregnancy, antithrombin III deficiency and intravenous cannula
Progression of thrombus	Growing in a retrograde direction from the point of attachment	Venous thrombi formed in the lower extremities propagating toward the heart may cause pulmonary artery embolization
Vessel occlusion	Partial or complete occlusion of vessels	Occlusive and often creating a long cast of the lumen of vein
Gross morphology	Gray white friable with alternating pale and red areas	Dark red with a higher concentration of red blood cells along with fair number of platelets and leukocytes
Light microscopy	▪ Lines of Zahn prominent (pale area composed of platelets held together by fibrin; and red areas composed predominantly of RBCs) ▪ Inflammatory changes in arteries absent	▪ Lines of Zahn not prominent or absent ▪ Inflammatory changes in veins present known as 'thrombophlebitis
Complications of thrombus	Infarction of organs such as heart, brain, kidney, spleen, lower limb	Pulmonary thromboembolism, edema, skin ulcers and poor wound healing
Therapy	Aspirin and anticoagulant therapy	Anticoagulants heparin and warfarin

Thrombosis is diagnosed by taking medical history, physical examination, ultrasonography, CT and MRI scan, hematologic tests and venography.

Table 3.24 Differences between thrombus and postmortem blood clot

Features	Thrombus	Postmortem Clot
Cause	Pathologic disorders	Dead person, sedimentation and settling down of blood components due to gravity
Location	Present in any blood vessel in the body	Present in dependent part in relation to the body kept after death
Shape	May or may not fit their vascular contours	Taking the shape of vessels or its bifurcation
Attachment to the vessel wall	Strong attachment of thrombus to vessel wall	Weak attachment of postmortem clot to vessel wall
Gross morphology	Dry, granular, firm, and friable	Gelatinous, soft and rubbery; two layer—currant jelly dark appearance in red cell-rich lower and a chicken fat appearance in cell-poor upper layer
Light microscopy	Lines of Zahn present	Lines of Zahn absent

other deep and superficial veins of the body. Less common sites of venous thromboembolism include upper extremities, liver, kidneys and brain.

- Risk factors of venous thromboembolism include family history of venous thromboembolism, obesity, older age, chronic diseases (e.g. heart disease, lung, disease, inflammatory bowel disease and cancer), injury to a vein due to trauma or major surgery, use of a central venous catheter, bed confinement, sitting for long time with crossed legs and intake of estrogen based contraceptive pills.

Clinical Pearls: Venous Thromboembolism

The symptoms of venous thromboembolism depend on location of embolus, extent of blood flow obstruction and ischemia of organ.

- **Deep vein thrombosis (DVT) in legs:** Patient presents with throbbing, cramps, swelling, pain, hardened veins, warmth and redness in the affected leg.
- **Pulmonary embolism:** Patient presents with sudden shortness of breath, rapid breathing, chest pain, hemoptysis and bluish discoloration of the lips or finger (cyanosis).

- **Central nervous system venous thrombosis:** Patient presents with headache, facial dropping, limb weakness, difficulty in speaking and in some cases, seizures.
- **Portal vein thrombosis:** Patient presents with pain and swelling in the upper abdomen, nausea and persistent fever.
- **Renal vein thrombosis:** Patient presents with pain in the flank or lower back, decreased urine output, hematuria and swelling of the lower limbs.

Deep Vein Thrombosis

Deep vein thrombosis (DVT) is most common in the deep veins of the calf region that frequently propagates in the femoral and iliac veins, from where it embolizes to the lungs leading to life-threatening pulmonary thromboembolism characterized by sudden onset of shortness of breath and clear lungs. Venous thrombi may cause venous inflammatory changes. Inflammation of veins with thrombus formation is known as '**thrombophlebitis**'.

Risk Factors

Risk factors of deep vein thrombosis include joint replacement, immobility, surgical trauma, intravenous cannula and catheterization, varicose veins, polycythemia, disseminated malignancies; and thrombophilia (factor V mutation, lupus anticoagulant, protein C and S deficiency, antithrombin III deficiency).

- In pregnant women, weight of the fetus on femoral blood vessels slows down blood venous return. Hormonal changes and stress precipitates thrombi formation. During childbirth, thromboplastin released from amniotic fluid results into activation of coagulation system.
- Hormonal replacement therapy and therapy for carcinoma prostate increase the risk of deep vein thrombosis.
- Following orthopedic surgery (knee or hip replacement, pelvic surgery) or cardiac surgery increase the risk of DVT.

Clinical Features

Patient with deep vein thrombosis presents with swelling in one or rarely both legs, pain in calf region, red discoloration of affected leg and feeling of warmth in the affected leg. DVT can occur without noticeable symptoms in 50% of cases.

- In medicine, **Homan's sign** is considered to be sign of DVT that is defined by discomfort behind the knee upon forced dorsiflexion of the foot.
- One should keep in mind that pain and swelling in affected leg may also occur as a result of rupture of gastrocnemius muscle, osteoarthritic cyst (Baker's cyst) of knee joint and anterior compartment syndrome (skin split).
- **Superficial venous thrombus** causes swelling, edema and tenderness along the course of vein in the affected area, iliofemoral venous thrombus is formed during puerperium.
- **Migratory thrombophlebitis** is defined by formation of venous thrombus in the lower extremities, embolization and propagation as toward the heart leading to pulmonary embolization.

Diagnosis

Diagnosis of deep vein thrombosis (DVT) is established by a combination of clinical history, ECG (sinus tachycardia) and imaging techniques (e.g. radiographic venogram and ultrasonography), which provide the most specific diagnostic investigation. **Wells score system** for deep vein thrombosis consists of clinical criteria with a single point awarded for criteria. Wells score system for DVT is given in Table 3.25.

Management

High-risk patients of DVT should be identified and offered prophylactic treatment with oral anticoagulants or low-molecular heparin. Immediate systemic administration of heparin anticoagulant is the standard treatment for DVT. Prolonged warfarin anticoagulant therapy is advised in patients developing recurrent DVT.

Table 3.25 Wells score system for deep vein thrombosis (DVT)

Clinical Findings	Score System
Paralysis, paresis or recent orthopedic casting of lower leg	1
Recently bedridden >3 days or major surgery within past four weeks	1
Localized tenderness in deep vein system	1
Swelling of entire leg	1
Calf swelling 3 cm greater than other leg (measured 10 cm below the tibial tuberosity)	1
Pitting edema greater in the symptomatic leg	1
Collateral nonvaricose superficial veins	1
Active cancer or cancer treated within 6 months	1
Alternative diagnosis more likely deep vein thrombosis (Baker's cyst, cellulitis, muscle damage, superficial venous thrombosis, post-phlebitis syndrome, inguinal lymphadenopathy, external vein compression)	2

Total score points 3–8 have high probability of DVT; and 1–2 score points have moderate probability of DVT.

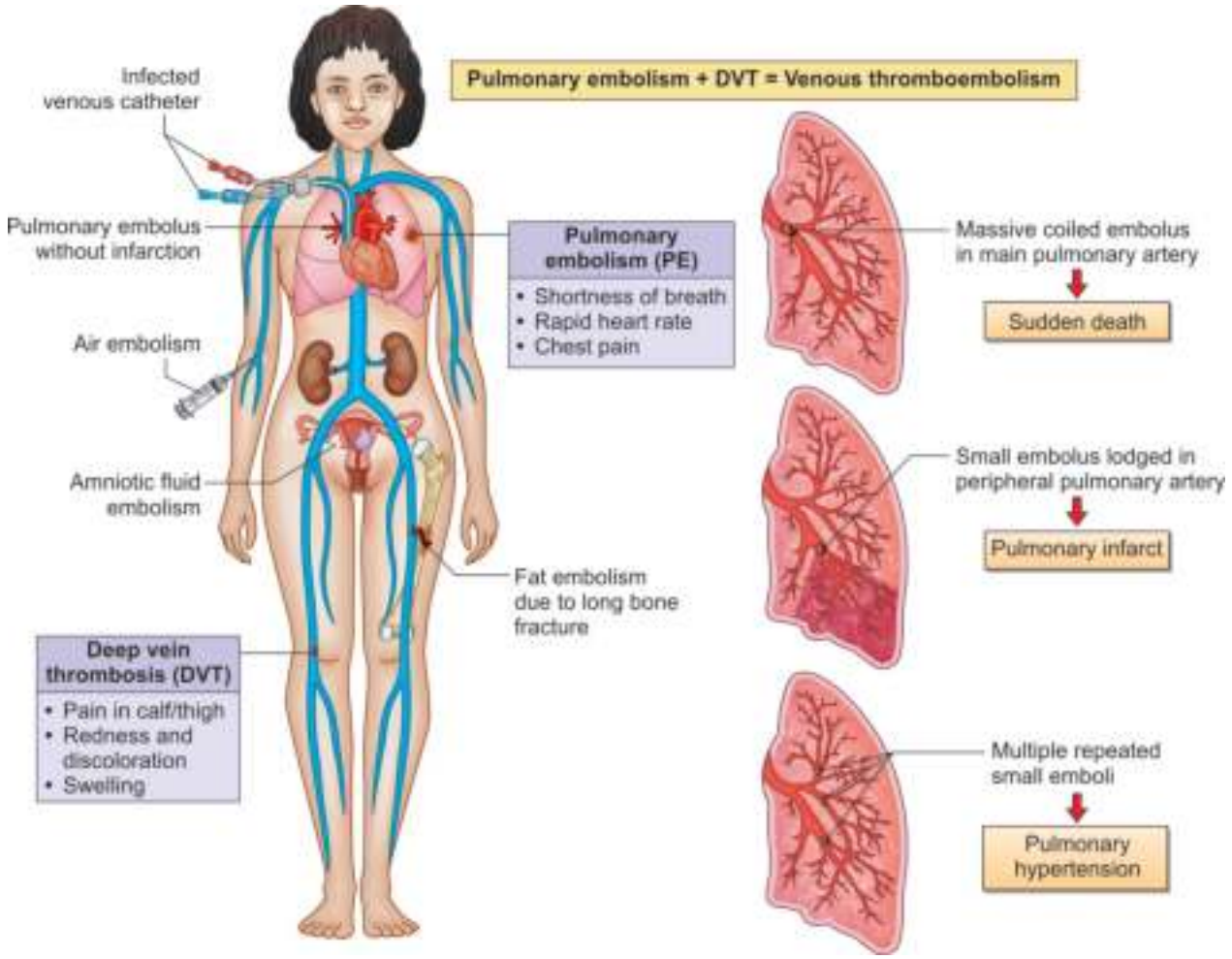


Fig. 3.26: Pulmonary embolism. It usually occurs when a blood clot called DVT in leg, travels through the bloodstream and becomes lodged in the blood vessels of the lung. This restricts blood flow to the lungs, lowers oxygen levels in lungs, increases blood pressure in the pulmonary arteries and affects other organs. Large or multiple blood clots in the lungs can be life-threatening.

Pulmonary Embolism

Pulmonary embolism (PE) is most important cause of sudden death, which most often results from thrombus formation in the deep veins of lower extremity or rarely from other deep veins of the body.

- Pulmonary embolism blocks pulmonary blood flow resulting in severe pressure increase in the pulmonary artery and right ventricle.
- Right heart strain occurs only with the most severe, large emboli that results in sudden onset of shortness of breath and fatal outcome. Pulmonary embolism is shown in Fig. 3.26. Wells diagnostic criteria of pulmonary embolism are given in Table 3.26.

Sources of Pulmonary Emboli

Sources of pulmonary emboli include thrombi in right heart (septal infarction, atrial fibrillation), tumor emboli, air embolism, pelvic veins thrombi (following pelvic or prostate surgery), fat emboli (fractures of long bones) and thrombi in deep veins of lower extremities.

Table 3.26 Wells diagnostic criteria of pulmonary embolism

Variables in Pulmonary Embolism	Wells Scoring System (Point)
Previous DVT or pulmonary thromboembolism	1
Present clinical signs of deep vein thrombosis (painful unilateral swollen warm leg)	1
Surgery or immobilization within the past four weeks (1 point)	1
Heart rate ≥100 beats/minute	1
Hemoptysis	1
Patient with active cancer disease	1
Alternative diagnosis less likely than pulmonary thromboembolism	1

Wells scoring system: 0–1 point: Low clinical probability of pulmonary thromboembolism; 2–6 points: Intermediate clinical probability of pulmonary thromboembolism; and ≥7 points: High clinical probability of pulmonary thromboembolism.

Predisposing Factors

Venous thrombosis is associated with stasis blood flow seen in elderly, debilitated, or chronically bedridden persons, joint replacement surgery, contraceptive oral pills, obesity, inherited and acquired hypercoagulable state, and congestive heart failure.

Pathophysiology

In immobilized patients, thrombi are formed most commonly in the lower extremities (deep veins of legs, femoral vein). Thrombi may also form in pelvic veins or inferior vena cava in some cases. Fragments of thrombi travel through the venous circulation and settle in branches of pulmonary artery.

Clinical Features

Pulmonary emboli may differ in size (small size, intermediate and large saddle thrombus). Clinical features of pulmonary embolism are related to the size of emboli reaching lungs.

- **Small pulmonary emboli:** Small emboli are less clinically significant. Patient presents with sudden onset of dyspnea and tachycardia. Auscultation may be normal or few rales and diminished breath sounds.
- **Intermediate size pulmonary emboli:** Intermediate size emboli increase pulmonary artery pressure leading to pulmonary hypertension. Patient may develop cor pulmonale (right-sided heart failure).
- **Large saddle thrombus:** A large venous embolus occluding the major pulmonary artery branches (bifurcation of the pulmonary artery) is known as saddle thrombus, which has sudden fatal outcome.
- **Emboli occluding pulmonary artery:** Emboli occlude pulmonary artery and decrease blood flow to pulmonary parenchyma and may cause hemorrhagic infarction in less than 10% cases of thromboembolism.
  - Pulmonary infarct is a wedge-shaped infarct located just beneath the pleura.
  - Patient presents with sudden shortness of breath, rapid breathing, chest pain, hemoptysis and bluish discoloration of the lips or finger (cyanosis).

Diagnosis

A computed tomography (CT) angiogram is standard imaging technique to establish pulmonary embolism in 95% of cases. Lower extremity Doppler ultrasound is excellent test. D-dimers testing is sensitive test in fresh new clots. D-dimers are metabolic breakdown products of fibrin. Plasmin chops up fibrin into the D-dimers.

Management

The standard of care in pulmonary embolism is administration of low molecular weight heparin and oxygen support. Later warfarin should be advised for 3–6 months after the use of heparin. Thrombolytic agents are administered in patients with hemodynamically unstable (hypotension), which activate plasminogen to plasmin. Plasmin dissolves only fresh clots. That is the reason thrombolytic agents are useful within 12 hours of myocardial infarction.

ARTERIAL THROMBOEMBOLISM

Arterial thrombi are formed in areas of active blood flow in cardiac chambers, large elastic (aorta) and muscular arteries (coronary, cerebral arteries) at the site of vascular endothelial injury, which tend to grow in a retrograde direction from the point of attachment.

- Arterial thromboembolism occurs when a blood clot or thrombus breaks off and causes partial/complete blockage of an artery that carries oxygen-rich blood from the heart to the rest of the body.
  - Partial blockage of an artery by a thrombus restricts blood flow and oxygen and induces ischemia of organ. Complete occlusion of an artery induces tissue death.
  - Arterial thromboembolism obstructing arteries supplying lower extremities below knee joints leads to ischemia and induce sudden pain, absence of arterial pulses, and a cold limb. In some cases, the limb must be amputated.
  - Arterial thromboembolism can also obstruct mesenteric artery supplying intestine produce hemorrhagic infarcts (gangrene gut). Some persons may develop myocardial infarction and cerebral stroke.
- Less common sites of arterial thromboembolism include kidneys, intestine and eyes. Arterial thromboembolism obstructing the end arteries supplying kidney and spleen produce wedge-shaped pale infarcts.
- Arterial thromboembolism is strongly linked to hyperlipidemia, hypertension, diabetes mellitus, cigarette smoking, sedentary lifestyle, recent surgery, previous stroke of cardiovascular disease, mitral stenosis and atrial fibrillation, stasis of blood flow (e.g. sickle cell anemia) and hypercoagulable state (e.g. antithrombin III deficiency, protein C deficiency, protein S deficiency, lupus anticoagulant).

Clinical Pearls: Arterial Thrombi inducing Tissue/Organ Infarction

Sudden pain is characteristic symptom of arterial thromboembolism. The most common forms of organ damage can be recognized by the following signs and symptoms.

Myocardial Infarction

- Cardiovascular disease is the leading cause of morbidity and mortality in developed countries.

- Most acute cardiovascular events are attributable to arterial thrombosis. Thrombi superimposed on ulcerated atheromatous plaque in coronary can cause myocardial infarction.
- Patient presents with chest pain, shortness of breath, profuse sweating, weakness, nausea, vomiting and palpitations.

- Atheromatous disruption through erosion or rupture creates a prothrombotic environment through activation of procoagulant factors, platelet activation by von Willebrand factor (vWF), platelet aggregation leading to thrombus formation. Simultaneously, enzymatically mediated processes that mediate endogenous fibrinolysis maintains the patency of the arteries.
- Interplay between the prothrombotic and antithrombotic pathways determines clinical outcome. If prothrombotic pathway predominates, the thrombus may propagate resulting in occlusion of the arteries.
- Thrombus achieves structural stability and resistance to dislodgement and thrombolysis through the cross-linked fibrin to vascular endothelium, thereby provide structural stability to the thrombus.

Cerebral Stroke

- Occlusion of the middle cerebral artery due to arterial thromboembolism is the most common site of arrest of arterial emboli in branches of the carotid artery resulting in cerebral infarction (liquefactive necrosis as brain lacks proteins).
- Thrombi at the junction of the internal and external carotid arteries are the causes of thrombotic brain infarcts and can also be a site of origin of emboli. Patient presents with sudden difficulty in walking, speaking and understanding, as well as paralysis or numbness of the face, arm and leg.

Peripheral Vascular Disease (Limb Infarction)

- Arterial thromboembolism obstructing arteries of leg leads to sudden pain, absence of pulses, and a cold limb.
- Patient presents with numbness, tingling sensation, pain, skeletal muscle weakness and spasm, and decreased arterial pulse in the affected lower extremity. In some cases, the limb must be amputated.

Thrombi in the Arteries

Arterial thrombosis occurs with partial or complete occlusion within large elastic and medium-sized arteries in the settings of ulcerated atheromatous plaques in abdominal aorta, coronary, cerebral and femoral arteries, which can cause systemic thromboembolism.

- Turbulence of blood flow at bifurcation of common iliac artery and abdominal aortic aneurysm can result in thrombosis. Atheromatous plaque with thrombus formation in coronary artery is shown in Fig. 3.27.
- Thrombi formed the junction of the internal and external carotid arteries may emboli to produce brain infarct (liquefactive necrosis). Ischemia in brain does not produce coagulative necrosis as it lacks proteins.

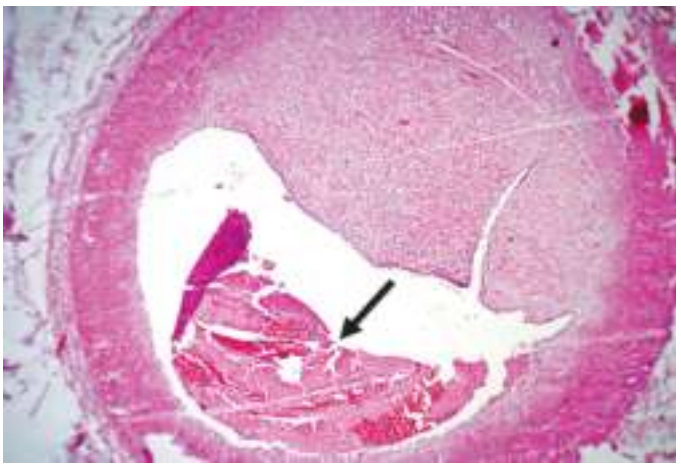


Fig. 3.27: Atheromatous plaque with thrombus formation in coronary artery (arrow) (400X). The development of atheromatous plaque and its rupture, erosion and calcification are hallmarks of atherosclerotic vascular disease. Atheromatous plaque rupture is defined by fibrous cap disruption whereby the overlying thrombus is in continuity with the underlying necrotic core.

- Arterial thrombi also undergo structural changes as thrombi grow.
  - Evolution of ST segment elevation myocardial infarction leads to doubling of the fibrin content in coronary artery thrombus each hour during clinically manifesting ischemia, whereas the relative platelet content halves each hour.
  - The structural changes in thrombus have been associated with formation of a dense, stiff fibrin network that impairs responsiveness to antiplatelet therapy and thrombolysis over time.
- Arterial thrombi are gray white and friable. Histologic examination of mature arterial thrombi demonstrates alternating pale and red areas. Pale areas of thrombus are composed of platelets held together by fibrin, while red areas are composed predominantly of red blood cells. This layering results in the '**lines of Zahn**'.

Mural Thrombi in Cardiac Chambers

Mural thrombi are formed in the left ventricle of patients due to transmural myocardial infarction.

- Mural thrombi are formed in the left atrium in rheumatic heart disease (RHD) patients with mitral stenosis and atrial fibrillation. Mural thrombi are also formed in congestive cardiomyopathy.
- Mural thrombi usually in cardiac chambers adhere to the endocardium and termed mural thrombi. Thrombi may also be formed on cardiac valvular vegetations such as infective endocarditis and nonbacterial thrombotic endocarditis.
- Mural thrombi can also develop in the settings of noninfective verrucous Libman-Sacks cardiac vegetations in systemic lupus erythematosus patients and myxoma in cardiac chambers.

EMBOLISM

Embolus is detached mass (e.g. thrombus, fat, gas, amniotic fluid), that is circulating to distant organs and eventual trapping within the vasculature resulting to partial or complete occlusion of cardiovascular system leading to dysfunction of organs. Origin of thrombi causing embolization in various organs are given in Table 3.27.

- Detachment of fragments of thrombi originating from veins (deep vein in legs, femoral vein) or heart (cardiac vegetations, myxoma) or atheromatous plaques travel in the arterial system. One-third of patients with a left atrial or left ventricular myxoma die from tumor embolization to the brain. Emboli obstruct the blood flow and cause ischemic damage of organs.
- Paradoxical emboli originate in the venous circulation and bypass the lungs, but gain access through an incompletely closed foramen ovale (most common) or atrial septal defect into the systemic circulation.
- Thromboembolism refers to obstruction of a blood vessel by a blood clot or thrombus that has dislodged from another site in the circulation.

PATHOPHYSIOLOGY OF EMBOLISM

The embolus may be a thrombus, fat globule (fat embolism), a bubble of air or other gas (gas embolism), amniotic fluid (amniotic fluid embolism) and embolism due to miscellaneous exogenous and endogenous materials. Two of the most serious conditions caused by an embolism are pulmonary embolism and cerebral stroke.

FAT EMBOLISM SYNDROME

Fat embolism syndrome refers to presence of emboli comprising of fat microglobules in the circulation, which is most often a consequence of severe trauma with long bone fractures such as femur with abundant fatty bone marrow. It can also be seen with extensive trauma to fat laden tissues, burns, and very rarely with orthopedic procedures. Fat embolic syndrome is shown in Fig. 3.28.

Pathogenesis

On long bone fracture, bone marrow fatty fragments enter the circulation, lodge in small blood vessels of the lung, skin, kidneys, brain and other organs producing ischemia and hemorrhage, which results in the clinical manifestations. Fat microglobules are converted into fatty acids, which damage vascular endothelium resulting in formation of platelet thrombi in areas of injury. Causes of fat embolism are given in Table 3.28.

Clinical Features

Patient develops potentially fatal fat embolism syndrome within 24–72 hours, which is characterized by pulmonary distress, cutaneous petechial hemorrhages, and various neurologic manifestations. Gurd and Wilson diagnostic criteria for fat embolism syndrome and laboratory findings are given in Table 3.29.

- **Pulmonary manifestations:** Patient develops dyspnea and tachypnea due to presence of fat microglobules in pulmonary capillaries leading to hypoxemia.
- **Petechial hemorrhage:** Patient develops petechial hemorrhage over the chest and upper extremities due to thrombocytopenia from platelet adhesion to microglobules of fat.
- **Neurologic manifestations:** Numerous petechial hemorrhages are produced by fat emboli in the brain, particularly in white matter known as '**brain purpura**'. Patient develops neurologic manifestations such as loss of consciousness, cerebral edema and herniation within a week in less than 10% of cases with fatal outcome. Fat emboli inducing petechial hemorrhages in brain are shown in Fig. 3.29.

Table 3.27 Origin of thrombi causing embolization in various organs

Origin of Thrombi	Embolization to Organs
Thrombi in deep veins of leg	Pulmonary thromboembolism
Mural thrombi in left ventricle	Systemic thromboembolism
Trauma to bone marrow	Fat embolism
Entry of excess air during intravenous fluid infusion, cardiothoracic surgery, delivery and trauma to veins during surgery of head and neck	Air embolism
Divers and ascent during flights	Decompression sickness
Amniotic fluid entry in circulation during delivery	Amniotic fluid embolism
Ulcerated atheromatous plaques	Atheroembolism
Tumor fragments (myxoma heart)	Tumor fragment emboli

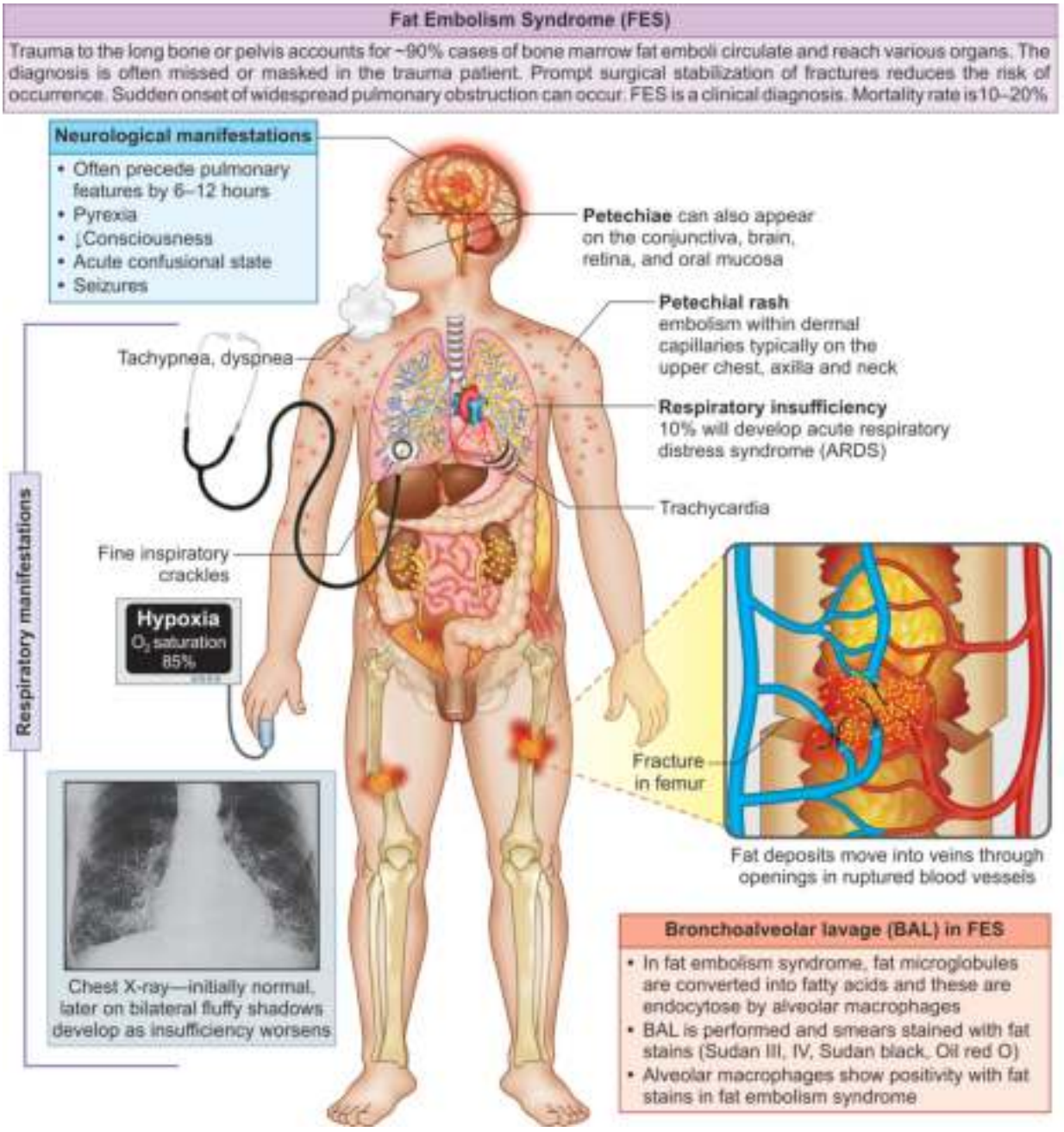


Fig. 3.28: Fat embolic syndrome. Fat embolism is characterized by fat globules within microcirculation, while fat embolic syndrome is the systemic manifestation of fat embolism, characterized by pulmonary insufficiency due to occlusion of blood flow, neurologic symptoms, anemia, thrombocytopenia and a rash. Fat embolic syndrome is most often associated with orthopedic trauma.

Laboratory Diagnosis

Bronchoalveolar lavage is done to demonstrate fat in alveolar macrophages by staining with fat stains, e.g. Sudan III, Sudan V, Sudan black and Oil red O. In fat embolism syndrome, fat microglobules are converted into fatty acids, which are taken by the alveolar macrophages. In autopsy specimens, frozen section examination reveals fat globules within the pulmonary arterioles demonstrated by fat stains. Examination of paraffin-embedded sections stained with hematoxylin and eosin reveals rounded clear holes due to dissolution of fat during processing in the small pulmonary arterial branch of lung characteristic for fat embolism syndrome.

AIR EMBOLISM

Air embolism results from the introduction of excess of air into the circulation leading to blood flow obstruction.

Table 3.28 Causes of fat embolism

Fracture of long bones
Soft-tissue trauma
Liposuction
Bone marrow infarction complicating hemoglobinopathy
Bone marrow harvest
Fatty liver induced by viral hepatitis
Pancreatitis
Intravenous injection of various oils by accident or intention
Breast augmentation
Periurethral injection to treat stress incontinence

Table 3.29 Gurd and Wilson diagnostic criteria for fat embolism syndrome and laboratory findings

Major Diagnostic Criteria
Skin (petechial hemorrhages)
Respiratory symptoms (tachypnea, dyspnea, bilateral inspiratory crepitations, hemoptysis, bilateral diffuse patchy shadowing on chest radiograph)
Neurological signs (confusion, drowsiness, coma)
Minor Diagnostic Criteria
Tachycardia (heart rate >120 per minute)
Pyrexia >38.5°C
Retinal petechial hemorrhages and fat emboli
Renal changes (anuria or oliguria)
Jaundice
Unexplained thrombocytopenia and low hemoglobin
Erythrocyte sedimentation rate increased
Fat macroglobulinemia
Fat globules in urine
Fat globules in sputum

- Patient presents with distal ischemic injury, pulmonary air embolism—sudden death, convulsions and deep coma.
- Air embolism occurs as a consequence of penetrating chest injury, clumsily performed criminal abortion, intravenous infusion of fluids and deep-sea divers.
- Air embolism occurs when atmospheric air may enter the blood circulation as a result of incision of intracranial vein during head and neck surgery. Inspiration process produces a suction effect by causing negative pressure in these veins leading to frothing of blood in right ventricle and impairment of cardiac functions.
- Penetrating chest injury leads to entry of air into the ruptured vessels resulting in air embolism.

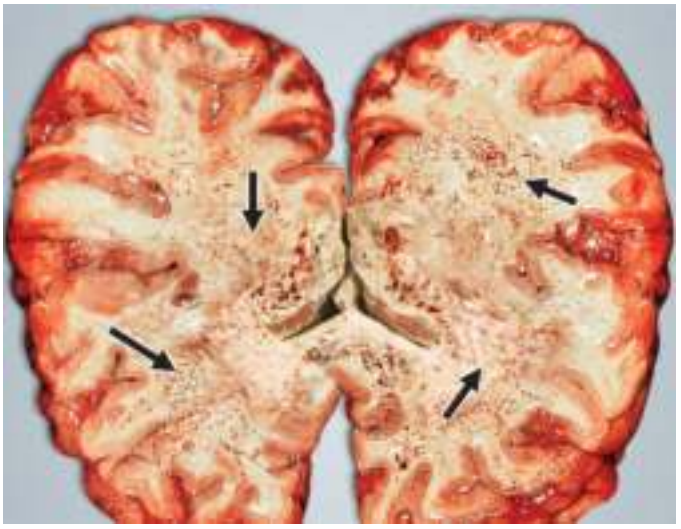


Fig. 3.29: Fat emboli inducing petechial hemorrhages in brain. The patient died due to multiple fractures of both legs in a motorcycle accident. The fat emboli caused petechial hemorrhages in the white matter throughout the brain, resulting in coma and death. The macroscopic appearance of cerebral malaria is identical to this (arrows).

Pneumothorax due to rupture of a pre-existing subpleural bleb or pulmonary embolism is responsible for dyspnea and pleuritic pain. Due to improper monitoring of intravenous infusion, excess of air (more than 100 cc) may result in air embolism.

Clinical Pearls: Decompression Sickness (Caisson Disease)

- Decompression sickness is a form of air embolism observed in deep-sea divers due to sudden change in atmospheric pressure, who return to the surface too rapidly.
- Insoluble nitrogen coming out of blood forms multiple gas emboli obstructs blood flow to skeletal system, central nervous system and other tissues.
- Patient presents with musculoskeletal pain in joints, muscle and bones called 'bends'.
- Obstruction to circulation leads to small infarcts. Nitrogen has an affinity for adipose tissue; hence obese persons are at increased risk for Caisson disease.

AMNIOTIC FLUID EMBOLISM

Amniotic fluid embolism refers to the entry of amniotic fluid containing fetal squamous cells, lanugo hair, vernix, and mucin following tear in the placental membranes may gain access to uterine and cervical veins into the maternal circulation, which occurs during labor or immediate postpartum period.

- Patient develops abrupt onset of dyspnea, cyanosis, hypotension and bleeding manifestations as a result of amniotic fluid emboli contents.
- Amniotic fluid embolism is most often confirmed at autopsy by histopathologic examination, which

reveals presence of squamous epithelial cells, lanugo hair, fat globules, mucinous material, marked pulmonary edema, diffuse alveolar damage and fibrin clots in the blood vessels.

Neonatal Respiratory Distress Syndrome in Amniotic Fluid Embolism

Diffuse alveolar damage with hyaline membranes is a feature of neonatal respiratory distress syndrome in amniotic fluid embolism.

- Severe damage to alveolar-capillary membrane by amniotic fluid emboli contents leads to formation of hyaline membrane and pulmonary edema.
- Due to impaired exchange of gases across alveolar-capillary membrane, patient develops dyspnea and cyanosis.
- Pulmonary edema in neonatal respiratory distress syndrome does not resolve. Neonate presents with signs of respiratory distress.

Disseminated Intravascular Coagulation in Amniotic Fluid Embolism

Amniotic fluid embolism is one of the major obstetric causes of disseminated intravascular coagulation (**DIC**) as a result of activation of coagulation system. Patient presents with bleeding manifestations due to consumption of coagulation factors and fibrin (i.e. defibrinating syndrome) results in formation of microthrombi with ischemic changes in various organs. There is sudden onset of cyanosis and shock, followed by coma and death.

EMBOLISM DUE TO EXOGENOUS AND ENDOGENOUS MATERIALS

Embolism may be caused by fragments of tumors such as clear cell carcinoma of kidney, lung carcinoma and malignant melanoma.

- Atherosclerosis means hardening (sclerosis) or loss of elasticity of large elastic arteries and medium-sized arteries due to atheromatous plaque formation. Emboli of atheromatous plaque material to distant sites may result in infarction (spleen, kidney, small intestine).
- Various exogenous and endogenous substances may act as emboli, which include fragments from tissue and placenta.
- Other emboli are formed by parasites, bullets, sutures and barium contrast media.

INFARCTION

An infarct is an area of necrosis resulting from ischemia caused by obstruction of arterial or venous blood supply in a particular tissue or organ such as myocardium, brain, lung, kidneys, spleen, bowel, ovaries, testes, and lower limb.

- Ischemia produces coagulative necrosis in solid organs rich in proteins such as heart, kidney and spleen. Coagulative necrosis refers to light microscopic changes in the dead or dying cells as a result of ischemia.
- Cellular outlines are maintained but structural details are lost. Widespread tissue necrosis is called an 'infarction'. **Liquefactive necrosis** occurs in brain (proteins deficient in brain) and suppurative infections.
- Intravascular causes of organ infarction include arterial occlusion by thrombotic or embolic events (99% of all infarcts), local vasospasm and expansion of an atheromatous plaque occluding lumen.
- Extravascular causes of organ infarction include arterial occlusion due to compression by a tumor, twisting of the blood vessels: testicular torsion and bowel volvulus, edema inducing ischemia of hernial sac entrapment, and traumatic rupture of the blood vessels. Causes of infarction depending on blockage of blood vessel are given in Table 3.30.

FACTORS INFLUENCING INFARCT FORMATION

Factors that influence development of organ infarct include the nature of blood supply, rate of development of the vascular occlusion and the vulnerability of tissue or organ to hypoxia and the blood oxygen content.

ANATOMICAL PATTERN OF ARTERIAL SUPPLY

Extent of tissue injury due to ischemia depends on the anatomical pattern of arterial supply of the tissue or organ. There are four patterns of arterial supply to various organs described as under.

Organs Infarct with Single Arterial Supply Without Rich Anastomoses

Some organs such as kidney, spleen and retina have single arterial supply without significant anastomosis. Occlusion of arteries supplying these organs produce white (pale) infarcts.

Table 3.30 Causes of infarction depending on blockage of blood vessel

Lesion	Artery Involved	Infarct in Organ
Blockage of arteries		
Atherosclerosis	▪ Coronary arteries ▪ Cerebral arteries ▪ Renal arteries	▪ Myocardial infarction ▪ Cerebral stroke ▪ Renal infarct
Thromboembolism in arteries (originating in heart and blood vessels)	▪ Coronary arteries ▪ Renal arteries ▪ Splenic artery	▪ Myocardial infarction ▪ Renal infarct ▪ Splenic infarct
External pressure on arteries	Arteries of organ	Organ infarct
Blockage of arteries and veins		
Strangulated hernia	Mesenteric vessels	Gangrene intestine
Volvulus intestinal loop	Mesenteric vessels	Gangrene intestine
Intussusception	Mesenteric vessels	Gangrene intestine
Torsion testes	Testicular vessels	Testicular infarct
Blockage of small arteries		
Microthromboemboli (originating from infective endocarditis)	Renal artery branches	Renal infarct
Sickle cell anemia	Renal peritubular capillaries in the medulla due to the low O_2 tension in the medulla	Renal infarct
Bed sores	Occlusion of skin, small vessels	Gangrene of affected skin
Decompression sickness	Dissolved gases forming microemboli occluding vessels	Infarcts of organs

Organ Infarct with Single Arterial Supply with Rich Anastomoses

Intestine supplied by superior and mesenteric arteries has multiple anastomoses. Large bowel has arterial blood supply from more than one artery. Splenic flexure and rectosigmoid junction are the most common sites of infarcts in colon. These are less vascularized areas known as 'watershed regions', which include splenic flexure (junction of the superior and inferior mesenteric arteries) and rectosigmoid junction (junction of left colic and sigmoid—superior rectal branches of the inferior mesenteric artery).

Organs Infarct with Dual Blood Supply

Lungs and liver have dual blood supply, hence less prone to ischemia.

- Liver receives blood supply from portal vein and hepatic artery. Occlusion of portal vein may produce **red infarct** as liver keeps on receiving arterial supply from hepatic artery.
- Lung is supplied by pulmonary and bronchial arteries. Occlusion of pulmonary artery by emboli produces red infarct, because lung keeps on receiving blood from bronchial artery. Hemorrhagic lung infarct is raised, wedge-shaped area with red blue discoloration that extends to the pleural surface. Majority of lung infarcts are located in the lower lobes. Perfusion is greater than ventilation in the lung's lower lobes. Patient develops dyspnea or pleuritic chest pain on inspiration, expiratory wheezing, tachycardia, productive cough (sputum may be blood-tinged), low-grade fever and pleural effusion (fibrinous exudate).

Organs Infarct with Parallel Blood Supply

Forearm is supplied by radial and ulnar arteries running in parallel pattern. Vitality of forearm due to occlusion of one artery is maintained by alternative another artery.

VULNERABILITY OF TISSUES TO HYPOXIA

Ischemic injury depends on the type of cells or tissues undergoing ischemic necrosis. Hypoxia adversely affecting tissues in descending order include neurons, myocardium, liver, skeletal muscle and fibroblasts. Vulnerability of cells/tissues/organs) to ischemic irreversible cell injury are given in Table 3.31.

Nervous System

Complete interruption of blood supply to brain causes irreversible damage to neurons within 3–5 minutes. Cerebellum's Purkinje cells and hippocampus area are more susceptible to ischemic injury.

Table 3.31 Vulnerability of cells/tissues/organs to ischemic irreversible cell injury

Organs	Cells/Tissues	Duration of Development of Irreversible Cell Injury
Nervous system	Neurons (cerebellum Purkinje fibers and hippocampus region most susceptible to ischemic injury)	3–5 minutes
Heart	Myocardium	20–30 minutes
Liver	Hepatocytes	1–2 hours
Skeletal muscle	Skeletal muscle fibers	Many hours
Fibrous tissues	Fibroblasts	Resistant to ischemic changes

Myocardium and Liver

Ischemic necrosis of myocardial cells and hepatocytes cause irreversible cell death within 1–2 hours.

Skeletal Muscles

Ischemic necrosis of skeletal muscle cells causes irreversible cell death within many hours.

BLOOD OXYGEN CONTENT

Blood oxygen content and cardiovascular system play important role in maintenance of arterial supply to the tissues. Disorders of blood and cardiovascular system adversely affect the arterial supply to the tissues or organs, which include blood loss, shock, sickle cell anemia, cardiac failure and atheromatous plaques in large elastic and medium size arteries. Complete occlusion of arteries produces more severe ischemic necrosis of tissues or organs.

RAPID DEVELOPMENT OF INFARCT

Sudden occlusion of arteries produces more severe and rapid ischemic tissue injury, because tissue or organ takes more time for development of collateral blood supply.

TYPES OF INFARCT

Infarct is gross manifestation of coagulative necrosis in organs, which is of two types, i.e. pale/white infarct and hemorrhagic/red infarct depending on the consistency of the tissue. Infarcts may be recent or healed lesion. Infarcts are classified based on the color, age, and presence or absence of microbial infection.

- Based on color, there are two types of infarcts, i.e. pale/white infarcts, and hemorrhagic/red infarcts. Pale/white infarcts occur in kidneys, spleen and heart. Hemorrhagic/red infarcts are common in lungs, small intestine, ovaries and testes. Differences between pale/white and hemorrhagic/red infarcts are given in Table 3.32.
- Septic (infective) infarcts occur when bacterial vegetations from a heart valve embolize or when microbes seed an area of necrotic tissue. Such infarcts are converted into abscess with a corresponding greater inflammatory response. Septic infarcts get

Table 3.32 Differences between pale/white and hemorrhagic/red infarcts

Comments	Pale/White Infarcts	Hemorrhagic/Red Infarcts
Arterial circulation	Single arterial supply	Dual arterial supply
Etiology	Occlusion of artery by thrombi, emboli, complicated atheromatous plaque and vasospasm	Occlusion of artery or vein by thromboembolic phenomenon or torsion
Consistency of organ	Solid consistency	Loose texture organs permitting blood
Organs involved	Heart, kidneys, spleen, digits of lower limb (cystic infarct in brain)	Lung, small intestine, testis and ovaries
Morphology of infarcts	▪ Small size infarcts ▪ Wedge shaped, with the occluded vessel at the apex and the periphery of the organ forming the base	▪ Large-sized infarcts ▪ Well circumscribed, firm and dark red infarcts
Shape of infarct	Zone of hyperemic at the periphery of infarct sharply defines margins	Margin of infarct not well defined. Fibrinous exudates present on serous membrane of organs
Edema in organ	Absent in organs	Present in organs
Light microscopy	Coagulative necrosis in all solid organs except brain*	Coagulative necrosis in all organs involved

**Liquefactive necrosis in brain occurs, because brain is deficient in proteins.*

organized. Bland infarcts are sterile (non-infective). No organisms are demonstrated in these infarcts.

PALE/ WHITE INFARCT

Pale/white infarct is secondary to the sudden occlusion of a vessel, which most common occurs in solid organs such as heart, kidney, and spleen. Increased density of solid tissue prevents RBCs from diffusing through necrotic tissue.

- Pale/white infarct produces wedge-shaped with apex pointing to the site of obstruction, and the base of infarct at the periphery of the organ.
- The cellular debris of pale/white infarct is degraded by neutrophils, and later by macrophages. Granulation tissue eventually forms, which is to be replaced ultimately by a scar.

Myocardial Infarct

Myocardial infarct is most often caused by ischemia related to coronary artery occlusion involving >75% of lumen by atheromatous plaque. Coronary artery occlusion causing myocardial infarction is shown in Fig. 3.30A and B.

- Major risk factors of atherosclerosis are hyperlipidemia, hypertension, tobacco smoking and diabetes mellitus.
- The coronary vessels most commonly involved in decreasing order of frequency are anterior descending branch of left anterior discending (LAD) of left coronary artery in 50%, right coronary artery (RCA) in 30% and left circumflex artery (LCA) in 20%. Atheromatous plaques are observed in proximal part of anterior descending artery.
- Complete occlusion of coronary artery produces transmural myocardial infarct. Partial coronary artery occlusion leads to subendocardial infarct limited to the interior one-third to inner half of the left ventricle. Due to severe reduction of blood flow in coronary artery causes circumferential myocardial infarct.

Renal Infarct

Renal infarct refers to ischemic necrosis due to occlusion of interlobar or larger branches of the renal artery most often caused as a result of thromboembolism. Patient presents with sudden onset of flank pain and hematuria.

Pathogenesis

The microthrombi originating from mural endocardium in the settings of acute myocardial infarction, and infective endocarditis can occlude renal artery resulting in renal infarct. Less common causes of renal artery occlusion inducing renal infarct include severe atheromatous plaque and sickle cell nephropathy. Sickling of red blood cells may occur in peritubular capillaries in the medulla due to the low O_2 tension in the medulla leading to renal ischemia. The glomeruli are conspicuously congested with sickle cells.

Gross Morphology

Irregular wedge-shaped pale/white infarcts are present in the renal cortex within one week. Old renal infarcts have a V-shaped appearance due to scar tissue. Apex of renal infarct is pointing towards cortex and base towards medulla.

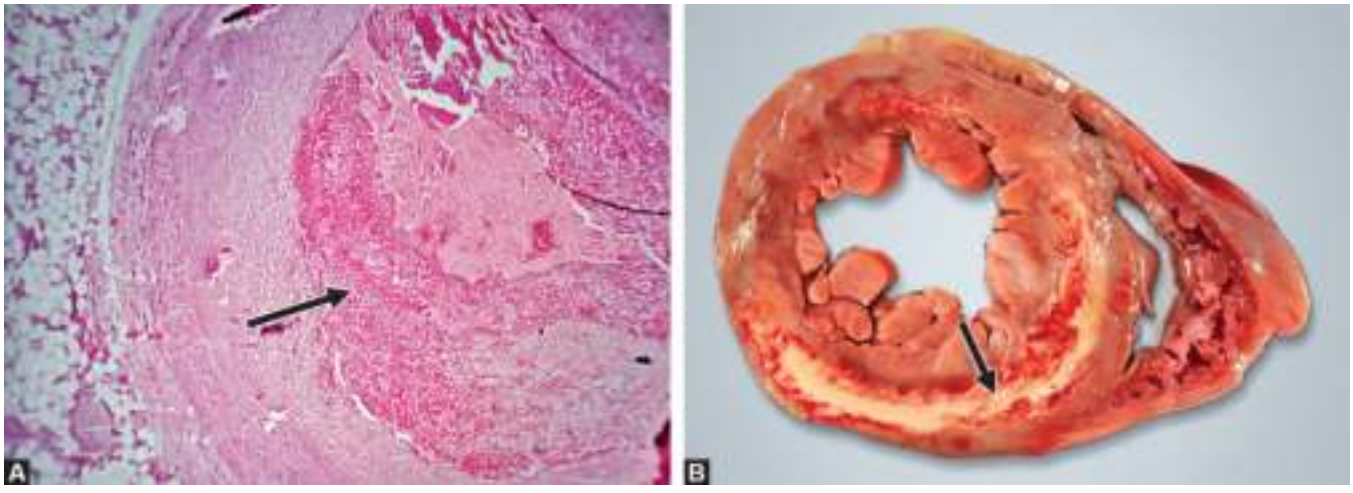


Fig. 3.30A and B: Coronary artery occlusion causing myocardial infarction. (A) Complicated atheromatous plaque with thrombus formation and complete occlusion of coronary artery (arrow) (400X), (B) myocardial infarct (arrow). Coronary artery occlusion can lead to myocardial infarction. The immediate and most common cause of coronary artery obstruction is the thrombus formation or rupture of atheromatous plaque. Patient presents with chest pain, pain radiating to jaw, neck or back and shortness of breath.

Light Microscopy

Histopathologic examination of renal infarct shows coagulative necrosis. Cellular outlines of renal tubules and glomeruli are maintained with loss of structural details. There is presence of polymorphonuclear cells in acute inflammatory phase followed by macrophages in later phase in the margin of renal infarct.

Splenic Infarct

Splenic infarct refers to ischemic necrosis due to occlusion of splenic artery and its branches most often caused as a result of thromboembolism.

- The microthrombi originating from the heart such as mural endocardium in the settings of acute myocardial infarction and infective endocarditis occlude splenic artery leading to splenic infarct.
- Splenic pale/white infarcts are most often multiple and wedge-shaped in appearance.
- Histopathologic examination of splenic infarct shows coagulative necrosis. Cellular outlines are maintained with loss of structural details. There is presence of polymorphonuclear cells in acute inflammatory phase followed by macrophages in later phase in the margin of splenic infarct. Gross morphology of pale/white infarct in spleen is shown in Fig. 3.31.

HEMORRHAGIC/RED INFARCT

Hemorrhagic/red infarct may result from either arterial or venous occlusion, which occurs principally in the loose-textured well vascularized organs such as lungs, small intestine, ovaries, and testis. Splenic vein occlusion due to thrombosis causes hemorrhagic/red infarct in spleen. Hemorrhagic infarct allows RBCs to diffuse through necrotic tissue. Gross examination reveals wedge-shaped hemorrhagic/red infarct with dark red to purple discoloration extending to the surface of organ.

Fig. 3.31: Gross morphology of pale/white infarct in spleen. Infarct is a localized area of tissue necrosis caused by arterial occlusion and is usually seen in the spleen, kidney and heart. Pale/white infarct lacks hemorrhage and limited red blood cells accumulation, when compared to hemorrhagic infarct (arrow). (*Courtesy:* Department of Pathology, Sapthagiri Institute of Medical Sciences, Bengaluru.)

Pulmonary Hemorrhagic/Red Infarct

Each lung receives blood supply from pulmonary and bronchial arteries. Pulmonary artery embolism produces wedge-shaped area of hemorrhagic/red infarct extending to the pleural surface. Hemorrhagic/red infarct area receiving constant blood supply from normal bronchial arteries, and allows RBCs to diffuse through necrotic tissue. Gross examination of pulmonary infarct reveals wedge-shaped area of hemorrhage extending to the pleural surface due to embolus in one of the pulmonary artery tributaries. Pulmonary hemorrhagic/red infarct is shown in Fig. 3.32.

Intestinal Hemorrhagic/Red Infarct

Small intestine has rich blood supply with extensive collateral circulation. Occlusion of mesenteric artery causes hemorrhagic/red infarct. Volvulus (segment of gut twisting on its mesentery) and strangulated hernial sac cause infarct in small intestine. Red blood cells diffuse through necrotic tissue. On gross examination, hemorrhagic/red infarcts are sharply circumscribed, firm, and dark red to purple. Gross morphology of

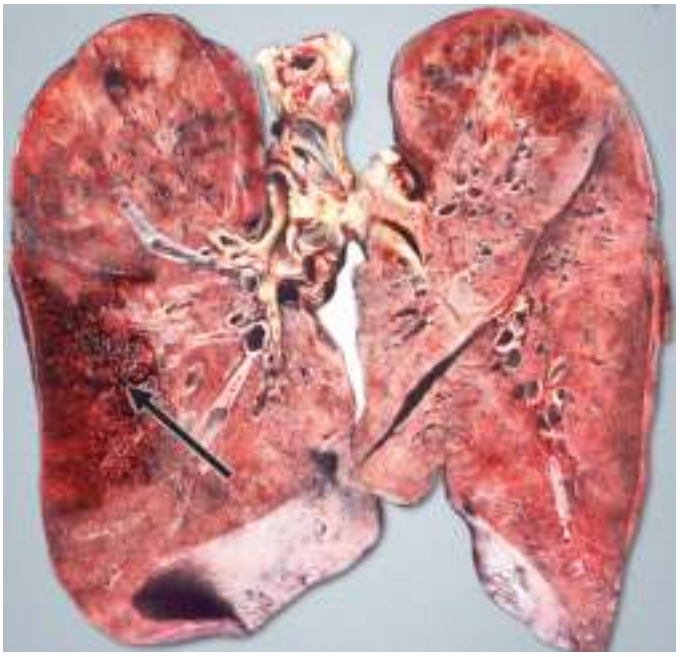


Fig. 3.32: Pulmonary hemorrhagic infarct. A wedge-shaped pulmonary hemorrhagic infarct is seen extending to pleural surface (arrow). Hemorrhage in pulmonary infarct is attributed to the dual arterial blood supply of lung. Deep vein thrombosis (DVT) is the most common source of pulmonary embolism.

Fig. 3.33: Gross morphology of ischemic bowel disease (gangrene intestine). External surface shows dusky blackish discoloration. Ischemic bowel disease represents an irreversible cell injury that occurs due to sudden occlusion of arterial blood supply by a thrombus leading to ischemia involving short or long segments of bowel. Splenic flexure (Griffiths point) and rectosigmoid junction (Sudek's point) are watershed areas prone to ischemia.

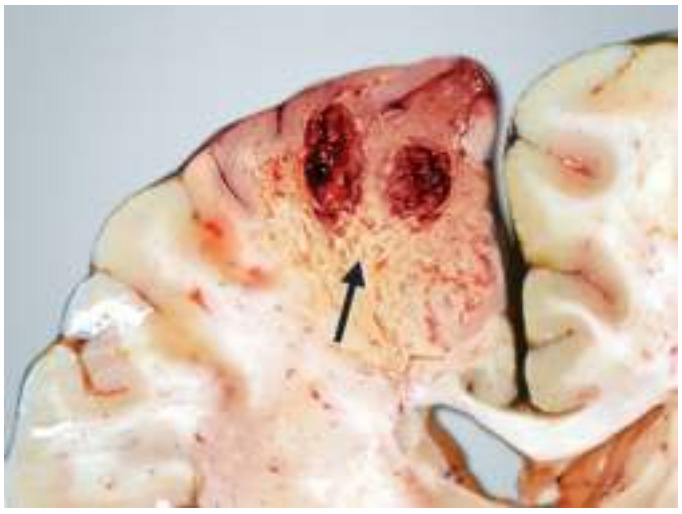


Fig. 3.34: Liquefactive necrosis in brain. Cut surface of the brain shows liquefactive necrosis with formation of cystic spaces on resolution of infarct (arrow). Liquefactive necrosis is type of necrosis which results in a transformation of the tissue into liquid viscous mass as a result of lysosomal release of digestive enzymes.

ischemic bowel disease (gangrene intestine) is shown in Fig. 3.33.

Hemorrhagic/Red Infarct of Testis or Ovaries

Torsion of testis or ovary and postoperative adhesions may cause venous occlusion results in hemorrhagic/red infarcts giving a bluish discoloration.

Pale/White Myocardial Infarct Undergoing Hemorrhagic/Red Infarct

Myocardial infarct is an example of white/pale infarct, but a hemorrhagic/red infarct occurs following spontaneous reperfusion of infarct area due to therapeutically induced lysis of the occluding thrombus.

BRAIN INFARCT

Cerebrovascular diseases are the most common group of central nervous system disorders. Complicated atheromatous plaques in arteries supplying brain at the bifurcation of carotid arteries, emboli arising from cardiac mural thrombi, vegetations of infected endocarditis valves, clumps of malignant tumor cells, bubbles of air, or droplets of fat into middle cerebral artery cause cerebral ischemia leading to liquefactive necrosis and formation of cystic spaces containing creamy necrotic debris in the brain.

Watershed Areas in Brain

Cerebral infarction occurs in the boundary zone between the territories supplied by middle cerebral and anterior cerebral arteries. Watershed areas of the arterial territory are most remote from the patent arterial stems and thus most vulnerable to the effects of cerebral underperfusion.

Gross Morphology

Brain does not contain proteins; hence ischemia produces liquefactive necrosis resulting in formation of cystic spaces containing creamy liquid necrotic debris by hydrolytic enzymes. Liquefactive necrosis in brain is shown in Fig. 3.34.

Light Microscopy

Microscopically, the cystic space in brain infarct contains necrotic cell debris, macrophages filled with phagocytosed material. The cyst wall is formed by proliferating capillaries, inflammatory cells, and neuroglial cells (gliosis) in brain.

SHOCK

Shock is a life-threatening clinical state as a result of decreased blood flow throughout the body resulting in reduced perfusion of tissues, impaired oxygenation of tissues and multiple organs failure. Shock may result from significant blood loss, severe allergic reaction, severe bacterial infections, severe/third degree burns,

Table 3.33 Type and causes of shock

Type of Shock	Causes	
Major types of shock		
Hypovolemic shock	▪ Severe trauma, major surgery, obstetrical related massive blood loss ▪ Severe burns ▪ Dehydration due to vomiting and diarrhea	▪ Diabetic precoma state ▪ Vasodilation (severe allergic reaction and bacterial sepsis)
Cardiogenic shock	▪ Myocardial infarction ▪ Ventricular arrhythmias ▪ Cardiomyopathy ▪ Cardiac tamponade (hemopericardium)	▪ Acute valve in competence ▪ Dissecting aortic aneurysm ▪ Massive pulmonary embolism
Shock associated with systemic inflammation	▪ Septic shock due to gram-positive, gram-negative bacterial infections resulting to vasodilatation, and fungal infections	▪ Anaphylactic shock: Type 1 hypersensitivity (excessive histamine resulting to vasodilatation)
Shock related to uncommon condition		
Neurogenic shock	▪ Spinal administration of anesthetic agent ▪ Spinal cord injury	▪ Head injury
Endocrine shock	▪ Adrenal failure	▪ Myxedema

Table 3.34 Pathologic findings in organs in shock

Organ	Pathologic Findings
Brain	Edema and focal ischemic changes
Heart	Subendocardial infarct
Lungs	Edema, hemorrhage, hyaline membranes
Kidneys	Acute tubular necrosis
Liver	Centrilobular congestion and necrosis
Intestine	Mucosal petechiae, foci of necrosis

dehydration, and cardiac failure. Type and causes of shock are given in Table 3.33. Pathologic findings in organs in shock are given in Table 3.34.

CATEGORIES OF SHOCK

There are mainly five categories of shock, which include hypovolemic shock, cardiogenic shock, septic shock, anaphylactic shock and neurogenic shock. Pathophysiologic findings in hypovolemic, cardiogenic and endotoxic shock are given in Table 3.35.

Table 3.35 Pathophysiologic findings in hypovolemic, cardiogenic and endotoxic shock

Characteristics	Hypovolemic Shock	Cardiogenic Shock	Endotoxic Shock
Cardiac output	Decreased	Decreased	Increased
Peripheral vascular resistance	Increased	Increased	Decreased
Left ventricle end diastolic pressure	Decreased	Increased	Decreased

HYPOVOLEMIC SHOCK

Hypovolemic shock is characterized by circulatory collapse due to acute reduction of blood volume resulting in circulatory dysfunction and inadequate tissue perfusion, which most often occurs due to acute blood loss >20% of blood volume or reduction of body fluids.

Etiopathogenesis

Hypovolemic shock is categorized into two subtypes: hemorrhagic hypovolemic shock and nonhemorrhagic hypovolemic shock.

- **Hemorrhagic hypovolemic shock:** Common causes of hemorrhagic hypovolemic shock include gastrointestinal tract bleed (e.g. esophageal varices, peptic ulcer disease, diverticulosis), trauma, ruptured abdominal atheromatous aneurysm, tumor eroding into major blood vessel and spontaneous bleeding due to prolonged anticoagulant use.
- **Nonhemorrhagic hypovolemic shock:** Common causes of nonhemorrhagic hypovolemic shock include severe/third degree burns, **Stevens-Johnson syndrome**, fluids loss from severe diarrhea or vomiting, intestinal obstruction, peritonitis and acute pancreatitis. Shifting of fluid in pleural cavity, pericardial cavity or peritoneal cavity can also cause nonhemorrhagic hypovolemic shock.

Pathophysiology

In hypovolemic shock, body's compensatory mechanism cannot maintain circulation for a long time. Blood pressure starts declining dramatically.

- **Compensatory mechanism:** In the initial phase of hypovolemic shock, body tries to compensate for fluid loss and maintains tissue/organ perfusion by

increasing cardiac output, sodium and water retention, synthesis of antidiuretic hormone.

- Decreased blood supply to kidney leads to synthesis of renin, which stimulates liver to release angiotensinogen, which form angiotensin I and converted to angiotensin II in lungs.
- Angiotensin II increases peripheral resistance and stimulates aldosterone production by adrenal cortex.
- Aldosterone acts on distal convoluted tubules of kidneys to absorb sodium and water. Compensatory mechanism in controlling hypovolemic shock by regulating of blood pressure is shown in Fig. 3.35.

- **Failure of compensatory mechanism:** If compensatory mechanism fails, blood pressure continues to drop leading to hypotension and cessation of tissue perfusion. Cardiac ischemia and arrhythmias may develop.

Clinical Features

Patient develops metabolic acidosis, cyanosis, cold clammy skin, thready pulse, tachycardia, perspiration, and renal failure associated with decreased urine output (oliguria).

Laboratory Diagnosis

Blood biochemistry in hypovolemic shock shows increased potassium, blood urea nitrogen and creatinine. During hemorrhage, there is no initial effect on hemoglobin and hematocrit concentration. Plasma is replaced

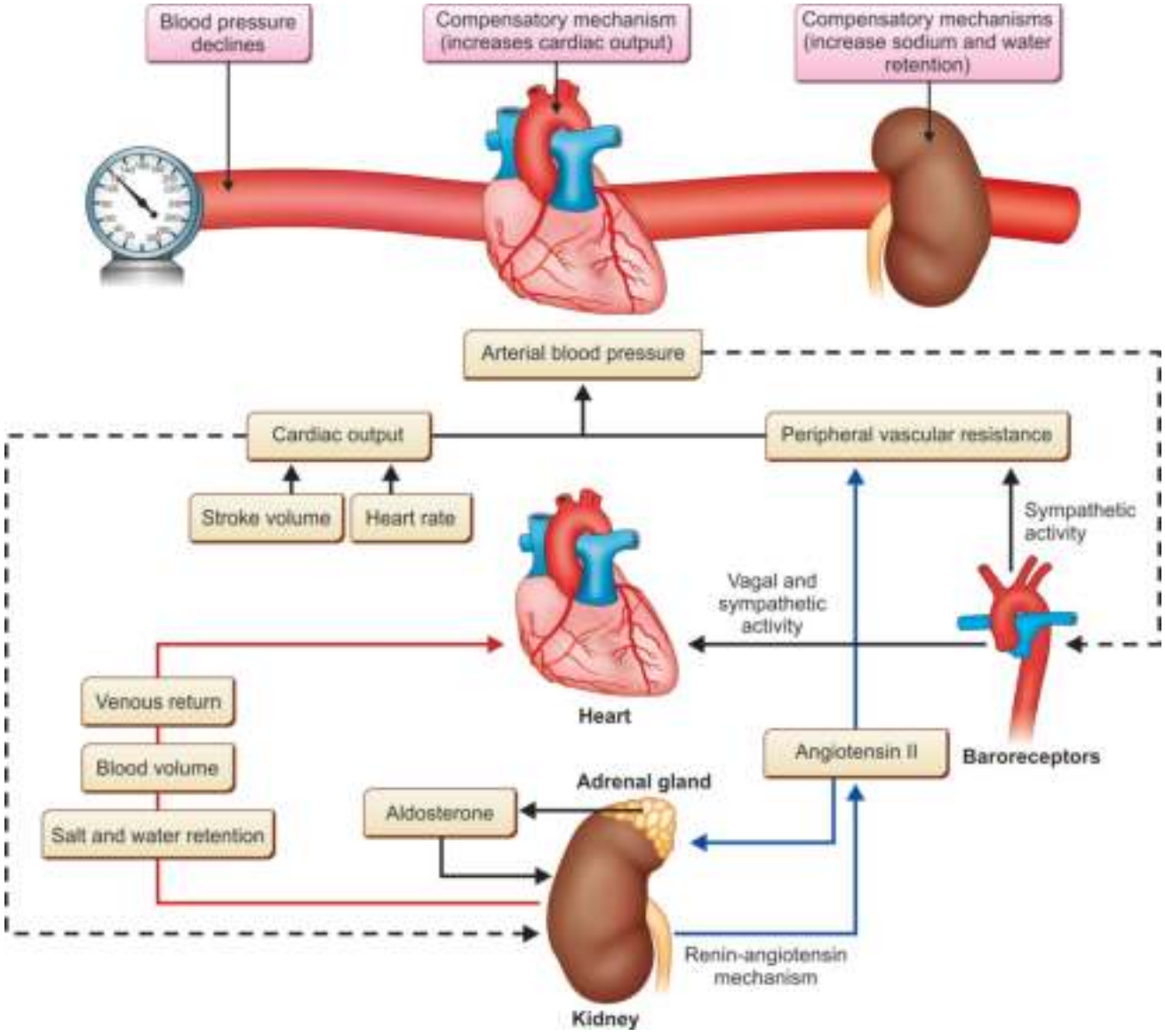


Fig. 3.35: Compensatory mechanism in controlling hypovolemic shock by regulating of blood pressure. The solid lines represent the mechanisms for kidneys and baroreceptor control of blood pressure through changes in cardiac output and peripheral resistance. The dashed lines represent the stimulus for regulation of blood pressure by baroreceptors and kidney.

first with fluid from interstitial space. Absolute neutrophilic leukocytosis is the first hematologic sign of hemorrhage. RBCs production response in the bone marrow begins in 5–7 days after hypovolemic shock.

CARDIOGENIC SHOCK

Cardiogenic shock is a life-threatening condition in which heart suddenly cannot pump out enough oxygen rich blood to the vital organs to meet body's demand most often caused by myocardial infarction.

- Other causes of cardiogenic shock include viral myocarditis, cardiomyopathies, cardiac arrhythmias, pulmonary embolism, severe mitral insufficiency, rupture of papillary muscles or chordae tendineae, rupture of ventricular free wall aneurysm, cardiac tamponade and rarely atrial myxoma.
- Patient presents with dyspnea as a result of pulmonary edema, cold, clammy skin due to vasoconstriction, hypotension, and rapid weak pulse due to compensatory response to decreased cardiac output. Clinical presentation varies related to organ involved and finally development of renal failure.

SEPTIC SHOCK

Septic shock is a serious condition that occurs when a body wide infection leads to life-threatening low-blood pressure, an altered mental state with organs dysfunction. Septic shock can be caused by bacteria and fungi.

- Septicemia with gram-negative organisms (*Escherichia coli*) originating from a urinary tract infection is the most common cause of septic shock. The invading bacteria are responsible for the release of endotoxin, a lipopolysaccharide (LPS), which binds to the surface of monocytes/macrophages.
- Septic shock due to gram-positive bacteria occurs in immunocompromised patients and persons undergoing invasive procedures. *Staphylococcus aureus* releases toxic molecules (so-called superantigens) resulting in 'toxic shock syndrome'. Septic shock causes multiple organs dysfunction syndrome (MODS) characterized by systemic shut down of vital processes and lactic acidosis, which requires major intervention to maintain homeostasis. Multiple organs dysfunction is most common cause of death.
  - At first, septicemia (bacteremia) begins with weakness, chills, and a rapid heart and respiratory rate. Left untreated, toxins produced by bacteria can damage the small blood vessels resulting in leakage of fluid into the surrounding tissues.
  - Final diagnosis of septicemia (bacteremia) is made by culturing the organisms from the blood. Major pathogenic pathways of septic shock are shown in Fig. 3.36. Synthesis of chemical mediators in varying quantity in septic shock stages are given in Table 3.36.

Pathogenesis of Septic Shock

Infectious microorganisms in the blood circulation induce dysregulated homeostatic response by synthesis of proinflammatory and counter-inflammatory cytokines causing hemodynamic decompensation in septic shock. Both immune activation and immunosuppression are present in septic shock.

- Severity of immune response depends on interactions between host innate response and pathogen characteristics. Closely interrelated host's inflammatory response and coagulation system play central role in the pathophysiology of septic shock.
- Abnormalities in macrovascular, microvascular endothelial cells and mitochondrial function contribute to the hemodynamic changes and organs failure in septic shock patients. Sepsis mortality increases with successive failure of organs.

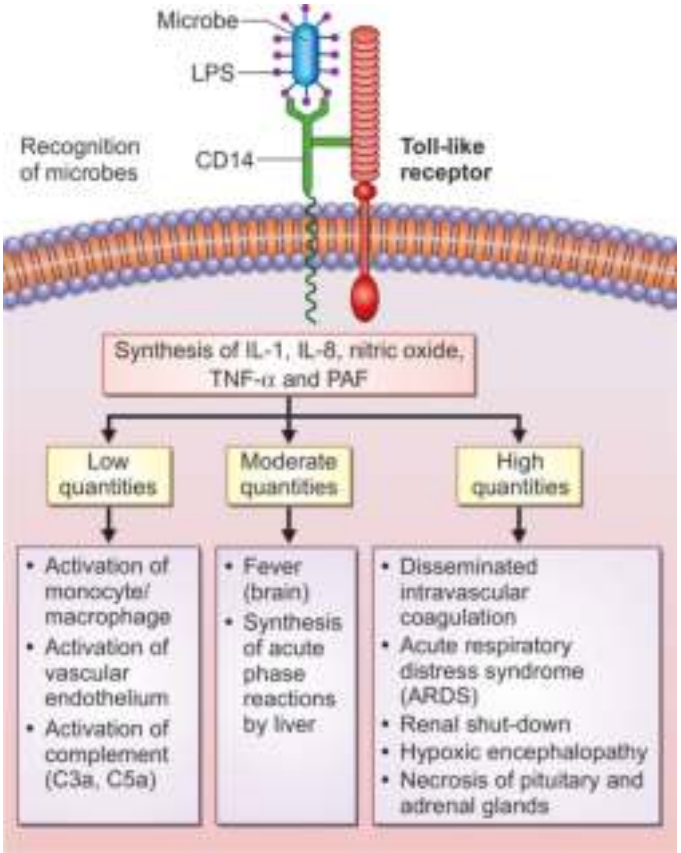


Fig. 3.36: Major pathogenic pathways of septic shock. Microbes release pathogen-associated molecular patterns (PAMPs) that bind to receptors belonging to the family of pattern recognition receptors (PRRs) and initiate cell signaling leading to enhanced activation of cytokines in varying quantity and reactive oxygen species (ROS). Depending on the quantity of cytokines, patient develops clinical manifestations.

Table 3.36 Synthesis of chemical mediators in varying quantity in septic shock stages

Quantity of Chemical Mediators	Chemical Mediators Actions	Net Effect
Chemical mediators in low quantity	▪ Activation of neutrophils and monocytes, endothelial cells and complement system ▪ Endothelial injury releases nitric oxide (NO) and prostacyclin, which cause vasodilatation and increase blood flow of skin vessels	Local inflammation (increased cardiac output and rebounding pulse)
Chemical mediators in moderate quantity	▪ Fever (cytokine action on brain) ▪ Acute phase reactants synthesis ▪ Leukocytosis (cytokine action on bone marrow)	Systemic effects of inflammation
Chemical mediators in high quantity	▪ Low cardiac output and decreased peripheral resistance ▪ Endothelial injury activates both coagulation pathways forming thrombi in small vessels and disseminated intravascular coagulation (DIC) ▪ Acute respiratory distress syndrome (damage to alveolar-capillary damage) ▪ Activation of alternative complement pathway stimulates mast cell release of histamine (vasodilator), which causes significant pooling of blood resulting in relative hypovolemia and impaired tissue perfusion	Septic shock

Proinflammatory and Anti-inflammatory Cytokines Synthesis Role in Septic Shock

Microorganisms contain pathogen-associated molecular patterns (PAMPs), which bind to the family of pattern recognition receptors (PRRs), i.e. toll-like receptors and mediate innate and adaptive immune responses.

- The best-known human pattern recognition receptors are ubiquitous toll-like receptors (TLRs), a highly diversified family of cell surface of cytoplasmic receptors for a number of microbial invaders and endogenous danger signals.
- Toll-like receptors activation, via multiple signaling pathways, leads to translocation of nuclear factor κB (NF-κB), a central regulatory transcriptor factor for several inflammatory target genes, including proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) and costimulatory molecules that instruct the type of immune response and direct antimicrobial response and tissue injury. TNF-α and IL-1 cytokines trigger the pathologic manifestations of septicemia: leukocytic activation and transmigration, severe vascular endothelial injury and increased capillary permeability leading to hypovolemia and exposure to tissue factor (TF) and other circulating coagulation factors.
- Injurious stimuli that induce severe cellular damage results in rapid cell rupture with consequent release of intracellular DAMPs to the extracellular space. Circulating necrotic cells release damage-associated molecular patterns (DAMPs), which can engage cells of immune system and promote inflammation leading to similar manifestations of septicemia *via* pattern recognition receptors (PRRs), i.e. DAMP receptors. Since ischemia and necrosis most often accompany infection.
- Proinflammatory cytokines play important role in the pathophysiology of septic shock. Immunosuppression in septicemia partially results from synthesis of anti-inflammatory cytokines and lymphocyte apoptosis. Toll-like receptors activation results in upregulation of signal transduction pathways, which leads to endonuclease activation and apoptosis.
- Interestingly, immune cells recognize foreign danger signals within themselves by toll-like receptors (TLRs) and nod-like receptors (NLRs) having similar functions and induce synthesis of cytokines like IL-1, IL-6, IL-8, IL-18, TNF-α, IFN-β and high morbidity group box-1 products (HMGB-1). There is also increased production of reactive oxygen species (ROS), prostaglandins and platelet activating factor (PAF), which induce vascular endothelial injury triggering the pathologic manifestations of septicemia: leukocytic activation, transmigration, cytokines synthesis, activation of complement system, severe vascular endothelial injury, increased capillary permeability leading to hypovolemia and exposure to tissue factor (TF), other circulating coagulation factors and thus causing cellular and multiple organs damage.

Inflammation and Activation of Coagulation System Cascade

There are substantial cross-links between inflammation and coagulation system. Vascular endothelial cell injury leads to release of tissue factor that activates coagulation system cascade resulting in rapid generation of thrombin, that converts fibrinogen to fibrin.

- Coagulation system cascade is balanced by early fibrinolysis via increased expression of plasminogen activator, that is counterbalanced by upregulation of plasminogen activator inhibitor 1 (PAI-1).
- Coagulation system is an essential protective response to microbial infection. The formation of fibrin nets trap activated platelets, microorganisms and leukocytes. Bacteria trapped by fibrin nets in a region of decreased nutrient supply, bacterial trapping in fibrin nets limits both local bacterial growth and hematogenous spread to organs. The majority of human microorganisms employ fibrinolytic properties to evade these fibrin nets.
- When overwhelmed, procoagulant and anticoagulant responses to microbial infection can accelerate consumptive coagulopathy and disseminated intravascular coagulation (DIC) leading to blockage of small blood vessels and thus multiorgan failure.

Vascular Endothelial Activation and Injury

Toxic microbial products of infection, proinflammatory mediators, reactive oxygen species (ROS), activated host leukocytes, complement activation (C3 and C5a), platelet activating factor synthesis, and inducible nitric oxide synthase, all exert direct effects on vascular tone, integrity and vascular endothelial cell signaling resulting in vasodilatation and massive leakage of capillaries and thus resulting in systemic hypotension.

Metabolic Abnormalities in Septic Shock

Glucose uptake by insulin-dependent tissues is mediated by insulin, which activates GLUT-4 transporters.

- Hyperglycemia is one of the most common metabolic derangements in patients presenting with septicemia resulting from altered glycogen metabolism and profound insulin resistance.
- Hyperglycemia modifies inflammatory response by enhancing production of proinflammatory tumor necrosis factor-α (TNF-α) cytokine and reactive oxygen species (ROS). Hyperglycemia decreases neutrophil function leading to decreased bactericidal activity.
- **Waterhouse-Friderichsen syndrome** is a life-threatening disorder due to severe bacterial infection, and characterized by disseminated intravascular coagulation (DIC), bleeding into adrenal gland associated with adrenal insufficiency and profound septic shock. Metabolic abnormalities in septic shock are given in Table 3.37.

Multiple Organs Failure in Septic Shock

The common denominator in septic shock is impaired utilization of oxygen by the tissues resulting from systemic hypotension, thrombosis in small blood vessels, disturbed perfusion of microcirculation, interstitial edema and inflammatory and immune reaction-mediated tissue injury. Septic shock patients most often manifest single or multiple organs failure such as kidneys, lungs and liver. Mortality in these patients has been roughly double for each additional organ system failure. Recovery of organ system failure is possible on reversal of the underlying insult and associated host inflammatory response.

- **Renal manifestations:** Septic shock patients experience acute kidney injury as a result of impaired blood flow. Renal parenchymal cells express toll-like receptors (TLRs), which may be activated by endotoxin. Elevated levels of cytokines (e.g. TNF-α), coagulation factors and inflammatory chemical mediators and neuroendocrine mediators in septic patients. Prognosis depends on severity of acute kidney injury.
- **Pulmonary manifestations:** Septic shock patients can manifest with acute pulmonary injury induced by pathogens reaching either from environment or systemic circulation.
  - Binding of endotoxin to toll-like receptors induce signaling pathways leading to necrosis of pulmonary parenchyma and disruption of

Table 3.37 Metabolic abnormalities in septic shock

Physiological Changes in Septic Shock	Metabolic Impact	
↑Gluconeogenesis, glycolysis	Hyperglycemia	
↑Protein catabolism	Altered circulating amino acids	
↑Lipolysis	▪ ↑Triglycerides	▪ ↓Lipoprotein
↓Micronutrients	Oxidative stress	
↑Neuroendocrine activation	▪ ↑Catecholamines	▪ ↑Counter-regulatory hormones
↑Cortisol	Hyperglycemia	
↑Catecholamine release	▪ ↑Gluconeogenesis	▪ ↑Glycolysis
↑Cytokine release	Hyperglycemia and insulin resistance	
Impaired oxygen utilization	↑Reactive oxygen species	

alveolar-capillary membrane leading to non-cardiogenic edema.
- There is increased production of danger-associated molecular patterns (DAMPs), which cause leukocyte activation, vascular endothelial cell dysfunction and interstitial edema as well as activation of platelets and coagulation factors.
- Progressive pulmonary dysfunction leads to pulmonary shunt with refractory hypoxemia, contributing to multiple organs dysfunction.

- **Cardiac manifestations:** Sepsis disrupts functions of cardiovascular system such as vascular tone and capillary integrity.
  - Increased synthesis of inflammatory cytokines contributes to peripheral arterial dilatation, diffuse capillary leakage, decreased contractility and reflux tachycardia.
  - Patient manifests with dilatation of both ventricles, decreased ejection fraction, diastolic dysfunction and decreased cardiac output. Elevated concentrations of TNF-α, IL-2 and IL-6 can induce adverse effects on myocardium.
- **Neurological manifestations:** Septic shock patients develop encephalopathy due to blood–brain barrier disruption, central nervous system inflammation, leukocyte recruitment, increased production of nitric oxide (NO) and vasoactive substances, and neuromotor dysfunction.

ANAPHYLACTIC SHOCK

Anaphylactic shock is a clinical syndrome of severe type 1 hypersensitivity reaction mediated by immunoglobulin E (IgE) resulting in cardiovascular system collapse and severe bronchospasm as a result of massive release of histamine.

- Immediate type 1 hypersensitivity can occur within seconds to minutes after presentation of the inciting antigen (allergen) such as drugs (e.g. nonsteroidal anti-inflammatory drugs, penicillin), food (e.g. milk, fish, shellfish, eggs) and insect stings.
- Patient develops hypotension, dizziness, respiratory distress, tachycardia, weak and rapid pulse, circulatory failure, and shock.

NEUROGENIC SHOCK

Neurogenic shock is most often associated with acute injury to the brain or spinal cord. The underlying mechanism is the disruption of the autonomic nervous system pathway resulting in decreased vascular resistance and changes in vagal tone.

- In high spinal cord injury, there is failure of sympathetic outflow and adequate vascular tone. There is loss of function below the site of spinal cord injury.
- Clinical examination reveals flaccid paralysis distal to injury site, loss of sympathetic nervous system, relative bradycardia, hypotension, vasodilatation,

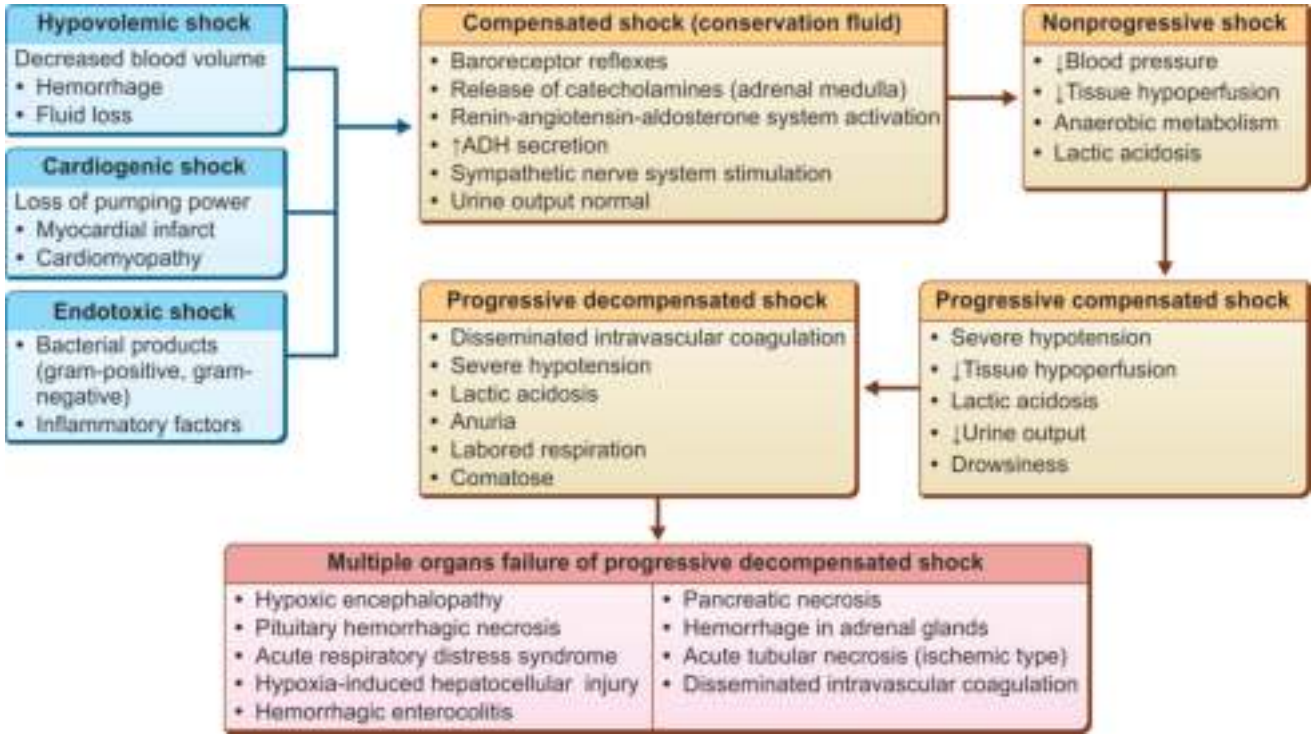


Fig. 3.37: Schematic representation of shock. The illustration shows etiology, pathogenesis and consequences of shock. Shock is a life-threatening medical condition as a result of insufficient blood flow throughout the body. There are many causes of shock, each of which can be caused by a number of different events.

warm, pink, dry skin, loss of urinary bladder control and priapism.

STAGES OF SHOCK

Stages of shock, etiology, pathogenesis and consequences are shown in Fig. 3.37.

- **Compensated shock stage:** In compensated shock stage, neurohormonal mechanism plays important role in this stage.
  - Compensatory mechanisms such as baroreceptor reflexes, release of catecholamines by adrenal medulla, activation of renin-angiotensin axis, antidiuretic hormone release and generalized sympathetic stimulation of tachycardia, increased peripheral vasoconstriction and conservation of fluid by kidneys lead to maintenance of perfusion of vital organs.
  - On clinical examination, level of conscious, respiratory rate, blood pressure and urine output are normal. There is mild lactic acidosis.
- **Nonprogressive shock stage:** Nonprogressive shock stage is characterized by persistent hypotension, vasodilatation, tissue hypoperfusion, anaerobic glycolysis, lactic acidosis. Clinical examination reveals low blood pressure, increased pulse rate, increased respiratory rate and decreased urine output.
- **Progressive decompensated shock stage:** Progressive decompensated shock stage is characterized by widespread tissue hypoxia, anaerobic glycolysis, excessive production of lactic acid, metabolic lactic acidosis, decreased tissue pH, blunting of vasomotor response, peripheral pooling of blood and decreased cardiac output. Compensatory mechanisms are no longer adequate. Clinical examination reveals reduced urine output, drowsy state, increased respiratory rate, increased pulse rate and hypotension.
- **Irreversible decompensated shock stage:** Irreversible decompensated shock stage is characterized by anoxic vascular endothelial cell injury, disseminated intravascular coagulation (DIC), widespread tissue anoxia, lysosomal enzyme leakage, aggravation of shock and complete renal shut down. Clinical examination reveals anuria, severe hypertension, comatose state, labored respiration, increased pulse rate and lactic acidosis. Multiple organs damage and metabolic disturbances are so severe that survival is not possible. Multiple organs involvement in irreversible decompensated shock are given in Table 3.38.

Table 3.38 Multiple organs involvement in irreversible decompensated shock

Organ	Morphology of Organ
Brain	Hypoxic encephalopathy
Pituitary gland	Pituitary hemorrhagic necrosis
Lungs	Acute respiratory distress syndrome
Liver	Hypoxia-induced hepatocellular injury
Intestine	Hemorrhagic enterocolitis
Pancreas	Pancreatic necrosis
Adrenal glands	Hemorrhage in adrenal glands
Kidneys	Acute tubular necrosis (ischemic type)
Blood	Disseminated intravascular coagulation

CHAPTER 4

Immunopathology

Vinay Kamal, Anubhav and Vigyat

LEARNING OBJECTIVES

IMMUNE SYSTEM

- Types of immune responses
  - Innate (inborn, natural, nonspecific) immune response
  - Adaptive immune response
- Active versus passive immunity
  - Active immunity
  - Passive immunity
- Components of immune system
  - Communicating body compartments
  - Cells of immune system
  - Lymphatic system

INNATE IMMUNE RESPONSE

- Components of innate immune system
  - Host three lines of defense
  - Barriers for pathogens
  - Immune cells of innate immune system
  - Soluble mediators
  - Cellular receptors for microbes and damaged cells
  - Responses of innate immune system

ADAPTIVE IMMUNE RESPONSE

- Antigens and immunogens
  - Antigenicity and immunogenicity
  - Categories of antigens
  - Antigen and immunogen
  - Superantigens
  - Molecular mimicry and autoimmunity
  - Antigen and host relationship
- Components of adaptive immune system
  - Immune cells
  - Lymphoid tissues/organs
  - Major histocompatibility complex
  - Cytokines
- T cell- and B cell-mediated immune responses
  - Development of B cells and T cells
  - Functions of B cells and T cells
  - Creation of antigen-binding T cell receptors and B cell receptors
  - Exposure of host to antigens
  - Co-operation in immune cells against antigens
  - Antigen processing and presentation by antigen-presenting cells (APCs)
- T cell and cell-mediated adaptive immune response
  - CD4+ helper T cell and cell-mediated adaptive immune response
  - CD8+ cytotoxic T cells and cell-mediated cytotoxicity
  - CD4+, CD25+, FOXP3+ regulatory T cells
  - Natural killer cells kill viruses and cancer stem cells
- B cells and humoral adaptive immune response
  - B cell activation and differentiation
  - Synthesis of immunoglobulin
  - Classes of immunoglobulin
  - Primary and secondary immune responses
  - Selected monoclonal antibody-based drugs

VACCINES AND IMMUNIZATION

- Vaccines
  - Vaccines preparation
  - DNA vaccine
  - RNA vaccine

IMMUNODEFICIENCY DISEASES

- Primary immunodeficiency diseases
  - B cell defects: primary immunodeficiency diseases
  - T cell defects: primary immunodeficiency diseases
  - Combined T cell and B cell defects: primary immunodeficiency diseases
  - Phagocytic dysfunction: primary immunodeficiency diseases
  - Complement system defects: primary immunodeficiency diseases
- Secondary immunodeficiency diseases
  - Acquired immunodeficiency syndrome

HYPERSENSITIVITY REACTIONS

- Type 1 hypersensitivity reaction
  - Categories of IgE-mediated type 1 hypersensitivity reaction
  - Bronchial asthma
- Type 2 hypersensitivity reaction
  - Antibody-dependent cellular cytotoxicity
  - Complement-mediated with increased susceptibility to phagocytosis of target cells
  - Anti-receptor antibody-mediated type 2 hypersensitivity reaction
- Type 3 hypersensitivity reaction
  - Pathogenesis of immune complex-mediated tissue injury and organs damage
- Type 4 hypersensitivity reaction
  - CD8+ cytotoxic T cell and cell-mediated immune response
  - CD4+ helper T cells and macrophages-mediated type 4 hypersensitivity reactions
- Type 5 hypersensitivity reaction
  - Graves' disease

IMMUNOLOGIC TOLERANCE

- T cell immunologic tolerance
  - T cell immunologic tolerance in central lymphoid organs
  - T cell immunologic tolerance in peripheral lymphoid organs
- B cell immunologic tolerance
  - B cell immunologic tolerance in central lymphoid organs
  - B cell immunologic tolerance in peripheral lymphoid organs

AUTOIMMUNE DISEASES

- Specific autoimmune diseases
  - Systemic lupus erythematosus
  - Scleroderma
  - Sjögren syndrome
  - Polymyositis
  - Dermatomyositis
  - Mixed connective tissue disease
  - Polyarteritis nodosa
  - Rheumatoid arthritis
  - Goodpasture syndrome
  - Graves' disease
  - Myasthenia gravis
  - Pemphigus vulgaris

TISSUE/ORGAN TRANSPLANTATION IMMUNOLOGY

- Immunology of transplant rejection
  - Distinguishing self- and non-self-antigen
  - Pathophysiology of transplant rejection
- Clinical stages of transplant rejection
  - Hyperacute transplant rejection
  - Acute transplant rejection
  - Chronic transplant rejection
- Graft-versus-host disease (GVHD)
  - Pathogenesis
    - Acute and chronic graft-versus-host disease
- Finding eligible donor–recipient match
- ABO blood group compatibility
- Tissue typing
  - Cross-matching
  - Panel of reactive antibody test
  - Serological screening
- Prevention of transplant rejection
  - Immunosuppressive drugs
  - Adverse effects of immunosuppressive drugs

AMYLOIDOSIS

- Chemical nature of amyloid
  - Amyloid light chain amyloidosis
  - Amyloid-β precursor protein associated amyloidosis
  - Transthyretin protein associated amyloidosis
  - β_2-Microglobulin protein hemodialysis associated amyloidosis
  - Endocrine glands–proteins associated amyloidosis
- Organs involved in amyloidosis
  - Renal amyloidosis
  - Liver amyloidosis
  - Spleen amyloidosis
  - Cardiac amyloidosis
  - Gastrointestinal tract amyloidosis
  - Skin amyloidosis
  - Nervous system amyloidosis
  - Respiratory system amyloidosis
- Laboratory diagnosis of amyloidosis
  - Histochemical stains

IMMUNE SYSTEM

Immunology is the branch of medicine and biology concerned with development of immunity against invading pathogens (bacteria, viruses, fungi, parasites).

- Immune system is composed of a number of specialized organs, cells and molecules that conducts surveillance of the body, which is activated by inflammatory inducers that indicate the presence of pathogens or tissue damage.
- Immune system plays important role in providing defense against infectious microbes, noninfectious substances and products of damaged cells, which is mediated by sequential and coordinated immune responses that are called innate (natural or native) and antigen-specific adaptive immunity.
- When an antigen attacks the host system, two distinct, yet interrelated, branches of the immune system are active: innate (nonspecific) and adaptive (specific) immune response. It should be noted that both innate and adaptive immune responses do not work independently. Moreover, most of the immune responses involve the activity and interplay of both the humoral and cell-mediated immune responses, which recognize foreign materials, neutralize and eliminate them.
- Innate (nonspecific) immune response provides initial defense against infections that does not require previous sensitization that is mediated by neutrophils, macrophages, dendritic cells (DCs), natural killer cells (NK cells), mast cells and innate lymphoid cells. Adaptive immune responses develop later and require activation of T cells, B cells, natural killer cells (NK cells), dendritic cells (DCs). Dendritic cells act as an important conduit between the innate and adaptive immune systems.
- Adaptive/specific immune response has again two divisions: humoral immunity and cell-mediated immunity, which are induced by different types of lymphocytes and function to eliminate different types of pathogens.
  - In humoral immunity, B cells secrete antibodies that prevent infections and eliminate extracellular microbes.
  - In cell-mediated immunity, CD4+ helper T cells activate macrophages and neutrophils to eliminate phagocytosed pathogens, or CD8+ cytotoxic T cells directly kill viruses and cancer stem cells.
- Immune-mediated disorders may be localized or system to a particular organ.
  - Major immune-mediated disorders include: hypersensitivity reactions, autoimmune disease (systemic or limited to a specific organ), immunodeficiency diseases and immunologic complications of solid organs and hematopoietic stem cells (HSCs) transplantation.
  - Hypersensitivity reaction is an exaggerated, misdirected expression of certain immune responses. There are main four types of hypersensitivity reactions including fifth type of hypersensitivity reaction.
  - Autoimmunity involves abnormal immune response to self-antigens against own proteins.
  - A deficiency or loss in immune function is called immunodeficiency.
- Cellular and humoral components of innate and adaptive immune responses are shown in Fig. 4.1. Cellular components of innate (nonspecific) and adaptive immune responses are shown in Fig. 4.2. Overview of diseases of the immune system is shown in Fig. 4.3. Importance of the immune system in health and disease is given in Table 4.1.

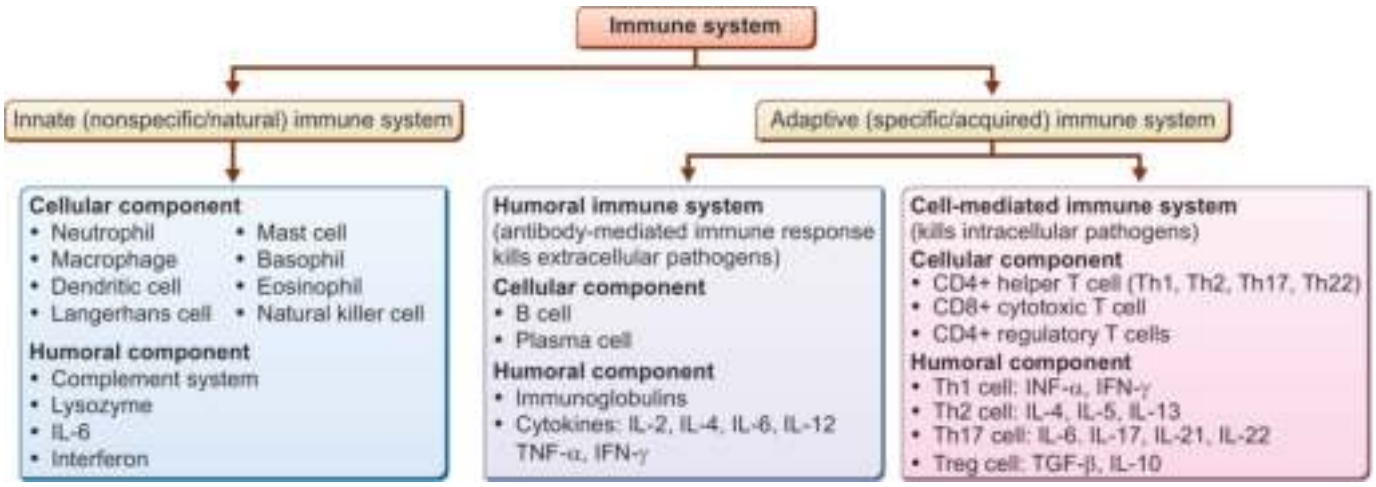


Fig. 4.1: Cellular and humoral components of innate and adaptive immune responses. The innate immune system is the first to respond to pathogens and does not retain immunologic memory of previous exposure to pathogens. Innate system consists of physical and chemical barriers, and cellular components such as monocytes/macrophages, mast cells, neutrophils, eosinophils, basophils, dendritic cells and natural killer cells. Components of the adaptive immune system include fundamental immune cells and molecules: B cells, T cells, immunoglobulins and major histocompatibility complex (MHC).

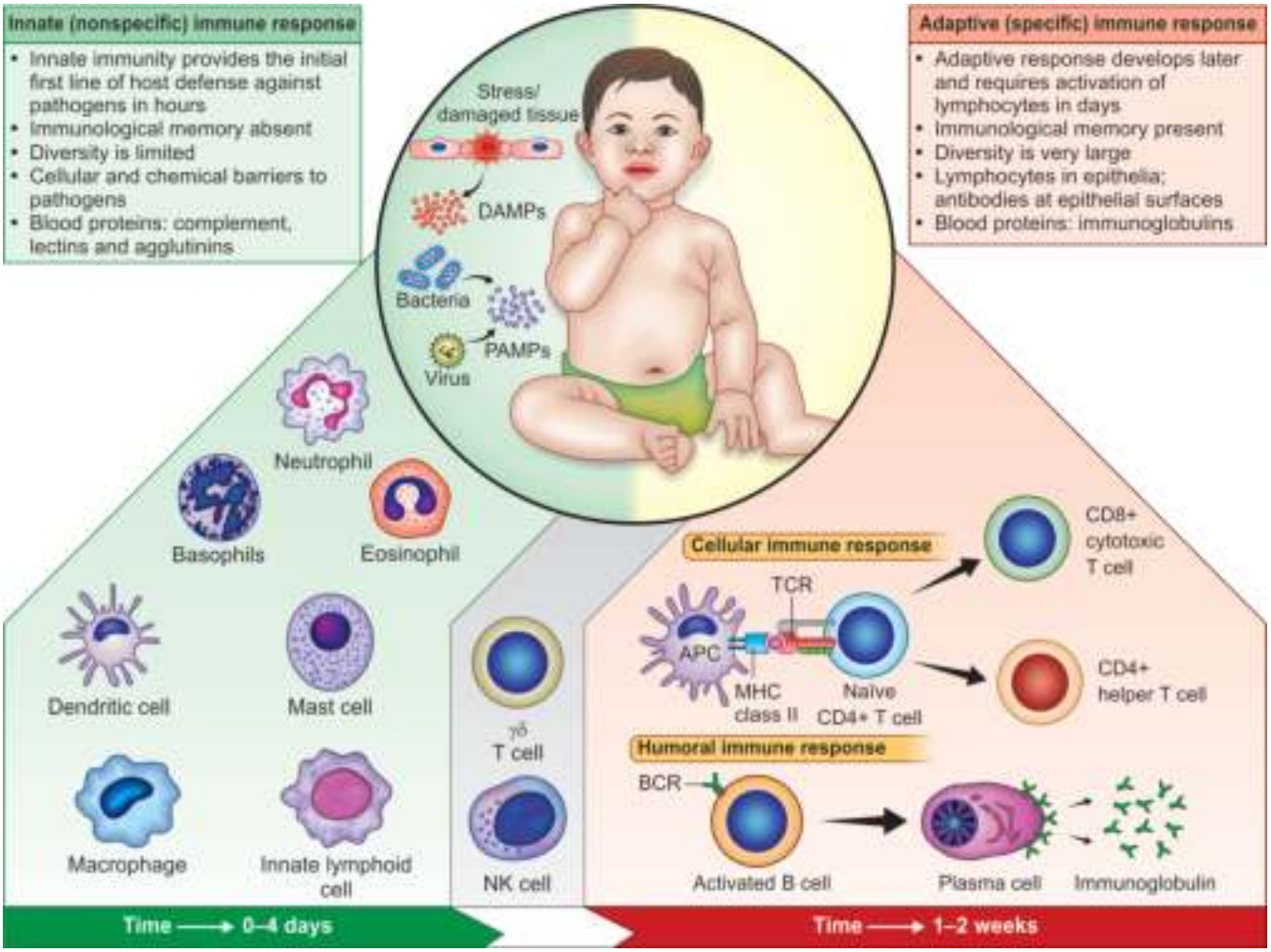


Fig. 4.2: Cellular components of innate and adaptive immune responses. Immune cells of the innate (nonspecific) immune system include macrophages, dendritic cells, neutrophils, basophils, mast cells and innate lymphoid cells. Cells involved in the adaptive immune response include T cells (e.g. CD4+ helper T cells, CD8+ cytotoxic T cells and CD4+ regulatory T cells) and B cells including antibody secreting plasma cells.

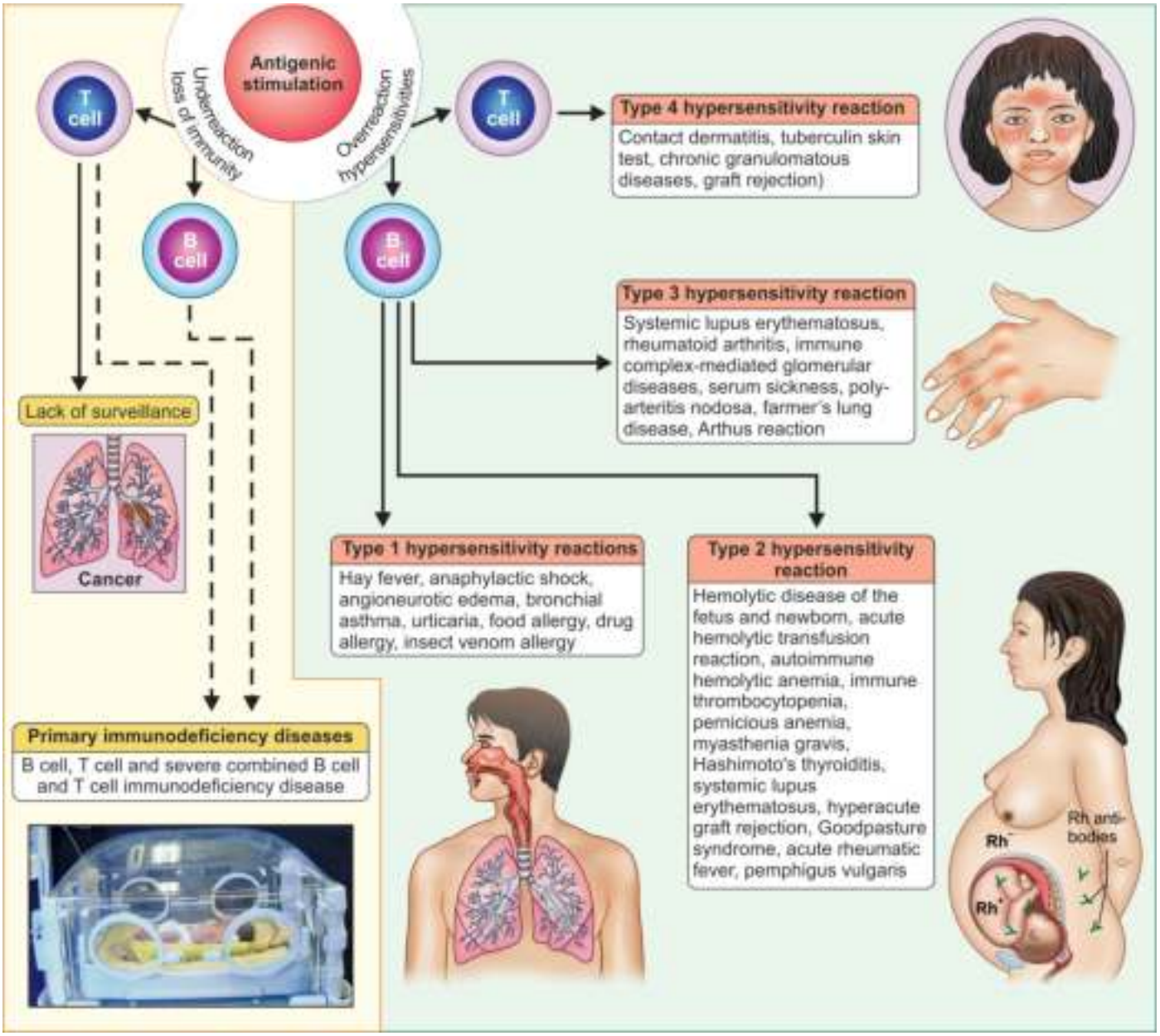


Fig. 4.3: Overview of diseases of the immune system. T cells and B cells provide necessary protection against infection and disease. Immune system can cause serious and debilitating disorders to immune stimuli. Lack of surveillance of immune system causes immunodeficiency diseases, hypersensitivity reactions, autoimmune diseases and carcinomas. Types 1, 2 and 3 hypersensitivity reactions are mediated by antibodies (IgG or IgM). Type 4 hypersensitivity reaction is mediated by T cells, which release cytokines, chemokines, macrophages and CD8+ cytotoxic T cells rather antibodies.

Table 4.1 Importance of the immune system in health and disease

Immune System Functions in Health	Immune System Impairment and Associated Disease
Defense against infections	Susceptibility to infections that can protect against infections
Defense against malignancies	Potential for immunotherapy of malignancies
Immune system can induce cell injury and inflammation	Immune system can cause hypersensitivity reactions (types 1, 2, 3, 4), autoimmune disorders and other inflammatory disorders
Immune system recognizes and responds to tissue/organ graft and newly introduced proteins	Immune system responses are barrier to tissue/organ transplantation and gene therapy

Pathology Pearls: Terminology used in Immunology

- **Adaptive immunity:** It refers to immune response to the first exposure to a pathogen or vaccination, that has memory.
- **Antigen:** It is nonself or foreign protein that binds to an antibody and triggers the immune response.
- **Antigen determinant (epitope):** It is one of the chemical groups recognized by a single type of lymphocyte.
- **Antigen-presenting cell (APC):** It is an immune cell that detects, engulfs and informs the adaptive immune response about an infection by presenting the processed antigen on the cell surface.
- **Antigen presentation:** It is binding of processed antigen to the protein-binding cleft (groove) of a major histocompatibility complex molecule.
- **Antigen processing:** It refers to internalization and digestion of antigen in an antigen-presenting cell (APC).
- **Antigen receptor on lymphocytes:** It has two-chain by which lymphocytes recognize antigen.
- **Allergen:** It is an antigen that induces allergic reaction.
- **Antibody-dependent cell cytotoxicity:** The cytotoxicity of certain lymphocytes that attack a target cell bound by IgG.
- **Autoimmune response:** It refers to inappropriate immune response to host cells or self-antigens.
- **Cell-mediated immune response:** It is adaptive immune response that is carried by T cells.
- **Clone:** It is a group of lymphocytes sharing the same antigen receptor.
- **Clonal expansion:** It refers to the growth of a clone of selected lymphocytes corresponding to specific BCR or TCR variant.
- **Clonal selection:** It refers to stimulating growth of lymphocytes that have specific receptors.
- **Constant region of immunoglobulin:** Constant region is a subdivision of a heavy chain that has the same amino acid sequences.
- **CD8+ cytotoxic T cell:** It has ability to induce apoptosis in target cells via perforin and granzyme and releases cytokine to enhance the immune response.
- **CD4+ helper T cell:** It is a cell of the adaptive immune system that binds to antigen-presenting cell (APC) via MHC class II molecules that secretes cytokines to enhance other immune responses involved in activation of both B cell and T cell.
  - **CD4+ helper T cell (Th1 cell):** It secretes cytokines that enhance the activity of macrophages and other cells.
  - **CD4+ helper T cell (Th2 cell):** It secretes cytokines that induce B cells to differentiate into antibody secreting plasma cells.
- **Dendritic cell:** It is immune cell that processes antigen material and presents it on the surface of other cells to induce an immune response.
- **Effector T cell:** Lymphocyte that has differentiated to effector cell, which has direct adverse effect on pathogen.
- **Epitope:** Epitope is a small component of an antigen that is specially recognized by antibodies, B cells, and T cells; the antigenic determinant.
- **Humoral immune response:** It is adaptive immune response that is regulated by activated B cells and antibodies.
- **Immunological memory:** It is the ability of the adaptive immune response to mount a stronger and faster immune response upon re-exposure to a pathogen.
- **Immunologic tolerance:** It is acquired ability to prevent an undesirable or harmful immune response to a detected foreign body known not to cause autoimmune disease or to self-antigens.
- **J-chain (joining chain):** J-chain is a protein produced by B cells and plasma cells that connects a monomer to form dimer or pentamer.
- **Lymph:** It is a watery fluid that bathes tissues and organs with protective white blood cells and does not contain red blood cells.
- **Major histocompatibility complex (MHC):** It is a gene cluster whose proteins present antigen to T cells.
  - **Major histocompatibility complex (MHC) class I:** It is found on most cells of the body, it binds to molecule on CD8+ cytotoxic T cells.
  - **Major histocompatibility complex (MHC) class II:** It is found on macrophages, dendritic cells, Langerhans cell and B cell, it binds to molecule on CD4+ helper T cells.
- **Memory T cell:** It is antigen specific long-lived immune cell (B cell or T cell) that does not differentiate into effector cell during the primary immune response but can immediately become effector cell upon future re-exposure to the same antigen.
- **Opsonization:** Opsonization refers to coating of antigen by antibody that enhances phagocytosis by a macrophage.
- **Papain:** Papain is cysteine protease commonly found in papaya, that digests proteins into fragments.
- **Polyclonal immune response:** It refers to response by multiple clones to a complex antigen with many determinants.
- **Plasma cell:** It is an immune cell that arises from B cell and secretes antibodies.
- **CD4+ regulatory T cell (Treg):** CD4+ regulatory T cell suppresses local inflammation and inhibits the secretion of cytokines, antibodies and other stimulatory immune factors; involved in immune tolerance.
- **Secretory piece:** Secretory piece is a polypeptide that attaches to a dimer (IgA) when it passes through epithelial cells in mucosal regions.
- **Somatic recombination:** Somatic recombination or V(D)J recombination is the process for generating antibody diversity.
- **Variable region of immunoglobulin:** Variable region is subdivision of a heavy chain or light chain that has different amino acid sequences, responsible for antigen recognition and binding.

TYPES OF IMMUNE RESPONSES

There are two fundamental types of immune responses: innate (inborn, natural, nonspecific) and adaptive (specific) immune response. Innate immune response

recognizes pathogen-associated molecular patterns (PAMPs). These PAMPs are recognized by pattern recognition receptors (PRRs), mainly expressed in the innate immune cells. PRRs can also recognize host molecules containing damage-associated molecular patterns (DAMPs) that are often released from damaged cell by invading pathogens. Innate and adaptive immune responses are shown in Fig. 4.4. Comparison of innate and adaptive immune responses is given in Table 4.2.

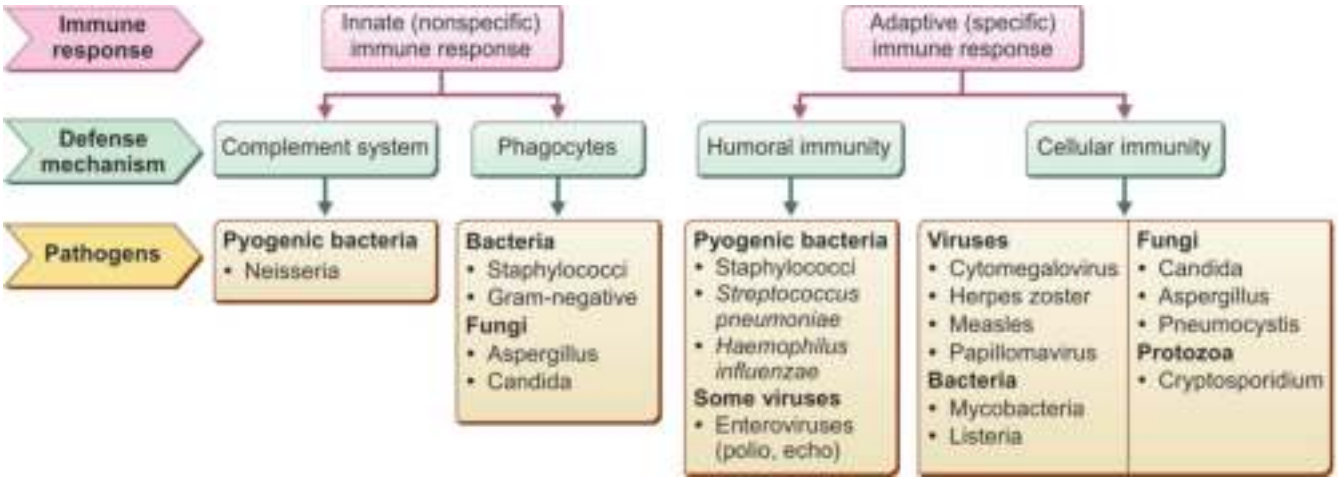


Fig. 4.4: Host innate and adaptive immune responses. Innate immunity is the defense system with person is born that protects against all pathogens. Innate immune system involves first line of defense physical and chemical barriers that keep harmful substances entering body. Adaptive immune responses are carried out by T cells and B cells. There are two broad classes of adaptive immune responses: humoral immunity (B cell) and cell-mediated immunity (T cell).

Table 4.2 Comparison of innate and adaptive immune responses

Characteristics	Innate Immune Response	Adaptive Immune Response
Presence	Innate immunity (inborn) exists in the body	Adaptive immunity is created in response to a foreign substance
Inheritance	Inherited from parents and passed to offspring	Adaptive immunity is not inherited from parents and passed to offspring
Specificity	Nonspecific immunity	Specific immunity
Diversity	Limited diversity to recognize pathogens and molecules encoded by germline genes	High diversity to recognize very large molecules encoded by somatic recombination of gene segments in lymphocytes generated randomly and there is specific immune response for each unique antigen
Speed of immune response	Faster response and fights any foreign invader	Slow response and fights only specific infection
Stimuli	Lipopolysaccharide (endotoxin), bacterial peptidoglycans	Protein antigens
Major cells involved	Macrophages, neutrophils, dendritic cells (DCs), natural killer cells (NK cells), mast cells and innate lymphoid cells	T cells, B cells, natural killer cells (NK cells), dendritic cells (DCs)
Cellular and chemical barriers	Skin, mucosal epithelia, antimicrobial molecules, phagocytes, cells, toxic granules, inflammation and fever	T cells and B cells in lymph nodes, spleen, mucosa-associated lymphoid tissue (MALT), antibodies secreted by plasma cells on the epithelial surfaces
Potency	Limited and lower potency	High potency
Time span	Innate immunity once activated against a specific antigen; innate immunity remains throughout the life	The span of developed adaptive immunity can be lifelong or short

Contd...

Table 4.2 Comparison of innate and adaptive immune responses (*Contd...*)

Characteristics	Innate Immune Response	Adaptive Immune Response
Defense	Epithelial lining (skin, mucous membranes), phagocytes, inflammation and fever	Cell killing, tagging of antigen by antibody for removal
Specificity	Nonspecific immune response (specificity for molecules and molecular patterns (PAMPs) associated with microbes, damaged host cells or foreign particles)	Highly specific immune response and can discriminate between microbes and nonmicrobial antigens, and their molecular structures
Pathogen recognition	General patterns on pathogens; nonspecific immune response	Specific immune response to individual microbes and antigens (antigens/antigen–antibody complex)
Self versus nonself discrimination	Innate immunity is based on self- versus nonself-antigens discrimination, so it has to be perfect	Adaptive immunity is not as good as the innate immunity, failure to discriminate self and nonself-antigens result in autoimmune diseases
Immunologic memory	Innate immunity cannot react with equal potency upon subsequent exposure to same antigen	Adaptive immunity has immunologic memory subsequent exposure to specific pathogens which have encountered before
Response to repeated infection	Similar immune response with each exposure	Immunologic memory, more rapid and efficient immune response with subsequent exposure
Blood proteins	Complement, lectins and agglutins	Immunoglobulins
Complement activation	Alternative and lectin pathways	Classical pathway
Molecular components	Cytokines (IFN-α, IFN-β, TNF-α, IL-1, IL-6, IL-10, IL-12, IL-15, IL-18), complement system, soluble chemical mediators, acute phase reactants	Antibodies, cytokines (IFN-γ, TNF-β, IL-2, IL-4, IL-l5, IL-13 lymphotoxin), complement system proteins
Effects	Local and systemic effects	Usually, local effects
Roles in disease	Systemic disorders	Local tissue injury
Inhibitors	Corticosteroids	Cyclosporine

INNATE (INBORN, NATURAL, NONSPECIFIC) IMMUNE RESPONSE

Innate immunity is essential in providing immediate and nonspecific first line defense for fighting against invading pathogens before adaptive immune response has developed.

- Components of innate immune response include physical barriers (i.e. tight junctions in the skin, epithelial and mucosal surfaces including mucus coating cells), epithelial and phagocytic cell enzymes (i.e. lysozyme), phagocytes (i.e. neutrophils, monocytes, macrophages, natural killer cells, dendritic cells and mast cells) and complement system to the site of tissue injury. It does not confer long-lasting immunity.
- Research on innate immune response has been focused on pathogen recognition receptors (PRRs) and signaling pathways. Necrotic cells release damage-associated molecular patterns (DAMPs), which lead to cell rupture with consequent release of intracellular DAMPs. Members of pathogen recognition receptors (PRRs) recognize diverse pathogens invasion or danger signals from release of DAMPs and initiate innate immune system signaling pathways, leading to proinflammatory cytokines synthesis, which in turn instructs development of adaptive response.
- Pathogen-associated molecular patterns (**PAMPs**) and damage-associated molecular patterns (**DAMPs**) mediated signaling and induction of an innate immune response usually results in resolution of infection.
- Toll-like receptors (**TLRs**) on phagocytes bind to bacterial lipopolysaccharide (LPS, or endotoxin) and induce innate immune response. Phagocytosis and activation of complement system are more rapid resulting in destruction of pathogens.

ADAPTIVE IMMUNE RESPONSE

Adaptive immune response is acquired that takes time for optimum reactivity, which is mediated by B cells and T cells and their products in response to specific antigens. Lymphocytes express highly diverse receptors that can recognize a vast number of antigens. Adaptive immune system mediated by different cells and molecules provide two types of immune responses dependent on the functions of B cells and T cells: humoral immune response (defense against extracellular microbes)

Table 4.3 Categories of adaptive immune response

Features	Humoral-mediated Immune Response	Cell-mediated Immune Response
Mechanism	Antibody-mediated immune response	Cell-mediated immune response
Cell type	B cells	T cells
Mode of action	Antibodies in serum	Direct cell-to-cell contact or soluble products screened by cells
Purpose	Primary defense against bacterial infection	Defense against viral and fungal infections, intracellular organisms; and graft rejection

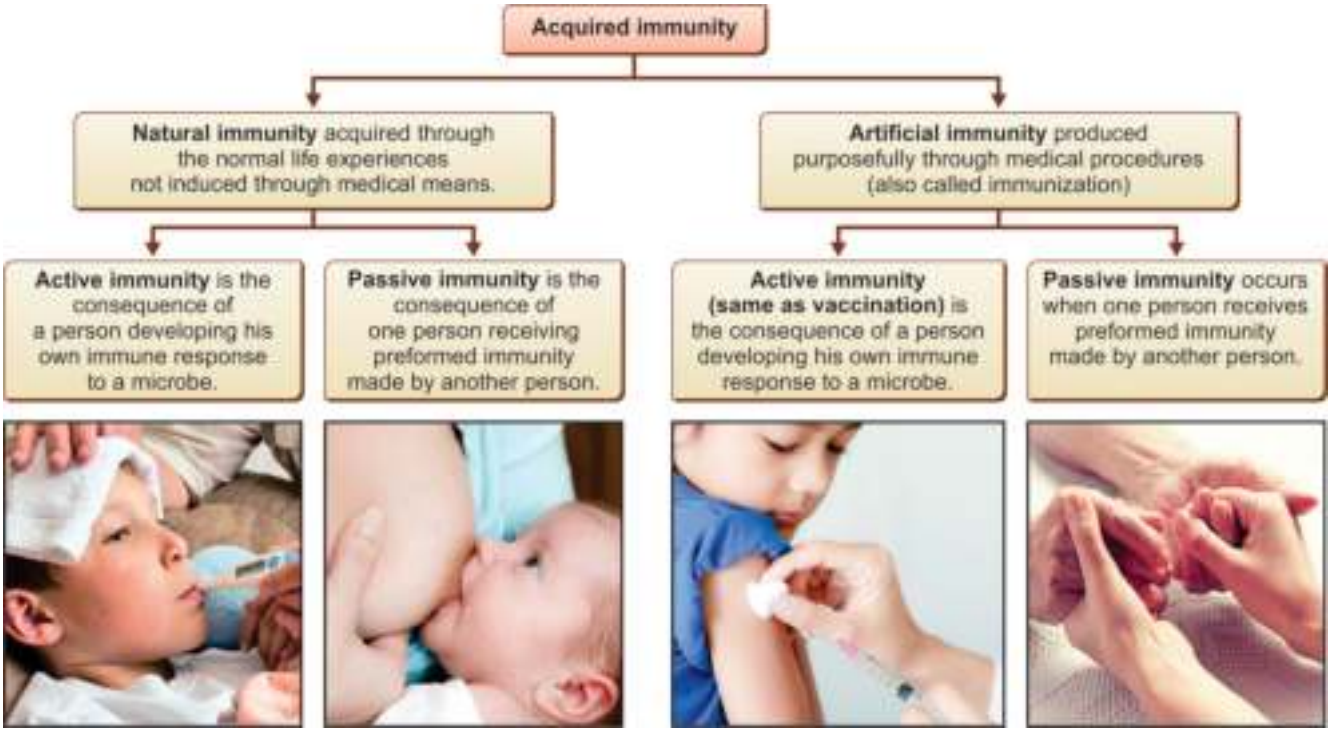


Fig. 4.5: Categories of acquired immunity. Natural immunity, which occurs during normal course of life, is either active (acquired from an infection and then recovering) or passive (antibodies donated by mother to fetus to her child). Artificial immunity is acquired through medical practices and can be active (vaccination with antigen), to stimulate an immune response) or passive (immune therapy with serum containing antibodies).

and cell-mediated immune system (defense against intracellular microbes). Categories of adaptive immune response are given in Table 4.3.

Adaptive Humoral Immune Response

Membrane-bound immunoglobulin on the surface of B cells serves as the cell's receptor for antigen, and is known as the B cell receptor (BCR). In humoral immune response, B cells recognize different antigens and induce immunoglobulins synthesis by plasma cells bind to antigens and prevent infections and eliminate extracellular microbes. B cell activation also initiates a cascade of intracellular signaling leading to the internalization of antigen for processing and presenting to CD4+ helper T cells. Categories of acquired immunity are given in Fig. 4.5.

Pathology Peals: Comparison of Acquired Immune Responses

Comparison of types of acquired immune response is given in Table 4.4.

Active Natural Type of Acquired Immune Response

- Active natural type of immune response is obtained in course of daily life. Antigens or pathogens enter body naturally.
- Body generates an immune response to antigens. Immunity may be lifelong (**chickenpox** or **mumps**) or temporary (**influenza** or **intestinal infections**).

Active Artificial Type of Acquired Immune Response

Active artificial type of immune response is obtained in response to an antigen. Immunity can be lifelong (oral polio vaccine) or temporary (tetanus toxoid) due to formation of antibodies.

Passive Natural Type of Acquired Immune Response

- Passive natural type of immune response is obtained by passage of neutralizing antibodies from mother to the fetus via placenta or breastfeeding (colostrum).
- There is no immune response to antigens.
- Immunity is usually short-lived (weeks to months). It provides protection for 6–12 months until child's immune system develops.

Passive Artificial Type of Acquired Immune Response

- Preformed antibodies (antiserum) are introduced into body by injection.
- Snake antivenom injection from horses or rabbits are administered in persons with snakebite.
- Immunity is short lived (half-life of three weeks). Host immune system does not respond to antigens.

Adaptive Cell-mediated Immune Response

In adaptive cell-mediated immune response, T cell receptor (TCR) on CD4+ helper T cell recognizes antigens (peptides) bound to the major histocompatibility complex (MHC/HLA) class II molecules on the surface of antigen-presenting cells (APCs) and activate macrophages to eliminate phagocytosed intracellular microbes and kill infected cells. CD8+ cytotoxic T cells recognize antigens on the virus infected cells and directly cause their elimination. CD4+ regulatory T cells (Tregs) suppress immune response to self-antigens.

Pathology Pearls: Immunologic Memory in Adaptive Immune System

- Immunologic memory to specific pathogens is most important characteristic of adaptive immunity that can provide long-lasting defense and protection against recurrent infections by inducing intense immune response on subsequent exposure to the same antigen.
- Vaccination is a good example of adaptive immune response. Vaccination exposes body to the antigen to induce antibodies synthesis specific to attenuated pathogen. Body acquires a memory to the virus without experiencing illness. The concept of immunologic memory is due to the body's ability to make antibodies against different antigens.
- Some breakdowns in the immunologic memory system can lead to autoimmune diseases.
- Molecular mimicry of a self-antigen by a infectious pathogen may trigger autoimmune diseases as a result of cross-reactive immune response against the infection. One example of a pathogen that uses molecular mimicry to hide from immunologic defenses is Streptococcus infection in which patient develops rheumatic heart disease.

ACTIVE VERSUS PASSIVE IMMUNITY

Active immunity is conferred by a host response to infectious microbes or microbial antigen, whereas passive immunity is achieved by transfer of antibodies or T cells specific for the infectious microbes. Both types of immune responses provide resistance to specific microbial antigens, but only active immune response generates immunologic memory. Therapeutic passive transfer of immunoglobulins, but not lymphocytes are performed routinely. Passive transfer of antibodies occurs during pregnancy from mother to the fetus. Differences between active and passive immunity are given in Table 4.5.

ACTIVE IMMUNITY

Active immunity means immune response caused by a disease agent. Natural active immunity develops as a result of disease process, which creates memory, takes time and is lasting. Artificial active immunity develops as a result of vaccination of person. Vaccines include inactivated toxins, killed microbes, parts of microbes, and viable but weakened microbes. Vaccines stimulate a protective immune response without causing the disease. Active immunity is usually permanent by creating memory.

PASSIVE IMMUNITY

Passive immunity develops, when a patient receives antibodies from another person. Natural passive immunity comes from the mother.

- Artificial passive immunity is acquired through medical procedures such as administration of human antiserum in patients (short-lived immunity).
- Artificial active immunity is the same as administering a vaccine (long lived immunity against an infectious agent that may be encountered in the future).

COMPONENTS OF IMMUNE SYSTEM

The immune system consists of three overlapping lines of defense: first line, second-line and third-line. A fourth component, the extracellular fluid surrounds the first three lines of defense and allows constant communication between all areas of the body. Lymph nodes, spleen, and thymus participate in defense mechanisms.

- White blood cells formed in the bone marrow participate in elimination of injurious agents by inducing inflammation and specific immune response. White blood cells travel freely due to

Table 4.4 Comparison of types of acquired immune response

Type of Immune Response	Mode of Acquisition	Immunoglobulin Synthesis	Duration of Immune Response
Active acquired immune response			
Active natural immune response	Infection by pathogens	IgG synthesis present (IgG half-life of 23 days)	Lifelong immune response in immunocompetent host (memory cells life span is in years)
Active artificial immune response	Vaccination	IgG synthesis present (IgG half-life of 23 days)	Lifelong immune response in immunocompetent host (memory cells life span is in years)
Passive acquired immune response			
Passive natural immune response	Transfer from mother *in vivo* to infant or colostrum	IgG synthesis absent	Short-long immune response
Passive artificial immune response	Transfer of antibodies preparations to non-immune persons	IgG synthesis absent	Short-long immune response

Table 4.5 Differences between active and passive immunity

Characteristics	Active Immunity	Passive Immunity
Duration of immune response	Active immunity is usually permanent that occurs when person comes in contact with living or dead microbes	Passive immunity lasts for a few weeks to months that occurs due to administration of ready-made antibodies for immunization
Immune response	Slow immune response because it takes time to generate antibodies	Immediate and quick immune response
Duration of immune response	Long lasting	Short lived
Side effects	No side effects	Passive immunity can induce reactions
Clinical aspect	Used for prophylaxis to increase resistance (BCG vaccination for prophylaxis of tuberculosis)	▪ Treatment of acute infections ▪ Breastfeeding (colostrum rich in abundant IgA) ▪ Fetus receives some antibodies from mother through placenta during pregnancy

close interrelationship among blood, lymphatic channels and reticuloendothelial system.

- Communication between cells of the immune system is facilitated by the release of chemical mediators from inflammatory cells and injured tissue, which drive inflammatory response, increased blood flow, recruitment of white blood cells to the site of tissue injury, initiate fever and destroy necrotic cells. Complement system acts in nonspecific or specific ways to lyse cells that has been identified as foreign.
- Pathogen-associated molecular patterns (PAMPs) on phagocytes (neutrophils and macrophages) recognize pathogens, phagocytose and degrade them.
- For purposes of immunologic study, the body is divided into three compartments: the blood, lymphatic system and the reticuloendothelial system. Fourth compartment is extracellular fluid compartment surrounding tissue cells.

COMMUNICATING BODY COMPARTMENTS

Tissue cells are in direct contact with blood vessel, lymphatic system, reticuloendothelial system and extracellular fluid. Blood cells formed in the bone marrow is called **hematopoiesis**, which begins in the yolk sac during embryogenesis. Later, the function is taken over by liver and lymphatic organs and finally bone marrow. Hematopoietic stem cells (HSCs) in the bone marrow differentiate to produce WBCs, RBCs and platelets. Blood cells are dispersed in the plasma.

CELLS OF IMMUNE SYSTEM

Principal cells of the immune system include lymphocytes (B cells and T cells), antigen-presenting cells—APC (e.g. dendritic cells, macrophages, follicular dendritic cells), and granulocytes (neutrophils, eosinophils and basophils).

- Immune cells originate in the bone marrow from pluripotent hematopoietic stem cells (**HSCs**), which give rise to a common myeloid progenitor cell leading to generation of major myeloid cell types (neutrophils, eosinophils, basophils, dendritic cells, mast cells, and monocytes/macrophages), the erythrocytes and megakaryocytes (which generate platelets).
- Pluripotent hematopoietic stem cells (HSCs) also give rise to common lymphoid progenitor cell, which

differentiates to major lymphoid cell types (B cells, T cells, and natural killer cells).

- The extracellular compartment is protected by humoral immune response, in which immunoglobulins produced by B cells induce elimination of extracellular pathogens and prevent spread of intracellular infections. The activation of B cells and their differentiation leads to formation of immunoglobulin-secreting plasma cells.
- Cell-mediated immune response is primarily mediated by mature T cells, antigen-presenting cells (macrophages, dendritic cells, Langerhans' cells) and the release of cytokines in response to antigen. T cells involved in cell-mediated immune response rely on antigen-presenting cells that contain membrane-bound MHC class molecules in order to recognize intracellular target antigens.
- Antigen-presenting cells (APCs) capture and display antigen to lymphocytes. Dendritic cells activate CD4+ helper T cells and induce cell-mediated immune response. Follicular dendritic cells display antigens to B cells to induce humoral immune response.
- Monocytes differentiate to become tissue macrophages. Macrophages function in the effector phase of cell-mediated immunity, which serve an important role as phagocytes in engulfing and killing microbes.

LYMPHATIC SYSTEM

The lymphatic system parallels the circulatory system and transports lymph through lymphatic channels to draining lymph nodes, which returns interstitial tissue fluid to systemic circulation and drains away excess inflammatory exudate fluid present in inflamed tissue. It also concentrates and processes invading pathogens and initiates specific immune response through phagocytes, lymphocytes and antibodies.

- Lymph is made up of water, dissolved salts and 2–5% proteins (antibody and albumin), which transports lymphocytes, fats, cellular debris and pathogens. Lymphatic channels permeate all parts of the body except the central nervous system, bone, placenta and thymus gland.
- Lymphoid organs and tissues with immune functions can be classified into primary and secondary.
  - Primary (central) lymphoid organs include bone marrow and thymus gland.
  - Secondary (peripheral) organs and tissues include lymph nodes, spleen, MALT (mucosa-associated lymphoid tissue, SALT (skin-associated lymphoid tissue) and GALT (gut-associated lymphoid tissue in Peyer's patches).

EXTRACELLULAR FLUID COMPARTMENT

Extracellular fluid (ECF) surrounds all cells in the body, which is composed of blood plasma, interstitial fluid, lymph and transcellular fluid (e.g. cerebrospinal fluid, synovial fluid, aqueous humor, serous fluid and gastrointestinal tract fluid). The interstitial fluid and the blood plasma are the major components of the extracellular fluid. ECF contains nutrients from capillaries by diffusion and holding waste products as a result of cellular metabolism. In general, the elevation of extracellular fluid increases the preload, which eventually contributes to the generation of hypertension.

INNATE IMMUNE RESPONSE

Innate immune system is immediate (nonspecific, inborn, or natural) first line of defense system against infection that responds to pathogens in a generic way, which does not confer long-lasting or protective immunity. Anatomic and chemical barriers are the first line of defense against pathogens. Innate immune system is also required to initiate specific adaptive immune response. Two principal types of reactions of the innate immune system are inflammation and antiviral defence. The specificity of the innate immunity is different from adaptive (specific) immunity. It does not react against the normal host cells.

- **Type of immune response:** Innate immune system responds in the same way to repeated encounters with the same infectious microbe. On the contrary, adaptive immune response mounts stronger and effective immune responses to each successive encounter with an infectious microbe.
- **Specificity:** Innate immune system responds to a limited number of microbial molecules such as pathogen-associated molecular patterns (**PAMPs**). It also recognizes molecules called damage-associated molecular patterns (**DAMPs**), that are released from the damaged or necrotic host cells. The subsequent immune response to DAMPs serves to eliminate the damaged or necrotic host cells and to initiate the process of tissue repair. Innate immune response occurs even in sterile injury in acute myocardial infarction as a result of cessation of arterial supply to the heart.
- **Receptors encoded by genes:** Innate immune system recognizes particular identical antigen receptors

expressed on the host cells such as macrophages. These antigen receptors are encoded by inherited genes. On the contrary, T and B cells in adaptive immune system recognize antigen receptors encoded by inherited genes formed by somatic rearrangement of gene segment during development and differentiation of B cells and T cells.

- **Distribution of receptors:** There are about 100 types of innate immune receptors which can recognize 1000 PAMPs and DAMPs on all cells of the same lineage. On the contrary, there are only two types of specific receptors in the adaptive immune response. B cells possess immunoglobulin (Ig) receptors and T cells have T cell receptors (TCRs). Because of their diversity, B cell and T cell receptors can recognize millions of different antigens.
- **Unable to discriminate self- and nonself-antigens:** Sensor cells express pattern recognition receptors (PRRs) that provide an initial discrimination between self- and nonself-antigens. Innate immune response does not recognize healthy host cells. Molecules expressed on healthy host cells inhibit innate immune reactions. Sensor cells induce an inflammatory response by producing chemical mediators such as chemokines and cytokines. Adaptive immune response is initiated by the activation of antigen-specific T cells and B cells. T cell responses to self-antigens can inflict tissue damage either directly or indirectly.

COMPONENTS OF INNATE IMMUNE SYSTEM

Components of the innate immune system include physical barriers (tight junctions in the skin, epithelial and mucous membrane including mucus itself, anatomical barriers, phagocytic cell enzymes (i.e. lysozyme) and immune cells (i.e. neutrophils, macrophages, mast cells, natural killer cells and dendritic cells).

HOST THREE LINES OF DEFENSE

The multilevel, intercommunicating network of host protection against of pathogens invasion can be organized into three lines of defense.

- First line of defense is the anatomical barrier provided by the body's epithelial surfaces. Epithelial surfaces of the body provide the first line of defense barrier against infection. Epithelial cells and phagocytes produce several kinds of antimicrobial proteins.
- Second line of defense is the chemical barrier, enzymatic system and complement system which act as an immediate antimicrobial barrier near the epithelia.
- If the pathogen overcomes these barriers, third line of defense—adaptive (specific) immune system is brought handle invading pathogens. Major components of the host defenses are shown in Fig. 4.6 and Table 4.6.

First Line of Defense

First line of defense is an inborn and nonspecific immune system comprising anatomical, chemical barriers provided by skin and mucous membranes lining respiratory system, gastrointestinal tract, urogenital system and other systems.

- Anatomical and chemical barriers prevent entry of pathogens. A ciliated pseudostratified columnar epithelium with mucus producing goblet cells is important in trapping debris and bacteria. The bacteria are then propelled to the esophagus and swallowed. Alveolar macrophages are important part of the host defense. As a part of the cough reflex, the muscles of the chest wall and diaphragm are important in clearing secretions from the respiratory tree.

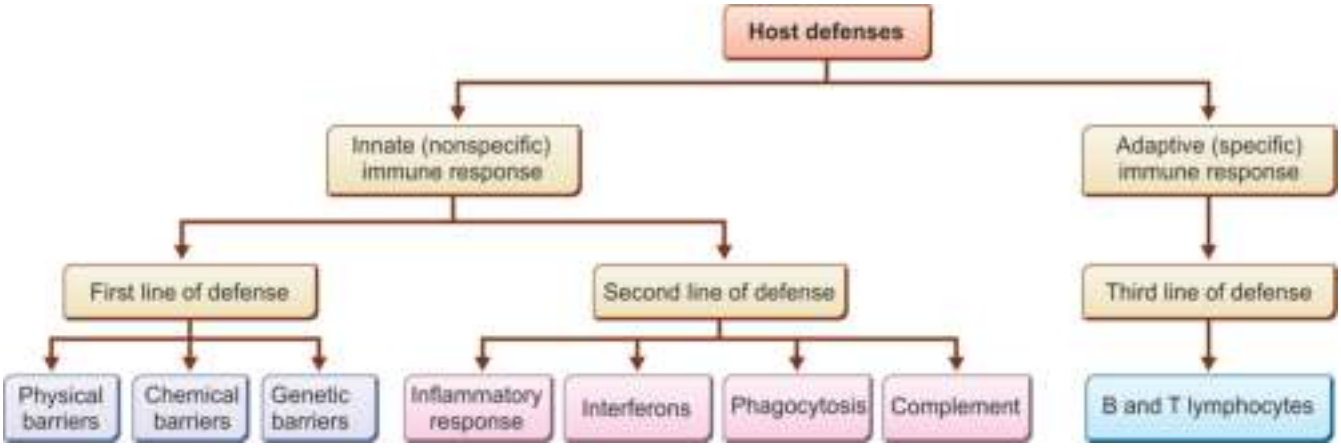


Fig. 4.6: Major components of the host defenses. Defenses are classified into two categories: innate and nonspecific; and acquired and specific. These can be further subdivided into the first, second and third lines of defense. Each of the defense is characterized by a different level and type of protection. The third line of defense is responsible for specific immunity.

Table 4.6 Major components of the host defenses

Lines of Defense	Comments
First line of defense: natural immune system	
▪ Physical barriers: skin, tears, coughing, sneezing ▪ Chemical barriers: low pH, lysozyme, digestive enzymes ▪ Genetic barriers: resistance inherent in genetic makeup of host (pathogen cannot invade)	First line of defense (innate host defense) prevents entry of pathogens in body
Second line of defense: natural immune system	
▪ Cellular component: mast cells, neutrophils, macrophages ▪ Humoral component: complement system, lysozyme, interferon	▪ Direct cell-cell contact results in secretion of soluble products ▪ Defense against fungal infections, intracellular organisms, tumor antigens and organ graft rejection
Third line of defense: adaptive immune system	
▪ Cellular component (B cells, T cells, plasma cells) ▪ Humoral component (antibodies, cytokines)	Adaptive (specific) host defense against pathogens

- Viral infections can disrupt the function of the respiratory epithelium allowing bacteria to enter areas that are normally sterile. A bronchogenic carcinoma can obstruct a bronchus. Superadded infection can arise distal to the obstruction. Compromise of the action of the chest muscles by trauma or abdominal surgery can affect the cough reflex.
- Infectious agents that successfully penetrate the physical barriers are then engaged by the cells and soluble factors of the innate immune system. There is no creation of memory cells that normally react immediately if same pathogen enters the body in the future.
- The innate immune system is also responsible for triggering activation of the adaptive immune system. The immune cells and products of the adaptive immune system reinforce the defense mounted by the innate immune system.

Second Line of Defense

Second line of defense comprises phagocytic cells (mast cells, neutrophils, macrophages), complement system, inflammatory chemical mediators and antimicrobial proteins such as complement, lysozyme, interferon and antibodies. Second line of defense does not create memory cells that normally react immediately if same pathogen enters the body in the future.

Third Line of Defense

Third line of defense is an acquired and specific immune response against invader pathogens. It is dependent on the function of cellular component (T cells, B cells, plasma cells), and humoral component (antibodies and cytokines). Third line of defense also creates memory cells that permit the immune system to immediately react if same pathogen enters the body in the future. Lymphoid organs such as the spleen, lymph nodes, and thymus are also intimately involved in these defense mechanisms.

BARRIERS FOR PATHOGENS

Nonspecific inborn or innate immunity is present at the time of birth, which provides different types of barriers to entry of the foreign agents in our body. Disruption in barriers for pathogens leads to infection.

- Patient with severe burns is susceptible to infections. Blockage of ducts in the salivary glands and lacrimal glands, intestine and genitourinary tract are at greater risk for infection.
- Bacteria in trachea are easily aspirated into the lung. Antibiotic use selects out bacteria such as *Pseudomonas aeruginosa*, *Enterobacter* species, and *Klebsiella* species.
- Supine position and reduction of stomach acidity allow bacteria to traverse the stomach and ascend the esophagus.
- Endotracheal ventilation gives direct access for organisms to the lungs. Abdominal surgery compromises the diaphragm and the cough reflex. Unwashed hands of health care workers can readily transmit bacteria from patient to patient.
- A lung abscess may arise as a result of bacterial pneumonia; aspiration of stomach fluid and oral bacteria; compression of a bronchus by a carcinoma. Right middle lobe pneumonia progresses to abscess formation due to lung parenchyma necrosis. Usually aerobic/facultative bacteria, e.g. *Klebsiella* species., *Staphylococcus aureus*, or *Pseudomonas aeruginosa*.
- Barriers to portal entry of pathogens are shown in Fig. 4.7. Barriers in first and second line of defense in innate immunity are shown in Table 4.7.

Anatomical Barriers

The skin and mucous membranes of the respiratory and gastrointestinal tract have several defenses.

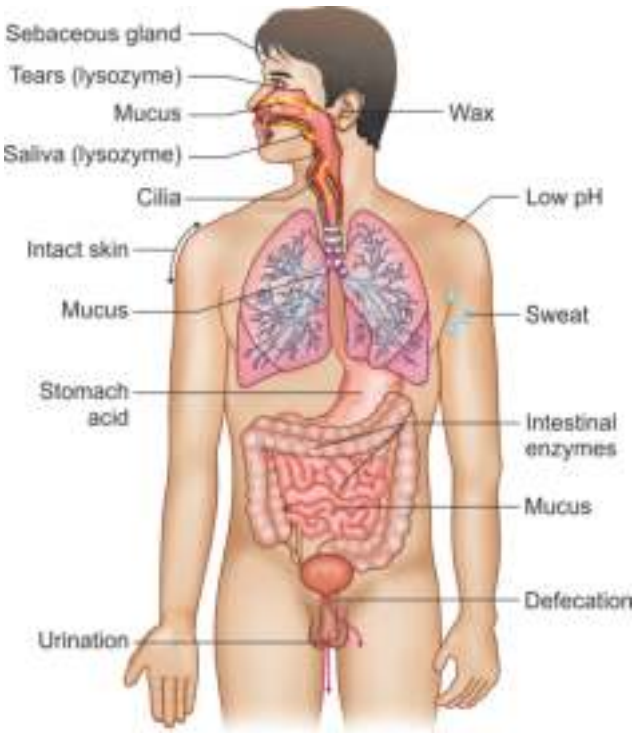


Fig. 4.7: Physical and chemical barriers to portal entry of pathogens. The skin, mucous membranes and endothelia throughout the body serve as physical barriers that prevent pathogens from reaching potential sites of infection. Chemical barriers such as enzymes in sweat, saliva and semen kill pathogens on body surfaces.

- Skin's outer most layer stratum corneum impregnated with insoluble keratin proteins is highly impervious to most pathogens. Sweat glands flush out microbes.
- The epithelial lining of the airways contains a brush border of cilia to entrap and propel particles upward toward the pharynx.
  - Sneezing due to irritation of nasal passages expels pathogens.
  - Coughing triggered by irritation of bronchi, trachea and larynx remove foreign irritants.
- Genitourinary tract gives partial protection by flushing out pathogens in urine.

Chemical Barriers

Chemical barriers against infection include enzymes in saliva, tears and mucous membranes and skin surface that break down the surface bacteria.

- **Skin: Dermcidin** synthesized by skin has antibacterial activity by lysing bacterial membranes. High lactic acid and electrolyte concentration of sweat and the skin's acidic pH and fatty acid content have antibacterial property. Sebaceous secretions have antimicrobial property.
- **Eyelids:** Meibomian glands of the eyelids lubricate the conjunctiva with an antimicrobial secretion.

Table 4.7 Barriers in first and second line of defense in innate immunity

Component	Function
Anatomical barriers prevent the entry of pathogens	
Skin	Skin epidermis
Nose	Hairs and cilia
Mucosa lining respiratory, gastrointestinal, and urogenital tracts	Mucosal lining secretes proteins and enzymes, absorbs metabolic substrates prevent microbial entrance
Antimicrobial peptides causing destruction of invading pathogens	
Skin	▪ Dermcidin (lysis of bacterial wall), sweat (rich in lactic acid, electrolytes), keratin, sebum (unsaturated fatty acids and hyaluronic acid): antibacterial actions ▪ Skin stratum corneum impregnated with insoluble keratin proteins is highly impervious to most pathogens
Eye	▪ Meibomian glands secretion lubricates the conjunctiva has antimicrobial property ▪ Lysozyme in tears has antimicrobial property
Salivary gland	Saliva contains lysozyme, which hydrolyzes the peptidoglycan in the wall of bacteria
Gastrointestinal tract	Hydrochloric acid by parietal cells and enzymes
Respiratory tract	Mucus secretion by lining mucosa in respiratory tract (mucociliary apparatus, sneezing and coughing)
Urogenital system	▪ Semen containing antibacterial secretion, flushing and acidity of urine ▪ Vaginal acidic pH (antibacterial action)
Phagocytic cells act as barriers	
Bone marrow	Macrophages, granulocytes, natural killer cells (NK cells kill infected cells and tumor cells, activation of macrophages through cytokine production)
Blood	Monocytes, neutrophils, eosinophils

Contd...

Table 4.7 Barriers in first and second line of defense in innate immunity (*Contd...*)

Component	Function
Lymph nodes	Tissue macrophages and natural killer cells (NK cells) kill carcinoma cells and virus infected cells
Liver	Macrophages (Kupffer cells)
Spleen	Macrophages, natural killer cells (NK cells) kill carcinoma cells and virus infected cells
Lymphoid cells	Innate lymphoid cells mediate immune response and regulate tissue homeostasis and inflammation
Endothelial/epithelial cells	Microbial recognition, cytokine production
Cytokines	
TNF-α, IL-1, chemokines	Mediate immune response and inflammation
IFN-α	Involved in resistance to viral infection
IFN-γ	Involved in resistance to intracellular pathogen infection and activation of macrophages
IL-12	Stimulates IFN-γ production by NK cells and T cells
IL-15	Stimulates NK cells
IL-10	Regulates and controls the inflammation process
TGF-β	Regulates and controls the inflammation process
Serum proteins	
Coagulation system	Localization of damage or infected tissue
Complement system	Opsonization, destruction of pathogens and T cell activation
Kallikrein-kinin system	Regulation of inflammation, coagulation system, pain cell proliferation, vasodilatation and blood pressure
C-reactive proteins and collectins	Opsonization of pathogens and complement activation
Cellular receptors	
Toll-like receptors (TLRs)	Recognize a variety of microbial components
NOD-like receptors (NDRs)	Sense bacterial components present in the cytoplasm
C-type lectin receptors (CLRs)	Recognize sugar moieties of bacteria and fungi
RIG-1-like receptors (RLRs)	Sense viral RNA

Cells derived preformed chemical mediators: histamine and serotonin.
Cells derived newly synthesized chemical mediators: prostaglandins, prostacyclin PGI2, TXA2, leukotrienes, lipoxins, cytokines (interleukins, TNF, chemokines, growth factors), oxygen-derived free radicals, nitric oxide, platelet activating factors and substance P.

Lysozyme enzyme in tears hydrolyzes the peptidoglycan in the wall of bacteria.

- **Salivary glands:** Lysozyme enzyme in saliva hydrolyzes the peptidoglycan in the wall of bacteria and protects from the pathogens.
- **Gastrointestinal tract:** Hydrochloric acid synthesized by gastric parietal cells that provides protection against many pathogens that are swallowed. Intestinal and bile secretions also cause destruction of pathogens.
- **Genital tract:** Semen contains an antibacterial chemical substance. Vagina has a protective acidic pH maintained by normal flora has protective against pathogens.

Reticuloendothelial System

Reticuloendothelial system is network of connective tissue reticular fibers enmeshing each cell inhabited by macrophages, which connects one cell to another within a tissue or organ. Macrophages attack and phagocytose pathogens that have managed to bypass the first line of defense. Monocytes migrate and differentiate into tissue macrophages, which are diffusely distributed in the connective tissues.

IMMUNE CELLS OF INNATE IMMUNE SYSTEM

There are many types of immune cells, that work to defend and protect the human body. Cells of the innate immune system originate from the pluripotent hematopoietic stem cells (HSCs) in the bone marrow that give rise to common myeloid progenitor and common lymphoid progenitor cells.

- Common myeloid progenitor cell gives rise to major myeloid cell types (neutrophils, eosinophils and basophils, monocytes/macrophages, dendritic cells and mast cells), erythrocytes and megakaryocytes.

- Common lymphoid progenitor cell gives rise to major lymphoid cells (T cells, B cells and natural killer cells).

Neutrophils

Neutrophils are produced from pluripotent hematopoietic stem cells (HSCs) in the bone marrow, that proliferate and differentiate through various stages, i.e. myeloblast, myelocyte, metamyelocyte, band form and mature neutrophils well equipped with an armory of granules, which release potent mixture of hydrolytic enzymes into phagolysosome which are lethal to microorganisms, but can also cause tissue damage.

- Neutrophils contain two types of granules: azurophil (primary) and specific (secondary) granules. Products of azurophil and specific granules of neutrophils participate in vascular permeability, chemotaxis and tissue damage.
  - Azurophil granules of neutrophils contain myeloperoxidase, lysosome-derived bactericidal factors, acid hydrolases, neutral proteases (collagenase, proteinase 3, elastase), nonspecific defensins, cathepsin D and phospholipase A_2.
  - Specific (secondary) granules of neutrophils contain high levels of iron-binding protein lactoferritin, collagenase, type 4 gelatinase, plasminogen activator, histaminase, alkaline phosphatase, leukocyte adhesion molecules and phospholipase.
  - Tertiary granules contain gelatinase of neutrophils.
- Neutrophils are pivotal effector cells of innate immune response and the first line of defense for eliminating pathogens in the tissues and clearing debris in acute inflammation. Neutrophils exit from the vascular compartment involving coordinated process: intravascular rolling, adhesion, transmigration along the activated vascular endothelial cells and basement membrane of the venules.
- Once neutrophils have traversed this vascular barrier, these are primed for the rapid detection of microbes and damaged tissue in the extracellular space. Neutrophils undergo sequential phases of highly coordinated chemotaxis towards tissue injury site leading to degradation of injurious agent. During mild tissue injury, inflammatory exudate is removed resulting to restoration of normal tissue architecture (resolution). Transition to chronic inflammation occurs in cases of persistent injurious agent.

Eosinophils

Eosinophil arises from the hematopoietic stem cells (HSCs) in the bone marrow and takes about eight days to mature through various stages, i.e. myeloblast, eosinophilic myelocyte, metamyelocyte, band form cell and mature eosinophil.

- The eosinophil migrates from the bone marrow into circulation leading to recruitment in target tissues/organs with the help of chemokine (eotaxin).
- Eosinophilic granules contain four major proteins: major basic protein (MBP) killing parasites and epithelial cells, eosinophilic cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN), which are involved in phagocytosis, killing invasive helminths, antigen presentation to reticuloendothelial cells and platelet interaction. The action of **EPO** also leads to oxidative burst, a crucial step in phagocytosis.

Macrophages

Macrophages can be found in almost all organs in the body such as lungs, liver, brain, lymph nodes, spleen, kidneys, skin, adipose tissue, peritoneum, synovium, placenta, and bone; they have special functions in each organ.

- Macrophages are efficient phagocytic cells of the innate immune system that phagocytose and secrete both proinflammatory chemical mediators (e.g. tumor necrosis factor, IL-1, IL-6, IL-8 and IL-12), and antimicrobial mediators. In addition, macrophages play an important role in eliminating damaged cells, necrotic debris, foreign materials and tumor cells.
- Macrophages and dendritic cells function as antigen-presenting cells (APCs), which present peptide antigens derived from the digested bacteria on the major histocompatibility complex (MHC) class II and activate specific adaptive cell-mediated immunity by activating CD4+ helper T cells.
- Macrophages present antigens to CD4+ helper T cells within tissues, while dendritic cells can activate naïve T cells to become effector CD4+ helper T cell, which are most powerful antigen-presenting cells (APCs).

Dendritic Cells

The mononuclear phagocytic cells such as dendritic cells and macrophages are closely related immune cells, which play central roles in antimicrobial defense and maintenance of organ integrity.

- Dendritic cells (DCs) are present in those tissues that remain in contact with the external environment,where dendritic cells sense self- and non-self antigens.
- Dendritic cells are present in skin, and inner lining of the nose, lungs and gastrointestinal tract. Dendritic cells can also be present in an immature state in the bloodstream.
- The different subtypes of dendritic cells include: Langerhans' cells (skin), plasmacytoid dendritic cells, interdigitating dendritic cells, and myeloid-

derived dendritic cells. Dendritic cells are characterized by distinct origins, receptors and varying functions.

- Dendritic cells can activate naïve T cells to become effector CD4+ helper T cells, and are most powerful antigen-presenting cells (**APCs**), while macrophages present antigens to CD4+ helper T cells.
- Dendritic cells also act as bridge between the innate immune system and specific adaptive immune response.
- The main differences between dendritic cells and macrophages include: (a) macrophages contribute to initiation of the inflammatory response, whereas dendritic cells get activated with inflammatory response to become antigen-presenting cells, and (b) macrophages do not die following activation while dendritic cells die after achieving their effector function.

Mast Cells

Mast cells are found in mucous membranes and connective tissues throughout the body, which originate from pluripotent progenitor cells in bone marrow, differentiate into progenitor cells and then mature mast cells under the influence of the c-KIT ligand, stem cell factor (SCF), and other cytokines. Under normal conditions, mature mast cells do not circulate in the bloodstream.

- The cytoplasm of the mast cell contains large granules that store inflammatory chemical mediators such as histamine, heparin, a variety of cytokines, chondroitin sulfate and neutral proteases.
- Mast cells can be activated by allergens, pathogens and physiologic chemical mediators. Activated mast cells release chemical mediators such as histamine and cytokines by two major pathways: (a) preformed chemical mediators are present in the granules, and (b) metabolism of arachidonic acid is produced through activation of a phospholipase. Intracellular Ca^{2+} and cyclic AMP are central to the initiation of these events. Mast cell degranulation may occur through C3a, C5a, and even by some microorganisms that can act directly on cell surface receptors.
- Mast cells play an important role in the physiologic functions (e.g. wound healing, defense against pathogens via the inflammatory response, vascular homeostasis, innate and adaptive immune responses, angiogenesis and venom detoxification), and pathophysiology of various disorders (e.g. allergy, bronchial asthma, anaphylaxis and autoimmunity).

Natural Killer Cells

Natural killer cells (NK cells) are derived from large granular lymphocytes. NK cells do not rearrange T cell receptor or immunoglobulin genes from their germline configuration. Immunophenotyping of natural killer cells includes CD16, CD56 and CD57.

- Natural killer cells express activating and inhibitory receptors to distinguish between healthy and infected cells. NK cells express activating receptors that recognize ligands on virus infected cells or malignant tumor cells.
- Natural killer cells are effector lymphocytes of the innate immune system, which control several types of malignant tumors and viral infections by limiting their spread and subsequent tissue damage. Recent studies revealed important insights into NK cells function in normal immune surveillance and NK cell receptor biology that has led to remarkable results in treated human malignancy.
- Natural killer cells do not require priming or prior activation in contrast to CD8+ cytotoxic T cells. NK cells can kill target cells by two major mechanisms: (a) cytotoxic granule-dependent signaling pathway and (b) death receptor signaling pathway. In both mechanisms, the target cell dies as a result of the activation of a battery of cytotoxic proteases within the target cell, called caspases.

Cytotoxic Granule-dependent Signaling Pathway of Natural Killer Cells

In the cytotoxic granule-dependent signaling pathway, binding of the natural killer cells to the surface of the virally infected cell triggers the extracellular release of perforin (pore-forming protein) and **granzyme** (diverse collection of proteases) from natural killer cell cytotoxic granules.

- Perforin polymerizes within the target cell membrane to form transmembrane channels that permit entry of granzyme into the target cell.
- Granzyme induces apoptotic cell death through activation of caspase protease cascade, either by directly processing and activating caspases, or through release of 'cytochrome c' from mitochondria that activates the 'apoptosome' pathway to caspase activation.

Death Receptor Signaling Pathway of Natural Killer Cells

In death receptor signaling pathway, membrane bound Fas ligand (FasL) on natural killer cell engages and trimerizes surface Fas receptor on the target cell.

- Engagement of surface Fas receptors on target cells recruits the adaptor protein Fas-associated death domain (**FADD**) to the death-inducing signaling complex (**DISC**) and followed by activation of caspase 8 on the cell surface receptors.

- Caspase 8 then promotes further caspase activation through directly processing other caspases, or via the mitochondrial apoptosome pathway similar to granzymes. Apoptosis occurs as a result of the activation of several 'executioner caspases 3, 6, 7, that coordinate apoptotic cell death through restricted proteolysis of numerous cellular proteins.

Intraepithelial Lymphocytes

Intraepithelial lymphocytes (IELs) contain subsets of innate-like T cell located above the basement membrane and in between intestinal epithelial cells, that evoke innate and adaptive immune responses to provide rapid protection to intestinal epithelium and promote sustained tolerance against dietary and microbial antigens. In the intestine, T cells express variable T cell antigen receptors with unknown antigen specificities.

SOLUBLE MEDIATORS

Soluble mediators of innate immune system include: cytokines, chemokines, pattern recognition receptors (PRRs), antimicrobial peptides, and complement system, which provide protection in the initial phase of contact with pathogens and prevent potentially harmful infections.

Opsonins

The process of engulfment of solid particulate material by phagocytic cells (neutrophils and macrophages) is known as phagocytosis, which is composed of four interrelated phases: chemotaxis, opsonization, engulfment and digestion.

- Opsonins are extracellular proteins, which react with bacteria and make them more susceptible to ingestion by phagocytes (neutrophils and macrophages).
- Examples of opsonins include specific IgG and C3b molecule of complement system. C3b is derived upon complement system activation by either classical pathway or the alternative pathway of complement system.

Complement System

Complement system consists of a group of soluble plasma proteins synthesized by liver present in its inactive form that interact with one another in three distinct enzymatic-activation cascades. These are numbered C1 to C9 (C3 most abundant).

- Complement system proteins play key role in innate and adaptive immunity. In innate immunity, complement proteins defend against foreign pathogens by opsonization, chemotaxis, and activation of leukocytes through cytolysis by C5b–C9 membrane attack complex.
- Recent studies revealed that complement system plays a key role in adaptive immunity by modulating and modifying the T cell response. All these studies suggest that complement system constitutes a critical link between the innate and adaptive immune responses.

Complement System Pathways

Complement system can be activated through three pathways: classical, alternative, and mannose-binding lectin, which have different triggers at starting points, but all converge at the same place, C3 convertase. This enzyme begins the formation of tiny openings in the cell membrane and the destruction of target pathogen. Pathways of complement system activation are shown in Fig. 4.8.

- **Classical pathway:** Classical pathway of complement system activation is also known as immunologic pathway, which involves fixation of antibodies (IgG and IgM) and complement (C1). C1q, C1, C1s are converted to form C4, C2, C3.
- **Alternative pathway:** Alternative pathway of complement system is antibody-independent pathway of complement system activation, which is accelerated by properdin in the presence of pathogens such as bacteria, fungi, viruses and parasites and venom. Factor B and factor D are converted to C3. Deficiency of C3 affects alternate pathway of complement activation resulting to recurrent infections with fatal outcome, until treated.
- **Mannose-binding lectin pathway:** The mannose-binding lectin pathway of complement system uses soluble receptors that recognize microbial surfaces to activate the complement system cascade, which is also antibody-independent pathway. It binds mannose (carbohydrate) on pathogen surfaces. It is nonspecific for bacteria and viruses. MBL, MASP-1, MASP-2 are converted to form C4, C2, C3.

Complement System Activation

All three activated complement system pathways converge to form C3. C3 convertase enzyme converts C3 molecule into an activator C3b. C3 convertase enzyme and C3b are the central features of complement system activation. Activation of complement system produces C3a, C5a and C5–9. Functional protein classes in the complement system are given in the Table 4.8. Mechanism of action of complement is described as under:

- **C3 convertase enzyme:** C3 convertase activates complement system and splits C3 into two functionally distinct C3a and C3b. C3a is released, while C3b binds to C5 convertase.

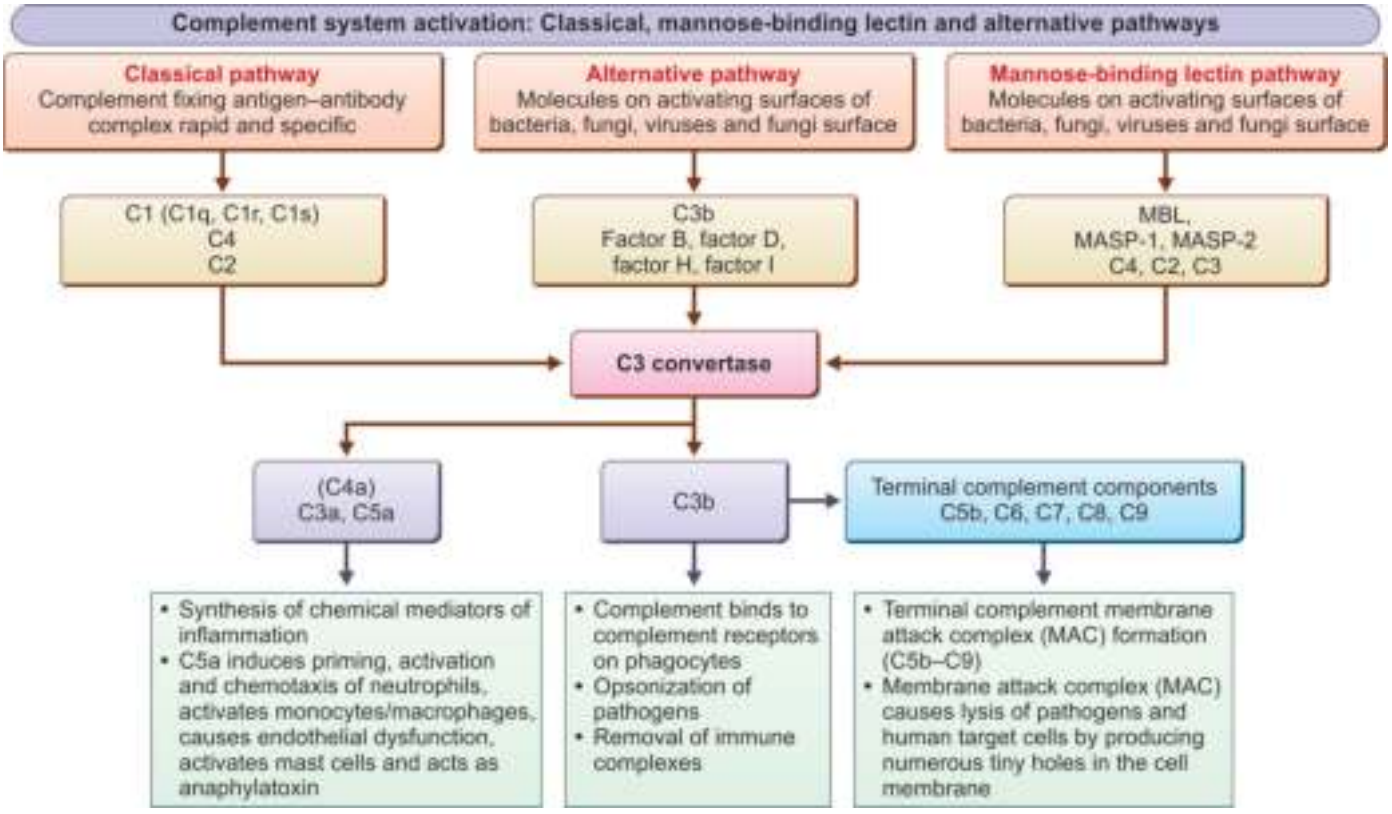


Fig. 4.8: Pathways of complement system activation. Complement system involves classical, alternative and mannose-binding lectin pathways. Common aim of complement system pathways is to yield C3 convertase, which facilitates the cleavage of C3, which in turn cleaves C5 to yield C5b. C5b hence forms a complex with C6, C7, C8 and C9 to form C5b–C9, also known as the membrane attack complex (MAC) that leads to lysis of pathogen plasma membrane and target cells.

Table 4.8 Functional protein classes in the complement system

Characteristics	Protein Classes of Complement System
Binding to antigen–antibody complexes and pathogen surface	C1q
Binding to mannose on bacteria	Mannose-binding lectin (MBL)
Activating enzymes	C1r, C1s, C2b, D, MASP1, MASP2
Membrane binding proteins and opsonins	C4b, C3b
Peptide mediators of inflammation	C5a, C3a, C4a
Membrane attack proteins	C5b, C6, C7, C8, C9
Complement receptors	CR1, CR2, CR3, CR4, C1qR
Complement-regulatory proteins	C1INH, C4bp, CR1, MCP, H, I, P, CD59

- **Binding of C3b to C5 convertase:** C3b–C5 convertase complex cleaves C5 to C5a and C5b. C5a is released, while remaining C5b binds to C5–C9. Other molecules include C3a and C5a, which are peptide clinical mediators of inflammation. These participate in chemotaxis of leukocytes and phagocytosis of bacteria.
- **Binding of C3b to C5–C9:** C5b is a reactive site for the final assembly of an attack complex. C5b fragment polymerizes with complement proteins C6, C7, C8, and C9, resulting in the formation of membrane attack complex (MAC). Insertion of MACs produces hundreds of tiny holes in the lipid bilayer cell membrane results in cell lysis.

Complement System: Actions

Breakdown products of complement system mediate acute inflammation by following mechanisms. Complement system cascade and its actions are given in Table 4.9.

- **Opsonization and phagocytosis:** Complement system activation generates cleavage products,

Table 4.9 Complement system cascade and its actions

Complement System Cascade	Actions
C3b	▪ Opsonization of microbes ▪ Phagocytosis of microbes
C3a and C5a	Increased vascular permeability
C5a	▪ Adhesion of leukocytes ▪ Transmigration of leukocytes ▪ Chemotaxis of leukocytes
C3a, C5a and C4	Anaphylactic shock (due to excessive release of histamine)
C5a	Synthesis of arachidonic acid metabolites (lipoxygenase pathway)
C5b–C9 membrane attack complex (MAC)	Degradation of microbes and enhancing arachidonic acid metabolism and producing reactive oxygen metabolites

Defective formation of membrane attack complex (MAC) leads to increased susceptibility to Neisseria organisms.

mainly C3b and C4b, which are deposited on the surface of the cells and recognized by phagocytes that express receptors for these proteins. C3b acts as opsonins, which coat the bacteria leading to phagocytosis by neutrophils and macrophages.

- **Increased vascular permeability:** Both C3a and C5a induce smooth muscle contraction and increased vascular permeability. C3a and C5a also act on endothelial cells lining blood vessels to induce expression of cell adhesion molecules.
- **Leukocytic adhesion, transmigration and chemotaxis:** C5a fragment is a chemotactic agent, which mediates the release of histamine from platelet-dense granules, which induces the expression of leukocyte adhesion molecules resulting in adhesion, transmigration and chemotaxis of leukocytes.
- **Anaphylactic shock:** C3a, C5a and C4 are called anaphylatoxins, which stimulate degranulation of mast cells and basophils liberating histamine. Histamine causes increased vascular permeability, vasodilatation and smooth muscle contraction. Patient develops 'anaphylactic shock'.
- **Synthesis of arachidonic acid metabolites:** C5a fragment activates lipoxygenase enzyme, which acts on membrane of neutrophils and monocytes resulting to synthesis of arachidonic metabolites via lipoxygenase pathway.
- **Membrane attack complex (MAC):** C5b–C9 is a lytic agent for bacteria and target cells, which stimulates arachidonic acid metabolism and produces reactive oxygen metabolites. Defective formation of membrane attack complex (MAC) leads to increased susceptibility to Neisseria organisms.

Inflammatory Cytokines: Messenger Molecules of Immune System

Cytokines released from innate immune cells play a vital role in regulating immune response in health and disease. The key proinflammatory cytokines such as IL-1, IL-6, IL-12, IL-16, TNF-α, IFN-α and IFN-γ are intracellular soluble messengers,which initiate signaling pathways via type 1 cytokine receptors (CCR1) and coordinate of all cell systems.

- **Chemokines** released by macrophages and dendritic cells recruit effector cells to the site of infection, which induce systemic reaction known as the acute-phase response.
- **TNF-α** is an important cytokine that triggers local containment of infection that induces shock when released into systemic circulation. Cytokines, principal source and cellular targets are given in Table 4.10.

Table 4.10 Cytokines, principal source and cellular targets

Inflammatory Cytokine	Principal Cell Source(s)	Principal Cellular Targets
Tumor necrosis factor-α (TNF-α)	Macrophages, T cells, mast cells	T cell proliferation, IL-2 production and cytotoxic to some malignant tumor cells
Tumor necrosis factor-β (TNF-β)	T cells	T cell proliferation, IL-2 production and cytotoxic to some malignant tumor cells
Tumor necrosis factor (TNF) family members	Macrophages, T cells, mast cells	▪ Vascular endothelial cells activation, neutrophils adhesion, activation, chemotaxis of leukocytes and phagocytosis ▪ Acute inflammation and activation of coagulation system ▪ Acute-phase proteins synthesis in liver ▪ TNF acts on hypothalamus to induce fever ▪ Muscle and fat catabolism (cachexia) ▪ Apoptosis of many cells

Contd...

Table 4.10 Cytokines, principal source and cellular targets (*Contd...*)

Inflammatory Cytokine	Principal Cell Source(s)	Principal Cellular Targets
Interleukin-1 (IL-1)	Macrophages, dendritic cells, endothelial cells, some epithelial cells, mast cells	▪ Endothelial cells activation, inflammation, coagulation ▪ IL-1 acts on hypothalamus to induce fever ▪ Acute-phase proteins synthesis in liver ▪ T helper 17 (Th17) cells differentiation
Interleukin-2 (IL-2)	Macrophages, T cells and natural killer cells	▪ Proliferation of B cells, T cells, and natural killer cells ▪ Monocytes activation
Interleukin-3 (IL-3)	T cells	Acts as growth factor for tissue mast cells and hematopoietic stem cells
Interleukin-4 (IL-4)	T cells	▪ Promotes growth of B cells and T cells ▪ Enhancing expression of MHC class II molecules
Interleukin-5 (IL-5)	T cells	Promotes end stage maturation of B cells into plasma cells
Interleukin-6 (IL-6)	T cells, monocytes/macrophages, and endothelial cells	▪ Promotes maturation of B cells and T cells ▪ Synthesis of acute-phase proteins from liver ▪ B cells proliferation and formation of antibody-producing plasma cells
Interleukin-7 (IL-7)	Stromal cells in bone marrow, dendritic cells, hepatocytes, keratinocytes, neurons and epithelial cells except normal lymphocytes	Acting as a growth factor for hematopoietic stem cells in bone marrow
Interleukin-10 (IL-10)	Macrophages, dendritic cells, T cells	Macrophages and dendritic cells: inhibition of cytokine and chemokine production, reduced expression of costimulators and class II MHC molecules
Interleukin-12 (IL-12)	Dendritic cells, macrophages	IFN-γ synthesis by natural killer cells and T cells, increased cytotoxic activity T cells and T helper 1 (Th1) cells differentiation
Interleukin-13 (IL-13)	T cells	Acting as a growth factor for hematopoietic stem cells in bone marrow
Interleukin-15 (IL-15)	Macrophages, others	Proliferation of natural killer cells and T cells
Interleukin-18 (IL-18)	Macrophages	IFN-γ synthesis by natural killer cells and T cells
Interferon-α (IFN-α)	B cells and macrophages	Antiviral activity, increased MHC class II molecules and natural killer cells activation
Interferon-β (IFN-β)	Fibroblasts	Antiviral activity
Interferon-γ (IFN-γ)	T cells and natural killer cells	Antiviral activity, activation of macrophages, increased MHC class II molecules
Chemokines	Macrophages, dendritic cells, endothelial cells, T cells, fibroblasts, platelets	▪ C–X–C (α-chemokine): chemotaxis of neutrophils ▪ C–C (β-chemokine): chemotaxis monocytes ▪ XC: (γ-chemokine): chemotaxis of lymphocytes ▪ CX3C: chemotaxis of monocytes and T cells
TGF-β	Many cell types	Inhibition of inflammatory T cells: differentiation of Th17, CD4+ regulatory T cells
G-CSF	Macrophages, neutrophils and T cells, fibroblasts and vascular endothelium	Hematopoiesis, activation and differentiation of neutrophils
GM-CSF	Fibroblasts and vascular endothelium	▪ Promoting the growth and development of macrophages from undifferentiated precursor stem cells ▪ Activation of natural killer cells and dendritic cells

Acute Phase Reactants Synthesis

Acute phase reactants are inflammatory markers synthesized in liver that exhibit changes in serum concentration by >25%, in response to inflammatory cytokines such as IL-6, IL-1 and TNF-α.

- Acute phase reactants cause several adverse systemic effects such as fever, anemia of chronic disease, anorexia, lethargy, weakness, somnolence, leukocytosis, amyloidosis, increased cortisol, decreased thyroxine, decreased serum iron and cachexia.
- Increased production of acute phase reactants is a sensitive indicator of inflammation, which can occur prior to the development of an inflammatory leukogram. Concentration of acute phase reactants during acute inflammation is given in Table 4.11.

CELLULAR RECEPTORS FOR MICROBES AND DAMAGED CELLS

Pathogen recognition receptors (**PRRs**) are a class of germline encoded transmembrane receptors found on macrophages, dendritic cells and epithelial cells that recognize different types of pathogen-associated molecular patterns (**PAMPs**).

- The activation of **PRRs** is critical for the initiation of innate immunity, which plays a key role in first line defense until more specific adaptive immunity is developed. PRRs differ in the signaling cascade and host defenses, which are activated by their engagement in various tissues.
- Currently identified PRR families are toll-like receptors (TLRs), nucleotide-binding and oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene-I-like receptors (RLRs) and AIM2-like receptor (ALR). AIM2-like receptors are found in hematopoietic cells, that recognize the presence of double-stranded DNA (dsDNA) of microbial or host cellular origin.

Table 4.11 Concentration of acute phase reactants during acute inflammation

Acute Phase Reactants	Function
Marked increase in concentration	
C-reactive protein	Fixation of complement and opsonization of pathogens
Mannose-binding lectin	Fixation of complement
α_1 Glycoprotein	Transpiration of protein
Serum amyloid P component	Precursor of amyloid component
Moderate increase in concentration	
α_1 Protease inhibitors	Inhibition of bacterial proteases
α_1 Antichymotrypsin	Inhibition of bacterial proteases
C3, C9 and factor	Enhancement of complement function, opsonization, destruction of pathogens and T cell activation
Ceruloplasmin	Oxygen scavenger
Fibrinogen	Localization of damage or infected tissue
Haptoglobin	Hemoglobin binding protein
Fibronectin	Cell attachment
Angiotensin	Blood pressure

Pathology Pearls: Cell-associated Pattern Recognition Receptors

- After entering the tissues, many microbes are recognized, ingested, and killed by phagocytes via a cell-associated pattern recognition receptors (PRRs).
- The cell-associated pattern recognition receptors enhance function of phagocytic cells. PRRs induce production of antimicrobial proteins, cytokines and chemokines, PRRs differentiate phagocytic cells to a more active state.
- G protein-coupled receptors on phagocytes link microbe recognition with increased efficiency of intracellular killing.
- Microbial recognition and tissue damage initiate an inflammatory response.
- Toll-like receptors represent an ancient pathogen-recognition system.
- Mammalian toll-like receptors are activated by many different pathogen-associated molecular patterns.
- TLR-4 recognizes bacterial lipopolysaccharide in association with the host accessory protein MD-2 and CD14.
- TLRs activate NF-κB, AP-1 and IRF transcription factors to induce the expression of inflammatory cytokines and type 1 interferons.
- The NOD-like receptors are intracellular sensors of bacterial infection and cellular damage.
- NLRP proteins react to infection or cellular damage through an inflammasome to induce cell death and inflammation.
- The RIG-I-like receptors detect cytoplasmic viral RNAs and activate mitochondrial antiviral signaling protein (MAVS) to induce type 1 interferon production and proinflammatory cytokines.
- Cytoplasmic DNA sensors signal through STING signaling to induce production of type 1 interferons.
- Activation of innate sensors in macrophages and dendritic cells triggers changes in gene expression that have far-reaching effects on the immune system.
- Toll-signaling in Drosophila is downstream of a distinct set of pathogen-recognition molecules.
- TLR and NOD genes have undergone extensive diversification in both invertebrates and some primitive chordates.

Toll-like Receptors

Macrophages, neutrophils, dendritic cells, microglial cells, eosinophils, mast cells, endothelial cells, mucosal cells and lymphocytes express toll-like receptors (TLRs) within wall of phagocytes.

- Toll-like receptors such as TLR1, TLR2, TLR4 and TLR6 are expressed on cell surface. Toll-like receptors such as TLR3, TLR7 and TLR8 are expressed in endosomes. Endosomal TLRs (TLR3, TLR7 and TLR8) respond only to nucleic acids.
- All TLRs contain a ligand-binding domain composed of leucine-rich motifs and a cytoplasmic signaling, toll-like interleukin-1 receptor (TIR) domain.
- Toll-like receptors on phagocytic cells provide defense against invasion by microorganisms. Different TLRs on phagocytic cells respond to many different structurally diverse products of microbes, which engulf and degrade pathogens. Binding of a particular class pathogen to TLRs initiates innate immune response against microbes by relaying a signal to the nucleus and activating synthesis of transcriptional factor resulting in synthesis of immune molecules known as interleukins. It is the first step to activate both the innate (nonspecific) and specific (adaptive) immune responses.
- Currently 10 TLRs are known to exist on human cells. Each TLR can recognize distinct microbial molecules known as pathogen-associated molecular patterns (PAMPs). Each TLR recognizes and binds distinct microbial molecules called pathogen-associated molecular patterns (PAMPs).
- Toll-like receptors and their actions are shown in Fig. 4.9. Toll-like receptors and their ligands are given in Table 4.12. Pathogen-associated molecular patterns (PAMPs) are given in Table 4.13.

NOD-like Receptors and the Inflammasome

The nucleotide-binding oligomerization domain-like receptors (NOD-like receptors, i.e. NLRs) sense pathogen-associated molecular patterns (PAMPs) that enter the cell cytoplasm via phagocytosis and also sense damage-associated molecular patterns (DAMPs) associated with severe cell stress.

- NOD-like receptors (NLRs) bind to PAMPs and DAMPs and play key role in regulating innate immune response. NLRs can cooperate with toll-like receptors (TLRs) and play key role in regulation of inflammatory response and apoptosis.
- NOD-like receptors (NLRs) are expressed in macrophages, dendritic cells and lymphocytes. NLRs constitute a large family of intracellular pathogen recognition receptors (PRRs) such as NOD1, NOD2 and NOD3. NOD1 and NOD2 recognize peptidoglycan components common to both gram-positive and gram-negative bacteria. Both NOD1 and NOD2 activate MAPK and NF-κB pathways in production of proinflammatory cytokines.

C Type Lectin Receptors

C type lectin receptors (CLRs) are expressed on monocytes, macrophages and dendritic cells, which recognize mannose, fucose and glucan carbohydrate moieties present in bacteria, fungi and viruses. Pathogen recognition by CLRs leads to pathogen internalization, and degradation; and subsequent antigen presentation. CLRs play key roles in regulation of innate and specific adaptive immunity.

RIG-1-like Receptors

RIG-1-like receptors (RLRs) are intracellular receptors for RNA viruses, which sense viral RNA and triggers

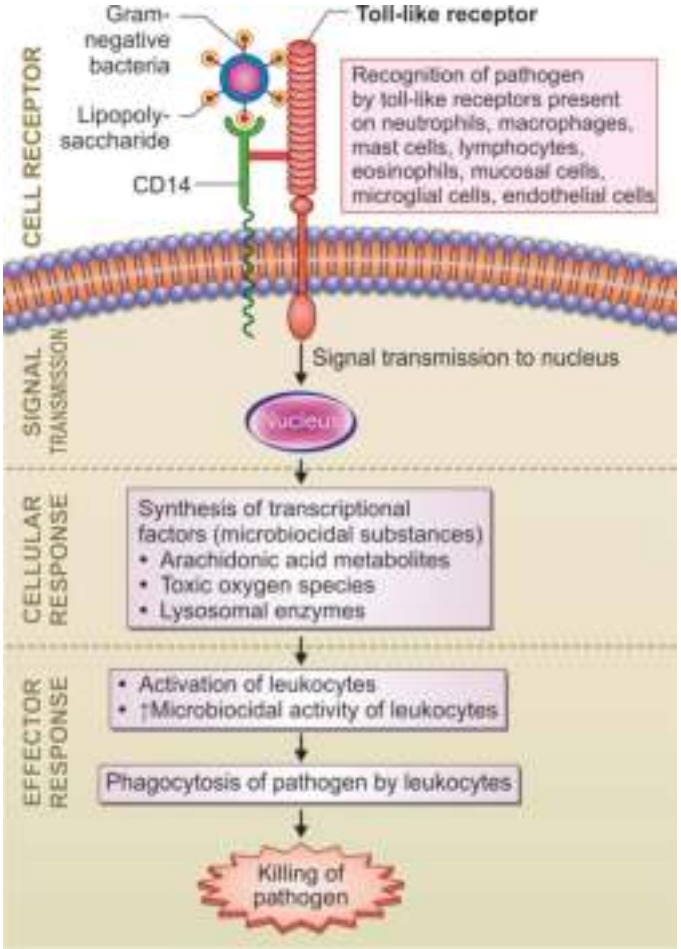


Fig. 4.9: Toll-like receptors and their actions. TLR binds and relays a signal to the nucleus resulting in activation of nuclear synthesis of transcription factors (chemical mediators) such as arachidonic acid metabolites and cytokines amplifying the inflammatory reactions, and production of microbiocidal reactive oxygen species and lysosomal enzymes killing the microbes.

Table 4.12 Toll-like receptors and their ligands

Receptor	Ligand (PAMPs)	Origin of Ligand	Location of Toll-like Receptors
TLR1	Triacyl lipopeptides and soluble factors	Bacteria, mycobacteria, *Neisseria meningitidis*	Plasma membrane
TLR2	Heat shock protein 70, peptidoglycan, lipoprotein/lipopeptides, HCV core and nonstructural 3 protein	Host gram-positive bacteria, various pathogens, hepatitis C virus	Plasma membrane
TLR3	Double-stranded RNA	Viruses	Endosome
TLR4	Lipopolysaccharides, envelope protein and Taxol	Gram-negative bacteria, mouse mammary-tumor virus	Plasma membrane
TLR5	Bacterial flagellin	Bacteria	Plasma membrane
TLR6	Zymosan, lipoteichoic acid, diacyl lipopeptides	Fungi, gram-positive bacteria, mycoplasma	Plasma membrane
TLR7	Single-stranded RNA (ssRNA), imidazoquinoline	Viruses, synthetic compounds	Endosome
TLR8	Single-stranded RNA (ssRNA), imidazoquinoline	Viruses, synthetic compounds	Endosome
TLR9	CpG-containing DNA	Bacteria, malaria and viruses	Endosome
TLR10	Not determined	Not determined	Plasma membrane
TLR11	Profilin-like molecule	*Toxoplasma gondii*	Plasma membrane

Toll-like receptors such as TLR1, TLR2, TLR4, LR5, TLR6, TLR10 and TLR11 are expressed on cell surface. Toll-like receptors such as TLR3, TLR7, TLR8 and TLR9 are expressed in the endosomes.

Table 4.13 Pathogen-associated molecular patterns (PAMPs)

Pathogens	Pathogen-associated Molecular Patterns
Gram-negative bacterial cell wall	Lipopolysaccharides (LPS)—endotoxin
Gram-positive bacteria cocci	Peptidoglycan such as lipoprotein, lipoteichoic acid
Bacteria with flagella	Flagellin
Viruses	Single-stranded or double-stranded RNA molecules
Fungi	Zymosan

innate and specific adaptive immunity mainly through the rapid induction of type 1 interferons (IFNs) and inflammatory cytokines. Viral RNA is a potent inducer of innate immune response, which is recognized by cytoplasmic RNA helicase or toll-like receptors (TLRs). The RLR family is composed of three members: RIG-1, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP-2).

G Protein-coupled Transmembrane Receptors

G protein-coupled transmembrane receptors are embedded in the plasma membrane of leukocytes.

- G protein-coupled transmembrane receptors bind to extracellular molecules (e.g. hormones and neurotransmitters) and transmits into intracellular responses leading to production of secondary messengers involved in regulation of many physiologic functions such as taste, smell, sight, neurotransmission, pain perception and cardiac output.
- Most leukocytes express more than one G protein-coupled transmembrane receptors on their surface and sense wide range of chemokines and chemoattractants leading to leukocyte movement during immune responses.

Mannose Receptors

Mannose receptors are pattern recognition receptors (PRRs) primarily expressed on tissue macrophages and dendritic cells, which recognize and bind to microbial structures bearing mannose, fucose and N-acetylglucosamine on their surface. Mannose receptors play key roles in host defense via phagocytosis and degradation of microbes, and provides a link between innate and specific adaptive immunity.

RESPONSES OF INNATE IMMUNE SYSTEM

Innate immune system is the first line of host defense against invasive pathogens by discrimination of 'nonself' from 'self'-antigens, which consists of physical, chemical, cellular and humoral defenses against pathogens. The main purpose of the innate immune response is to prevent the spread of pathogens throughout the body.

Signaling in Innate Immunity and Inflammation

Inflammation is the immune response to harmful stimuli such as pathogens, damaged cells, irradiation and toxic compounds, and acts by removing injurious stimuli and initiating healing process. Innate immune cells, neutrophils, macrophages, dendritic cells, mast cells and natural killer cells express pattern recognition receptors (PRRs), which bind to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) released from injured cells leading to recognition of invading pathogens and their destruction. Activated PRRs initiate signaling cascades that trigger the release of factors that promote recruitment of leukocytes to the tissue injury site.

Antiviral Defense

Innate immune response is the first line defense against invading viral pathogen. Interferon molecules such as IFN-α and IFN-β are produced and secreted from the infected cells upon virus infection and recognition. Interferon molecules, then act as signaling/communication molecules to induce antiviral and antiproliferative response in the neighboring cells so that those cells become refractory to infection.

Pathogen-associated Molecular Patterns Signaling inducing Innate Immunity

Pathogen recognition receptors (PRRs) are a class of germline encoded receptors that recognize pathogen-associated molecular patterns (PAMPs) of innate immune system. Examples of **PAMPs** include lipopolysaccharide (LPS) of gram-negative bacteria, lipoteichoic acids (LTA) of gram-positive bacteria, peptidoglycans and lipoproteins. Upon PAMP recognition, PRRs present on the cell surface or intracellularly signal to the host, the presence of infection by pathogens triggers pro-inflammatory and antimicrobial responses by activating a multitude of intracellular signaling pathways, including adaptor molecules, kinases and transcription factors. Pathogen-associated molecular patterns are shown in Fig. 4.10.

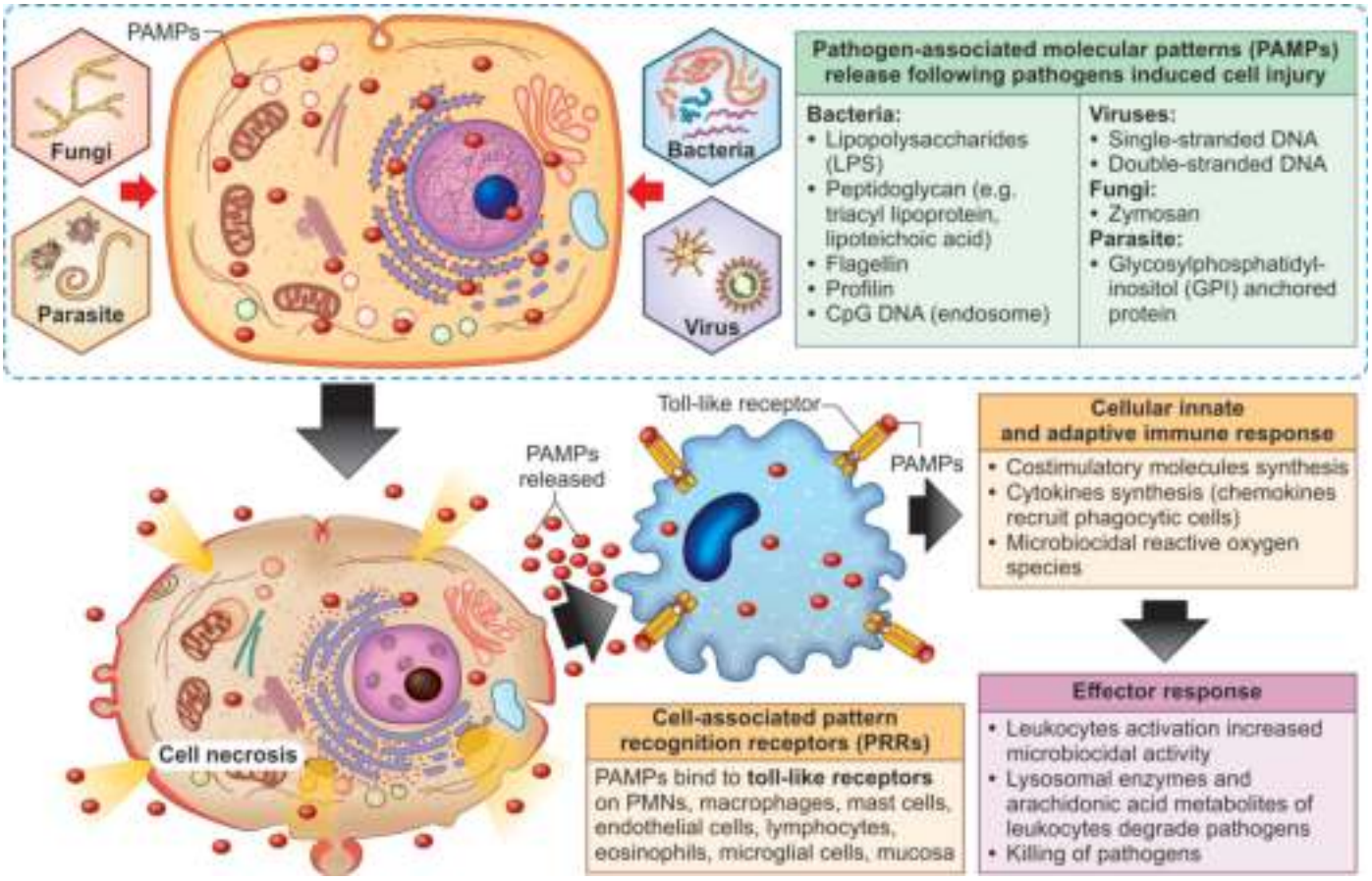


Fig. 4.10: Pathogen-associated molecular patterns. Microbes release PAMPs that bind to the family of pattern recognition receptors (PRRs), i.e. toll-like receptors and mediate innate and adaptive immune responses. Activation of toll-like receptors by specific ligands induces cytokine release and costimulatory molecules that instruct the type on immune response and direct antimicrobial response and tissue injury.

Damage-associated Molecular Patterns Signaling inducing Innate Immunity

Damage-associated molecular patterns (DAMPs) are endogenous danger molecules that are released from damaged cells that activate the innate immune system by interacting with pattern recognition receptors (PRRs). Necrotic cells release their cellular contents from organelles and nucleus (RNA, DNA and nucleotides) as well as IL-1. ATP, HMGB1, uric acid and heat shock proteins (HSPs) that recruit and activate neutrophil, macrophages and dendritic cells, thereby contributing to host defense and inducing a highly inflammatory response by binding to PRRs. Damage-associated molecular patterns (DAMPs) are shown in Fig. 4.11.

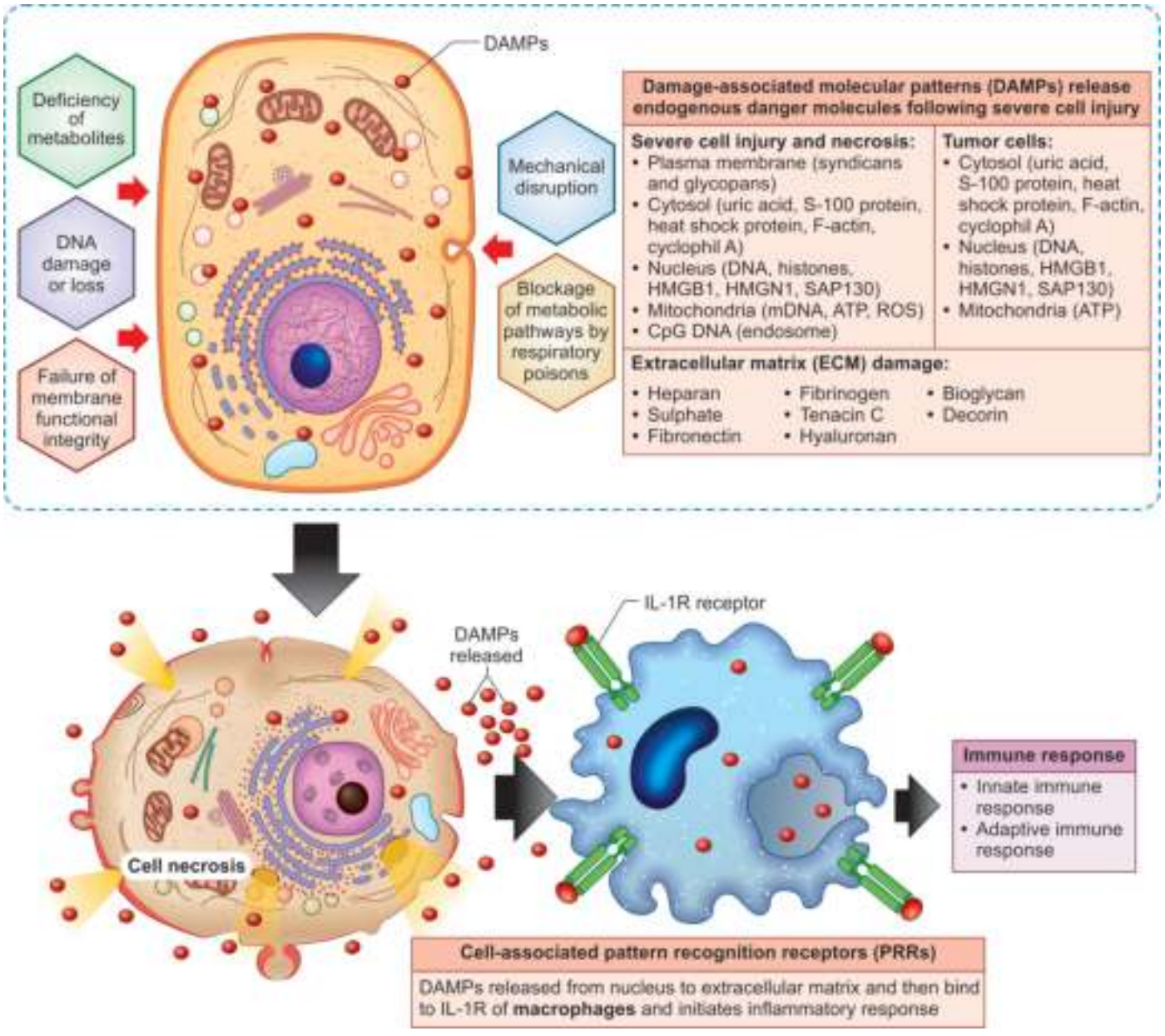


Fig. 4.11: Damage-associated molecular patterns (DAMPs). Necrotic cells release damage-associated molecular patterns (DAMPs), i.e. endogenous danger molecules from damage cells, whereas mild cell injury apoptosis induced cells do not. Stimuli that induce cell necrosis frequently cause severe cellular damage, which leads to rapid cell rupture with consequent release of intracellular DAMPs. Damage-associated molecular patterns can then engage cells of the immune system and promote inflammation. On the other hand, because stimuli that initiate apoptosis are physiologic and relatively mild, apoptotic cells do not rupture and their removal is coordinated by macrophages and other cells of the innate immune system, before release of DAMPs can occur. For this reason, apoptosis is not typically associated with activation of the immune system.

ADAPTIVE IMMUNE RESPONSE

Adaptive immune response provides highly specific protection against pathogens, which involves T cells, B cells, natural killer cells (NK cells), dendritic cells (DCs). T cells and B cells are indistinguishable by light microscopy using conventional stains but can only be distinguished by the presence of different surface proteins on T cells and B cells. Dendritic cells act as an important conduit between the innate and adaptive immune systems.

- Adaptive immune response consists of distinct steps: recognition of antigen, activation of T cells and B cells, their interrelationship with macrophages and elimination of antigen (effector phase). Immune response declines as a result of apoptosis of antigen-stimulated T cells or B cells leading to restoration of homeostasis. Antigen-presenting cells (APCs) which survive and are responsible for immunologic memory.
- Two types of adaptive (specific) immunity include: humoral immunity and cell-mediated immunity, which are induced by different types of lymphocytes and function to eliminate different types of pathogens.
  - In humoral immunity, B cells secrete antibodies that prevent against entry of pathogens and eliminate extracellular microbes. B cells recognize many different antigens and develop into antibody secreting plasma cells.
  - In cell-mediated immunity, CD4+ helper T cells activate macrophages and neutrophils to eliminate phagocytosed pathogens. CD8+ cytotoxic T cells directly kill infected cells. CD4+ helper T cells recognize antigens on the surfaces of the antigen-presenting cells (APCs) and secrete cytokines, which stimulate different mechanisms of adaptive immunity and inflammation.
- CD8+ cytotoxic T cells recognize antigens on infected cells and eliminate them. CD4+ regulatory T cells suppress immune responses to self-antigens.

Pathology Pearls: Key Features of Adaptive Immune Response

Recognition of Antigen

Adaptive immune system has been ability to identify antigen and distinguish between self- and nonself-antigens.

Specificity

- Adaptive immune system mounts a highly specific response against distinct antigens, which allows immune system to respond to newly encountered antigens.
- Fine specificity exists because individual lymphocytes express membrane receptors that can distinguish subtle differences in structure between distinct epitopes.

Specialization

- Adaptive immune system generates responses that are optimum for defense against different types of microbes.
- Cells of adaptive (specific) immune system include lymphocytes (B cells and T cells), monocytes/macrophages, Langerhans' cells of skin, and dendritic cells of lymphoid tissue.

Diversity

- Diversity is one of the key characteristics of adaptive immune system that enables immune system to respond to a large variety of distinct antigens.
- Lymphocyte repertoires of 3×10^7 different clonotypes protect human against infections while avoiding unwanted immune responses against self-peptides and innocuous antigens. Another important source of diversity in the immune system is due to the genes encoding major histocompatibility complex (MHC) molecules.
- For an adaptive immune response to be induced, the proteins of a pathogen need to be degraded into peptides, which are subsequently bound to major compatibility complex molecules on the surface of antigen-presenting cells (APCs).
- The resulting MHC peptides can be recognized by T cell receptors. Each person expresses three classical MHC class I (HLA-A, HLA-B, HLA-C) and three MHC class II gene pairs coding for the α- and β-chains of HLA-DP, HLA-DQ, HLA-DR.
- Diversity of the lymphocyte receptors occurs as a result of evolution of somatic diversification mechanisms. Genes coding for the V, D and J segments of the lymphocyte receptors are somatically rearranged and imprecise joining of the gene segments, addition of nucleotides and somatic hypermutation subsequently contribute to the diversity of lymphocytes that can bind their ligands with great specificity.

Clonal Expansion of Lymphocytes

- The clones of lymphocytes with different specificities are present in an unimmunized person. Lymphocytes activated by antigen give rise to clones of antigen-specific effector cells that mediate adaptive immunity. Clonal expansion increases number of antigen-specific lymphocytes from a small number of naïve B cells and T cells.
- Clone refers to population of B cells and T cells with identical antigen receptors are presumably derived from one precursor cell. Each antigen selects a pre-existing clone of specific B and T cells and stimulates the proliferative and differentiation of that clone.

Immunologic Memory

- Lymphocytes activated by antigen proliferate in the peripheral lymphoid organs, generating effector cells and immunologic memory.
- Adaptive immune system has memory that leads to enhanced responses to repeated exposures to the same antigens. Some of B cells and T cells become memory cells that recognise the same antigen in the future.

- The immune response to the first exposure to antigen called primary response that is initiated by naïve B and T cells that are encountered antigen for the first time. Subsequent encounter with same antigen leads to secondary immune response.

Nonreactivity of Adaptive Immune System to Host Cells (Self-tolerance)

- Adaptive immune system prevents injury to the host during immune responses to foreign antigens.
- Immunologic unresponsiveness is also called tolerance. Tolerance to self-antigens is maintained by many mechanisms: (a) elongation of lymphocytes that express membrane receptors specific for some self-antigens, and (b) inactivation of self-reactive lymphocytes or suppression of lymphocytes by the actions of regulatory cells.
- Abnormalities in the maintenance of self-tolerance lead to immune responses against the self (autologous) antigens, which may induce autoimmune disorders.

Contraction of Adaptive Immune Response and Homeostasis

Contraction of adaptive immune response and homeostasis prevent injury to the host during responses to foreign antigens.

ANTIGENS AND IMMUNOGENS

In immunology, antigen is a macromolecule of high molecular weight >10,000 daltons, which can trigger an immune response in the host by activating leukocytes that provide protection. Antigens may be present on invaders, such as microorganisms, cancer stem cells and transplanted organs.

ANTIGENICITY AND IMMUNOGENICITY

Antigen has two distinct properties: antigenicity (immunologic reactivity) and immunogenicity. Antigenicity is the ability to combine specifically with the final products of the immune response, i.e. secreted immunoglobulins and/or surface receptors on T cells. Immunogenicity is defined as the ability of cells/tissues to provide an immune response and its generally considered to be an undesirable physiologic response.

Antigenicity

Antigenicity refers to ability of an antigen to combine specifically with final products of antibodies and/or T cell receptors (TCRs). Antigenicity depends on recognition of antigen, size of antigen and complexity of cell or molecule.

- **Antigen motifs:** Each antigen may contain several motifs that are recognized by immune system and each motif is an epitope within an antigen to which antigen receptor binds. Epitope or antigenic determinant is the smallest unit of antigenicity present on antigen, that is capable of sensitizing T cells and B cells and reacting with specific site of B cell receptor (BCR) or T cell receptor (TCR). Antigen and its several motifs are shown in Fig. 4.12.
- **Antigen epitopes:** Antigen has two types of epitopes: (a) sequential or linear epitope with sequence of few amino acids residues, and (b) conformational or nonsequential epitope with flexible region of complex antigens having tertiary structures. T cells recognize sequential epitopes, while B cells bind to the conformational epitopes.
- **Hapten-carrier molecule:** Small molecules cannot act as antigen by themselves. Binding of antigen with small carrier molecule known as **hapten** produce immune response.
  - Hapten molecule has molecular weight of <1,000 MW. Hapten size is too small to trigger an immune response alone but can be immunogenic when it attaches to a larger carrier molecule, such as host serum protein.
  - Hapten-carrier complex is capable of inducing immune response in the body. Simple hapten contains only one epitope, while complex hapten contains two or more epitopes.

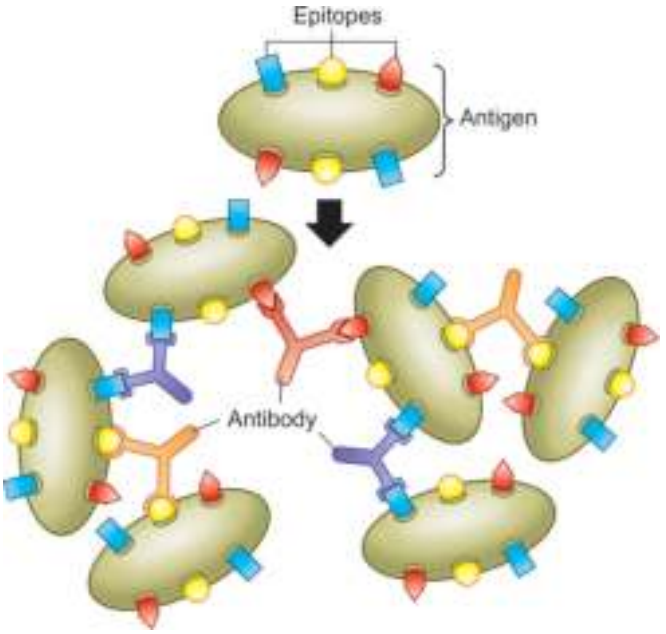


Fig. 4.12: Antigen and its several motifs. Antigen is a macromolecule that reacts with components of the immune system. Antigen may contain several motifs that are recognized by immune cells. Three different antibodies bind to different epitopes of the same antigen.

Immunogenicity

Immunogenicity is the ability of an antigen to induce humoral or cell-mediated immune responses. The substance that satisfies this property is called **immunogen**.

- **Adjuvant:** Adjuvant is a substance that enhances the immunogenicity of an antigen. Adjuvant is added to the **vaccine** to increase the immunogenicity of the vaccine antigen. Adjuvant delays release of vaccine, enhances phagocytosis, activates CD4+ helper T cells and induces granuloma formation.
- **Factors influence immunogenicity:** Factors influencing immunogenicity include: size of the antigen, chemical nature of antigen, susceptibility of antigen to tissue enzymes, structure complexity, foreignness to the host, optimal dose, route of antigen administration, repeated number of doses of antigens, multiple antigens, effect of prior administration of antibody and genetic factors.

Pathology Pearls: Properties of Antigens

Characteristics of antigens are shown in Fig. 4.13A and B. The hapten-carrier phenomenon is shown in Fig. 4.14A and B.

Chemical Nature of Antigen

Antigen and immunogen can be either proteins, polysaccharides, lipids or nucleic acids, which are cell surfaces or membrane bound antigens.

- **Proteins:** These are powerful antigens because of high-molecular-weight and structural complexity.
- **Lipopolysaccharides:** These are endotoxins, capsular polysaccharides of pneumococci.
- **Polysaccharides:** These are too small to be antigenic. But in the case of red blood cells, protein or lipid carrier gives rise to an increase in their size. Polysaccharides are present in the form of side chains and give rise to antigenicity. Red blood cell surface antigens are glycoproteins.
- **Nucleic acids:** These are nonimmunogenic except for single-stranded DNA, simpler structure, molecular flexibility, and rapid degradation.
- **Polypeptide hormones:** These hormones act as weak antigens.
- **Nucleoproteins:** These act as strong antigens.
- **Lipids:** These are nonimmunogenic except cardiolipin.

Molecular Weight of Antigen

- Higher the molecular weight, better the molecule acts as an antigen. The number of epitopes is directly proportional to the size of antigen.
- Haptens are small molecules, which need to combine with the larger carrier molecule to be immunogenic.
- Large molecules of antigens with molecular weight **>10,000** daltons act as good immunogenic because these antigens are easily phagocytosed and processed by antigen-presenting cells (APCs) resulting in increased synthesis of antibodies.

Molecular Structure

- Structural stability of the antigens is essential and always required.
- Antigens with structural stability act as strong immunogen. If the structure is unstable, it acts as poor immunogen.

Routes of Entry

- Route of entry of antigens also influences the immune response. Antigen interaction with skin results in localize reaction.
- Environmental antigens enter the body via respiratory system and gastrointestinal tract.
- Antigens in the form vaccines are administered via subcutaneous or intramuscular routes.

Dose of Immunogen

For adequate immune response, there is always a need for optimum dose of antigen, otherwise, there may be no reaction with a smaller dose.

Genetic Host Reaction

An immune response gene influences the immune response, that varies in different persons.

CATEGORIES OF ANTIGENS

Many native endogenous antigens present in the host play important role in both health and disease, which classified as autologous, heterologous, and homologous antigens (iso-antigens).

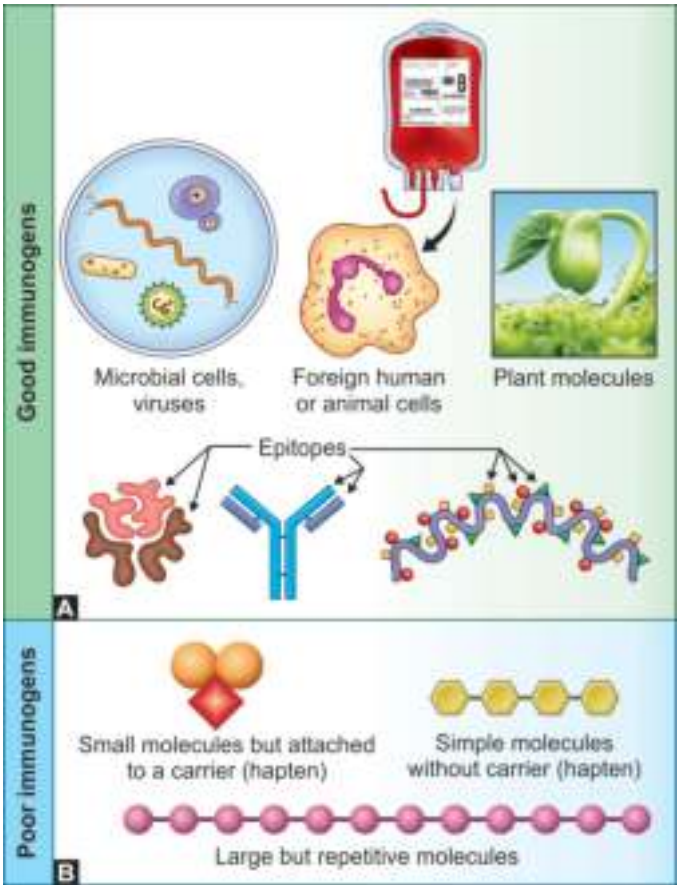


Fig. 4.13: Characteristics of immunogens. (A) Microbial cells, foreign human or animal cells and viruses make good immunogens, (B) complex molecules with several epitopes also make good immunogens. Poor immunogens include small molecules not attached to carrier molecule.

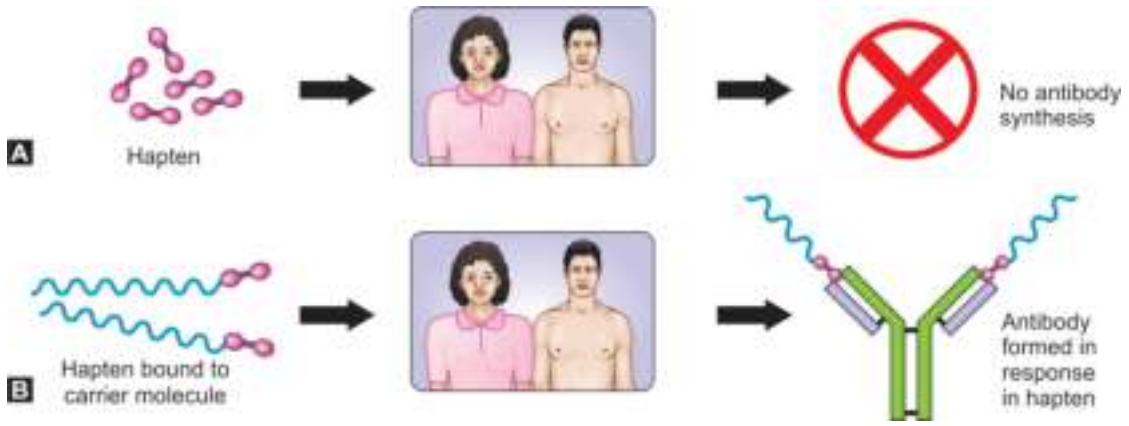


Fig. 4.14: The hapten-carrier phenomenon. (A) Hapten molecule is too small to trigger an immune response, (B) binding of hapten with carrier triggers an immune response.

Exogenous Antigens

Exogenous antigens enter the body through inhalation or ingestion, which are found on the surface of pathogens in the extracellular compartment. Molecular components of the pathogens inside cells are nonself antigens.

Endogenous Antigens

Endogenous antigens are generated inside the cell as a result of the cell metabolism, in which the epitopes are generated as a result of the regular metabolism of self-antigens. Autoantigens are already present within the host's own normal constituents. In normal person, autologous antigens are recognized as 'self-antigens', which do not elicit immune response. This state is known as tolerance. Endogenous autoantigens mistakenly are recognized as non-self-antigens, which are and not able to distinguish between self- and non-self-antigens by the immune system, that results in the destruction of self-tissues, causing autoimmune diseases.

Heterologous Antigens

Heterologous antigens enter the body from outside such as bacteria, viruses, snake venom, certain foods and cells from other persons. Allergens cause damage to host tissue as a consequence of the immune response. Heterologous antigens can induce cross-reaction resulting in genesis of certain disease. Cross reactivity occurs between M proteins present in certain strain of β-hemolytic *Streptococcus pyogenes* and determinant present in heart and joints resulting in genesis of acute rheumatic fever.

Homologous Antigens

Homologous antigens distinguish the tissue components of one person from another. Expression of homologous antigens is genetically regulated, which can be seen from the mode of inheritance of one important group of such antigens, e.g. the ABO blood group system.

Neoantigens

Neoantigens are expressed on the surface of the infected cells by oncogenic viruses. Tumor neoantigens may play an important role in helping the body to induce immune response against the cancer stem cells. Neoantigens arise via mutations that alter amino acid coding sequences. Some of these mutated peptides can be expressed, processed and presented on the cell surface and subsequently recognized by CD8+ cytotoxic T cells.

ANTIGEN AND IMMUNOGEN

The terms antigen and immunogen are most often used interchangeably. When an antigen binds to a receptor molecule of antibodies, B cells and T cells, it may or may not elicit a B cell (humoral/antibody) and/or a T cell-mediated immune response. Immunogens can be categorized into good and poor.

- Antigen that evokes a powerful immune response is called a good immunogen. Immunogenicity refers to the ability of an antigen to induce cellular and humoral immune response, whereas **antigenicity** the ability to bind with antibodies and/or T cell surface receptors (TCRs).
- Antigen–antibody interaction is optimal when the epitope, or antibody recognition/binding site on the antigen is open to the surroundings and therefore available for the antibody to bind.

Pathology Pearls: Good Immunogens Versus Poor Immunogens

Good Immunogens

- Microbial cells, foreign human or animal cells and viruses make good immunogens. Complex molecules with several epitopes also make good immunogens.

- Epitope is a small specific portion of antigen that is recognized by B cells and T cells. The epitope recognized by T cell receptors (TCRs) is often buried. The antigen must first be broken down into peptide fragments. The epitope peptide binds to a self-molecule, an MHC molecule.
- The T cell receptor (TCR) binds to a complex of MHC molecule and epitope peptide. Cells, viruses and large molecules can have numerous antigenic determinants. A given microorganism has many such epitopes, all of which stimulate individual specific immune response.

Poor Immunogens

- Poor immunogens include small molecules not attached to carrier molecule, which may be simple, complex and repetitive. Widely distributed, 'cytochrome c' is generally poor immunogen.
- Protein molecules have large number of different amino acid residues, whereas nucleic acids have a small number of different nucleotide bases. Therefore, large diversity can be obtained in epitopes of protein molecules than the nucleic acids.

Pathology Pearls: Antigens Versus Immunogens

Similarities between Antigens and Immunogens

- Antigen and immunogen are two types of molecules that bind to the components of the immune system, including antibodies, surface receptors of B cell and T cell.
- Moreover, antigen and immunogen can be either proteins, polysaccharides, lipids or nucleic acids.

Differences between Antigens and Immunogens

- **Inducing adaptive immune response:** An antigen refers to a substance specifically bind to antibodies or surface of receptors of B cells (BCRs) and T cells (TCRs), while, an immunogen is an antigen capable of inducing humoral or cell-mediated adaptive immune responses. It is the main difference between antigen and immunogen.
- **Immunogenicity:** Antigens can be either immunogenic or nonimmunogenic while immunogens are immunogenic. Not all antigens are immunogens, but all immunogens are antigens.
- **Type of molecules:** Antigens can be either proteins, polysaccharides, lipids or nucleic acids, while immunogens are normally proteins and large polysaccharides.
- **Haptens:** Haptens are low-molecular-weight molecules, which bind to antibodies, while haptens become immunogenic when binding to large carrier molecules.

SUPERANTIGENS

Conventional antigens are only recognized by specific CD4+ helper T cells having T cell receptor (TCR) with a corresponding shape that corresponds to a peptide of that antigen bound to MHC class molecules. Superantigens, on the other hand, bind directly to the outside of MHC class II molecules and TCR and activate many CD4+ helper T cells without being processed within antigen-presenting cells (APCs). Specific TCR is not required for activation of CD4+ helper T cells. Conventional antigen and superantigen presentation pathways are shown in Fig. 4.15A and B.

- Superantigens are a family of unusual antigenic proteins that interact with exceedingly large number of CD4+ helper T cells and excessive activation of immune system. Activation of very large numbers of CD4+ helper T cells results in the secretion of excessive amounts of inflammatory cytokine IL-2.
- Superantigens can span both MHC II receptors and some T cell receptors (TCRs) resulting in excessive secretion of TNF-α, platelet activating factor (PAF), IL-1, IL-2, IL-6 and IL-8. Excessive production of these cytokines leads to induce inflammatory response leading to massive influx of potent chemical mediators that cause endothelial damage, acute respiratory distress syndrome, disseminated intravascular coagulation (DIC), shock and multiple organs failure seen with pathogen-associated molecular patterns (PAMPs)-induced inflammation. It is also suggested that superantigens may be implicated in rheumatoid arthritis.
- Superantigens such as pyrogenic toxin bypass the standard antigen presentation mechanism and uncontrolled cytokine release results in toxic shock syndrome (TSS).
- Superantigens are actually a form of virulence factor present in group A *Streptococcus pyogenes*, *Escherichia coli*, Epstein-Barr virus, *Chlamydia pneumoniae*, *Campylobacter jejuni*, Coxsackie B4 nuclear antigen, hepatitis C virus and poliovirus, which overstimulate T cells leading to produce serious illness.
- Whole T cell subpopulations can be activated by superantigens independently of antigen specificity.

MOLECULAR MIMICRY AND AUTOIMMUNITY

Molecular mimicry is a mechanism by which infectious or chemical agents may induce autoimmunity, which occurs when similarities between foreign and self-peptides favor an activation of autoreactive B cells and T cells by a foreign-derived antigen in a susceptible person. Molecular mimicry is unlikely to be the only underlying mechanism for autoimmune responses; however, autoimmune responses can be induced by breach in central intolerance, nonspecific bystander activation, or persistent antigenic stimuli leading to autoimmune diseases. Host genetic constitution, exposure to pathogens and environmental chemical agents are additional links to understanding of molecular mimicry. Molecular mimicry and some examples of homologies and tissue antigens as potential cross-reacting T cell epitopes are given in Table 4.14.

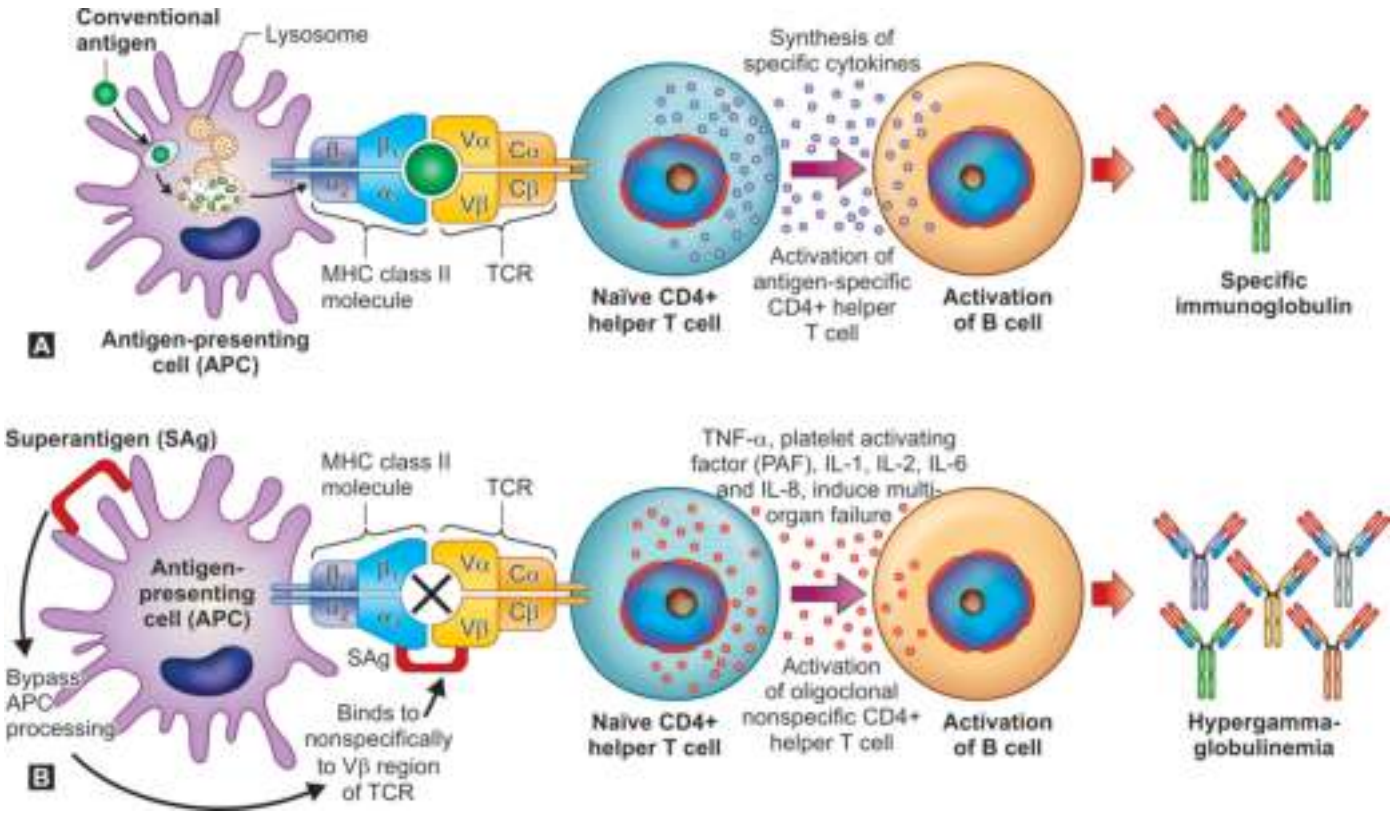


Fig. 4.15: Conventional antigen and superantigen presentation pathways. (A) Conventional antigen is processed into short peptides and presented on MHC class II molecules to one numerous naïve CD4+ helper T cells. (B) In contrast, superantigen bypasses antigen-presenting cell and directly crosslinks TCR and MHC class II molecules outside its peptide binding site leading to oligoclonal T cell activation, massive release of cytokines and multiorgan failure.

Table 4.14 Molecular mimicry and some examples of homologies and tissue antigens as potential cross-reacting T cell epitopes

Microbial Antigen	Self-tissue Antigens Cross-reactivity	Disease Produced as a Result of Molecular Mimicry
Group A *Streptococcus pyogenes*	Cardiac myosin	Rheumatic fever
Escherichia coli	HLA-DRB1* 04:01 allele	Rheumatoid arthritis
Epstein-Barr virus	Myelin basic protein	Multiple sclerosis
Chlamydia pneumoniae	Myelin basic protein	Myelin basic protein
Campylobacter jejuni	Myelin-associated gangliosides and glycolipids	Guillain-Barré syndrome
Coxsackie B4 nuclear antigen	Glutamate decarboxylase present in pancreas islets cells	Insulin-dependent diabetes mellitus
Hepatitis C virus	Glutamate decarboxylase present in pancreas islets cells	Insulin-dependent diabetes mellitus
Poliovirus	Acetylcholine receptor	Myasthenia gravis

Clinical Pearls: Molecular Mimicry and Rheumatic Fever

- Antibodies to the M protein of *Streptococcus pyogenes* cross-react with antigenic determinants of the host. By binding to the endothelium of the heart valves these antibodies initiate an inflammatory response resulting in healing with fibrosis of cardiac valves.
- Patient presents with pharyngitis due to group A Streptococcus. Antibody response occurs against specific antigenic determinant (M protein) on the bacterium.
- Antibodies cross-react with same antigenic determinant found in heart tissue, which promote complement deposition and the inflammatory response.
- Following inflammatory response, healing results in thickened and deformed cardiac valves.

ANTIGEN AND HOST RELATIONSHIP

Based on the antigen–host relationship, antigens can be classified into two groups: (a) self-antigens

(autologous antigens), and (b) nonself-antigens (foreign antigens).

- Biologic classes of antigens are categorized: (a) T cell-dependent antigens are presented by antigen-presenting cells (APCs) to CD4+ helper T cells which lead to T cell activation, and (b) T cell-independent antigens such as bacterial capsule, flagella and lipopolysaccharide do not require the help of APCs and CD4+ helper T cells. Differences between T cell-dependent and T cell-independent antigens are given in Table 4.15.
- Introduction of antigen into a host may give rise to one or more of basic reactions such as synthesis of immunoglobulins, clonal proliferation of T cells and immunologic tolerance.
- Immunity is categorized into humoral (antibody-mediated) and cell-mediated immunity. Immune responses acquired through B cells and T cells can be classified into natural immunity and artificial immunity.
- B cells and T cells are important components of the immune system, which possess cell surface receptors known as BCR and TCR respectively. Both **BCR and TCR** possess unique binding sites, but differ in the process of the recognition of antigens.
- B cell receptor (BCR) is a membrane-bound immunoglobulin molecule on the B cell surface serves as the cell's receptor for soluble antigen.
- T cell receptor (TCR) is a complex of integral membrane proteins that participate in the activation of T cells in response to an antigen when displayed on HLA class I and class II molecules. Recognition of antigens by the TCR and BCR of the immune system is given in Table 4.16.

Table 4.15 Differences between T cell-dependent and T cell-independent antigens

Characteristics	T Cell-dependent Antigens	T Cell-independent Antigens
Structure	Complex-protein in nature	Simple lipopolysaccharide, capsular polysaccharide and flagella
Immunogenicity	Immunogenic over wide range of dose of antigens	Dose-dependent immunogenicity
Immunologic memory	Memory present	No memory
Antigen processing step	Antigen processing required	Antigen processing not required
Antigen metabolism	Rapid metabolism of antigen	Slow metabolism of antigen
Activation of B cell clonality	Activation of B cell monoclonality	Activation of B cell polyclonally
Activation of B cell	Activation of cell mature B cells	Activation of cell mature and immature B cells
Affinity and switch-over	B cells stimulated T cell-dependent antigen undergo affinity and switch-over	B cells stimulated T cell-dependent antigen do not undergo affinity and switch-over
Antibody classes synthesis	All classes of immunoglobulin	IgM synthesis

Table 4.16 Recognition of antigens by the T cell receptor (TCR) and B cell receptor (BCR) of the immune system

Pattern Recognition Receptors	HLA System	T Cell Receptor (TCR)	B Cell Receptor (BCR)
Receptor location			
Cell surface, cytoplasmic secreted	Cell surface	TCR is integral membrane proteins	Transmembrane receptor protein secreted on cell surface
Receptor recognition			
PAMPs/DAMPs	Each HLA class molecule can bind many different peptides	HLA class molecule specifically recognized by TCR	Highly soluble antigen specifically recognized by BCR
Protein diversity in an individual			
10–100s	12 for classical HLA class molecule	Millions	Millions
Genes			
Each protein individually encoded, low polymorphism	Each protein individually encoded, extremely polymorphic	Genetic recombination creates diversity	Genetic recombination creates diversity

Humoral Immune Response

Humoral immune response is mediated by immunoglobulin molecules that are secreted by plasma cells. B cells that reognize antigens (e.g. proteins, lipids, polysaccharides amd small molecules) undergo proliferation and differentiation into plasma cells that synthesize different classes of antibodies (IgG, IgA, IgM, IgD and IgE) with distinct functions. Immunoglobulins circulate in tissue fluids (blood and lymph) and provide humoral immunity. Some of B cells become memory cells that recognise the same antigen in the future.

- Each clone of B cells expresses a cell surface antigen receptor, which is a membrane-bound form of antibody, with a unique antigen specificity.
- Many different types of antigens, including proteins, polysaccharides, lipids, and small molecules, are capable of eliciting humoral immune response.
- The response of B cells to protein antigens requires activating signals from CD4+ helper T cells. B cells can respond to many nonprotein antigens without the participation of CD4+ helper T cells.
- Each plasma cell secretes antibodies that have the same antigen-binding site as the cell surface antigen receptor that first recognizes the antigen.
- Polysaccharides and lipid antigens stimulate secretion of immunoglobulin M (IgM). Protein antigens induce the production of antibodies of different classes (IgG, IgA, IgE) from a single clone of B cells, which serve distinct functions.
- CD4+ helper T cells also stimulate B cell to produce antibodies with increased affinity for the antigen. This process, called 'affinity maturation', improves the quality of the humoral immune response, which combats microbes in many ways.
- Antibodies bind to microbes and prevent them from infecting cells, thus neutralizing the microbes. In fact, antibody-mediated neutralization is the only mechanism of adaptive immunity that stops an infection before it is established; that is why eliciting the production of potent antibodies is a key goal of vaccination.
- IgG antibodies coat microbes and target them for phagocytosis because phagocytes (neutrophils and macrophages) express receptors for parts of IgG molecules. IgG and IgM activate the complement system, and complement products promote phagocytosis and destruction of microbes.
- Immunoglobulin A (IgA) is secreted on mucosal epithelia and neutralizes microbes in the lumens of mucosal tissues, such as the respiratory and gastrointestinal tracts, thus preventing inhaled and ingested microbes from infecting the host.
- Maternal immunoglobulin G (IgG) is actively transported across the placenta and protects the newborn until the baby's immune system becomes mature. Most IgG antibodies have half-lives in the circulation of approximately 3 weeks, whereas other classes of antibodies have half-lives of just a few days.
- Some antibody-secreting plasma cells migrate to the bone marrow or mucosal tissues and live for years, continuing to produce low levels of antibodies, which provide immediate protection if the microbe returns.
- Antibodies have binding sites that affix tightly to an antigen and hold it in place for agglutination, opsonization, complement fixation, and neutralization.

Cell-mediated Immune Response

Cell-mediated immunity (CMI) attributable to population of specific T cells is the principal mechanism whereby intracellular bacteria are eliminated by macrophages activated by interferon-γ derived from T cells. When T cells are activated by an antigen, T cells differentiate into CD4+ helper T cells, memory T cells, CD8+ cytotoxic T cells and CD4+ regulatory T cells. CD8+ cytotoxic T cells eliminate exogenous viruses and cancer stem cells. Cell-mediated hypersensitivity is responsible for graft rejection (**heart, eye, liver, and kidney**). Because cell-mediated immune response is able to recognize self and nonself. Therefore, immunosuppression therapy is given lifelong in such cases.

Immunologic Tolerance

Immunologic tolerance is the prevention of an immune response against a particular antigen, which occurs in two forms: **central** and **peripheral**. Fetus-maternal immunologic tolerance is the prevention of a maternal immune response against a developing fetus. Introduction of antigen to the host during fetal or neonatal life can induce specific immunologic tolerance. A second exposure to such antigen is not followed by antibody synthesis or proliferation of sensitized lymphocytes.

COMPONENTS OF ADAPTIVE IMMUNE SYSTEM

The cells of the immune system originate in the bone marrow from pluripotent hematopoietic stem cells (HSCs), which give rise to two types of progenitors, which include: **common lymphoid progenitor** gives rise to major lymphoid cell types (T cells, B cells and natural killer cells) and **common myeloid progenitor** gives rise to major myeloid cell types (neutrophils, eosinophils, basophils, dendritic cells, mast cells, monocytes/macrophages) as well as erythrocytes and platelets generating megakaryocytes.

- Components of the adaptive immune system include: immune cells (T cells, B cells, macrophages, antigen-presenting cells, innate lymphoid cells), lymphoid

tissues/organs, major histocompatibility complex (MHC) and cytokines.

- Adaptive immune responses are initiated in the secondary lymphoid organs (i.e. lymph nodes, spleen and the mucosa-associated tissues in the Peyer's patches of gut) in response to interaction of recirculating T cells and B cells with cognate antigens, whether delivered by dendritic cells in the case of T cells or free antigens in the case of B cells.

IMMUNE CELLS

The adaptive immune system is highly dependent on cells of the innate immune system for the purpose of knowing when to respond, how to respond and for how long.

- Cells of adaptive immune system include monocytes-macrophages, Langerhans' cells of skin, dendritic cells of lymphoid tissue, and lymphocytes (B cells and T cells). Immunocompetent cells include T cells, B cells, natural killer cells (NK cells) and antigen-presenting cells (APCs).
- Adaptive immune response involves the interactions of these cells to foreign proteins or antigens found in pathogens. B cells and T cells develop specific protein receptors for antigen. Effector cells are T cells, granulocytes and macrophages that eliminate antigens.
- Adaptive immune system uses receptors that are generated *de novo* through random gene rearrangement and recombination in response to each infectious agent that is encountered these receptors of the immune cells. Immune cells of adaptive immune system require instruction from the cells of innate immune system for responding to particular antigen.
- Phagocytes (granulocytes and monocytes) that normally circulate in the blood are recruited at the site of tissue injury. Granulocytes phagocytose and eliminate microbes. Macrophages phagocytose and kill microbes.
- Antigen-presenting cell, naïve CD4+ helper T cell and B cell interactions are shown in Fig. 4.16. Some important cells of the immune system are given in Table 4.17. Differences between B cells and T cells are given in Table 4.18. Antigen-responsive T cells and B cells are given in Table 4.19.

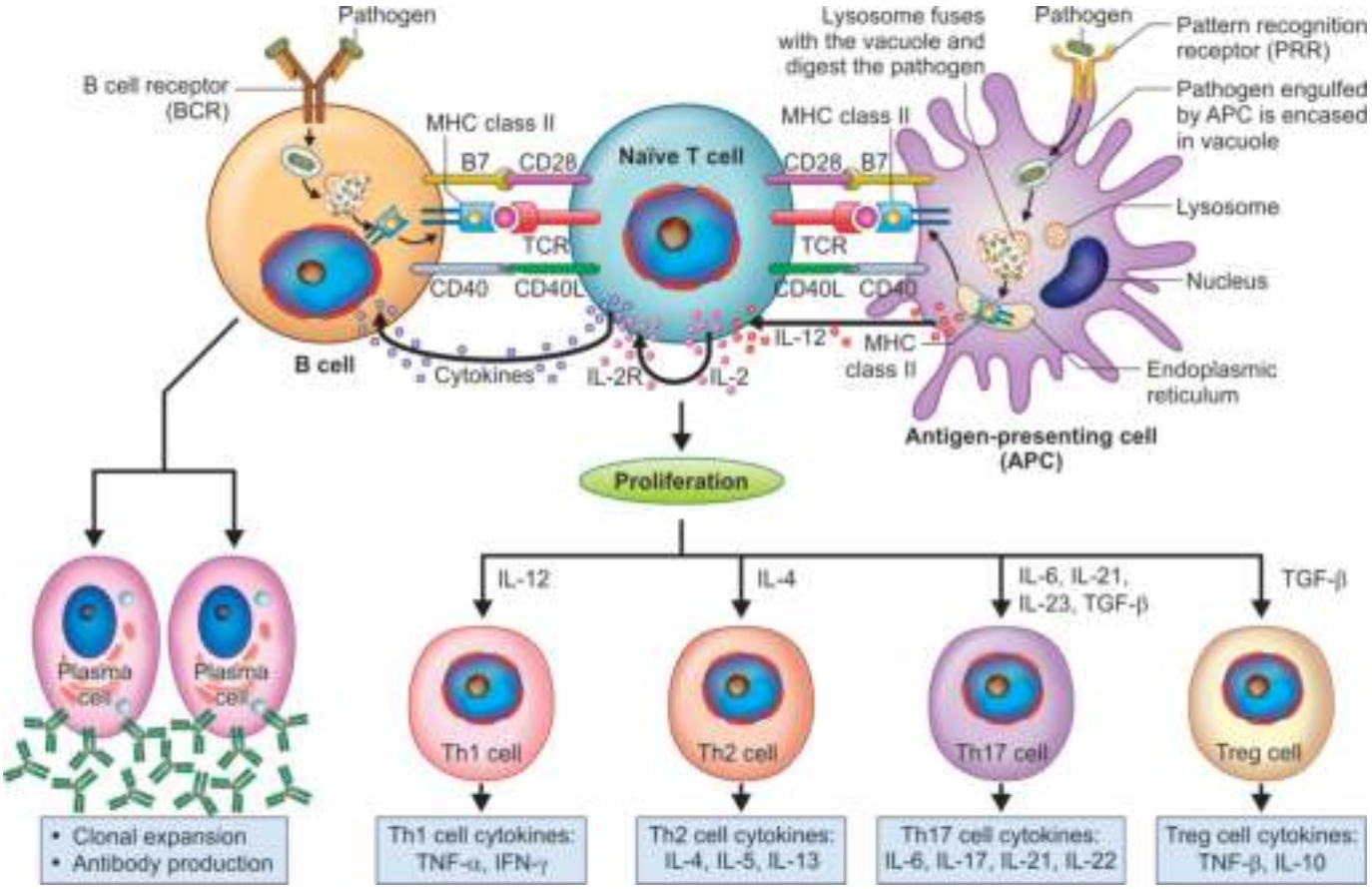


Fig. 4.16: Antigen-presenting cell, naïve CD4+ helper T cell and B cell interactions. When antigen-presenting cell activates naïve CD4+ helper T cell in the peripheral lymphoid tissue, T cell can differentiate into Th1, Th2, Th17 and CD+ regulatory T cells (Treg). Activated CD4+ helper T cell activates B cells, which differentiate to antibody secreting plasma cells. Activated CD4+ helper T cell also activates macrophages and CD8+ cytotoxic T cells.

Table 4.17 Some important cells of the immune system

Immune Cell	Mechanism	Function
Reticuloendothelial Cell		
Macrophage	Macrophage functions as antigen-presenting cell (APC) and synthesizes cytokine IL-1 that activates T cells	Phagocytosis and elimination of microorganisms
CD4+ Helper T Cells, CD8+ Cytotoxic T Cells and CD4+ Regulatory T Cells		
T helper cell (Th1)	Th1 cell synthesizes cytokines (TNF-α, IL-6 and IFN-γ), inhibits activation macrophage and cytotoxic T cells; and activate and differentiation of T cells and B cells	Phagocytosis and elimination of intracellular Mycobacterium extracellular bacteria and viruses
T helper cell (Th2)	Th2 cell synthesizes cytokines IL-4, IL-5, IL-6 and IL-13, which activate B cells	Phagocytosis and elimination of extracellular pathogens (helminths); allergic responses and bronchial asthma
T helper cell (Th9)	Th9 cell synthesizes cytokine IL-9	Allergic inflammation, autoimmunity and carcinoma
T helper cell (Th17)	Th17 cell synthesizes cytokines (IL-17, IL-21 and IL-22)	Phagocytosis and elimination of bacteria (Salmonella) and fungi; bronchial asthma
T helper cell (Th22)	Th22 cell synthesizes cytokine IL-22	Skin immunity, tissue regeneration, Crohn's disease and carcinoma
T helper cell (TFM)	T helper cell (TFM) synthesizes IL-4 and IL-21	Antibody cross-switching, affinity, maturation and auto-immunity
T regulatory cell (Treg)	Treg cell synthesizes cytokines IL-10 and TGF-β	Suppression of lymphocytes, maintenance of immune self-tolerance and tissue homeostasis; and prevention of autoimmunity
Memory T cell	Memory T cell protects against previously encountered pathogen	On re-encounter to specific invading pathogen, memory T cells are quickly converted into a large number of effector T cells to eliminate pathogen
Cytotoxic T cells	Infected cell presents microbial antigen to cytotoxic T cells	Killing of virus infected cells and cancer stem cells
γ and δ T cells	γ and δ T cells differentiate into CD8+ cytotoxic T cells	Killing of cancer stem cells
B Cell and Plasma Cell		
B cell	B cell acts as an antigen-presenting cell (APC) to T helper 2 (Th2) cell	B cell is precursor of plasma cell that synthesizes immuno-globulins, which neutralizes microbes, phagocyte microbes and activate complement
Plasma cell	B cell differentiates to form plasma cell, which synthe-sizes IgM and then IgG and IgA immunoglobulins	Plasma cell synthesizes immunoglobulins (IgG, IgA, IgM, IgD and IgE)

Table 4.18 Differences between B cells and T cells

Characteristics	B Cells	T Cells
Origin	Hematopoietic stem cells	Hematopoietic stem cells
Site of maturation	Bone marrow and lymphoid organs	Thymus gland
Distribution in lymph nodes	Cortex (germinal follicles and medullary cords) of lymph nodes	Paracortical areas of lymph nodes
Distribution in spleen	Germinal centers of follicles in spleen	Periarteriolar lymphoid sheaths in spleen
Differentiation (product of antigenic stimulation)	Plasma cells and memory cells	T-helper cells, T-suppressor cells, cytotoxic T cells and T memory cells

Contd...

Table 4.18 Differences between B cells and T cells (*Contd...*)

Characteristics	B Cells	T Cells
Circulation in blood	Low numbers (10–20%)	High numbers (60–70%)
Immune cell surface markers	Immunoglobulin receptor	T cell receptor
MHC molecules	MHC-I and MHC II	MHC-I and MHC II
Fc receptors	Present	Absent
Electron microscopy	Microvilli present	Smooth surface
Requires antigen-presenting cells with MHC	No	Yes
General functions	Production of antibodies to inactivate, neutralize target antigens	Cell function in regulating immune functions killing 'foreign cells', hypersensitivity, synthesize cytokines
Immunological markers	CD19, CD20, CD21, CD22	CD1, CD2, CD3, CD4, CD5, CD6, CD7, CD8

Table 4.19 Antigen-responsive T cells and B cells

Cells	Characteristics	Markers	Functions
Antigen-responsive T cells			
CD4+ helper T cells	CD4+ helper T cells activate APCs through peptide class II MHC complexes	CD2+, CD3+, CD4+, TCR+ (T cell receptor)	▪ **Distribution:** blood (35–60%), lymph node (50–60%) and spleen (50–60%) ▪ CD4+ helper T cells activate B cells proliferation, differentiation (class-switch antibody production), activate macrophages and stimulate inflammation
CD8+ cytotoxic T cells	Recognition of antigen presented by class I MHC antigens	CD2+, CD3+, CD8+, TCR+ (T cell receptor)	▪ **Distribution:** blood (15–40%), lymph node (15–20%) and spleen (10–15%) ▪ CD8+ cytotoxic T cell secrete lymphokines and kills viral, tumor nonself-organ transplant
CD4+ regulatory T cells	Recognition of antigen presented by class I MHC antigens	CD2+, CD3+, CD25+, FOXP3+ TCR+ (T cell receptor)	▪ **Distribution:** blood (rare), lymph node (10%) and spleen (10%) ▪ Suppression of T cell and B cell response, maintenance of immune self-tolerance and tissue homeostasis; and prevention of autoimmunity
Mucosa-associated invariant T cells	Helper and cytotoxic functions in GIT	CD3+, CD8+	**Distribution:** blood (5%), lymph node (rare) and spleen (rare)
γ and δ T cells	γ and δ T cells differentiate into CD8+ cytotoxic T cells	CD45 and CD27	Killing of cancer stem cells
Antigen-responsive B cells			
Follicular B cells	Humoral immunity (antibody production)	Fc receptors, class II MHC, CD19+, CD23+	▪ **Distribution:** blood (5–20%), lymph node (20–25%) and spleen (40–50%) ▪ Surface immunoglobulin diverse specifications for many types of molecules
Marginal zone B cells	Humoral immunity (antibody production)	IgM+, CD27+	▪ **Distribution:** blood (2–3%), lymph node (3–5%) and spleen (7–10%) ▪ Surface immunoglobulin limited specifications for a restricted set of molecules
B1 cell	Humoral immunity (antibody production)	IgM+, CD27+, CD20+, but CD70 negative	▪ **Distribution:** blood (1–3%), lymph node (rare) and spleen (rare) ▪ Surface immunoglobulin limited specifications for a restricted set of molecules

Immunological markers on T cells are CD1, CD2, CD3, CD4, CD5, CD6, CD7 and CD8.

T Cells

T cells are the major components of adaptive immune system. Once produced from pluripotent hematopoietic stem cells (HSCs) in the bone marrow, T progenitor cells migrate to the thymus gland to mature and become T cells.

- While in the thymus gland, the developing T cells start expression of T cell receptors (TCRs) and other CD4+ and CD8+ receptors. All T cells express TCRs and either CD4+ or CD8+, not both. Hence, some T cells express CD4+, and other express CD8+. Unlike antibodies, TCRs can bind to antigens directly.
- T cell receptors can recognize antigens that are bound to certain molecule, called major histocompatibility complex (MHC) class I and class II. Both MHC class I and class II molecules are membrane-bound surface receptors on antigen-presenting cells (**APCs**) such as dendritic cells (DCs) and macrophages. CD4+ and CD8+ receptors play key role in T cell recognition and activation by binding to either major histocompatibility complex class I and class II molecules.
- T cells undergo rearrangement causing limitless recombination of a gene that expresses T cell receptors. The process of rearrangement permits for binding diversity. The binding diversity of T cells could potentially cause accidental attacks against host self-cells and molecules due to some rearrangement configurations can accidently mimic a host's self cells, molecules and proteins. Mature T cells should recognize only foreign antigens combined with self-major histocompatibility complex molecules in order to mount an appropriate immune response.
- In order to ensure T cells will function properly, once they become mature and have been released from the thymus gland, T cells undergo two selection processes.
- The positive selection and negative selection processes protect host cells and tissues against own immune response. Without these selection processes, autoimmune disorders would be much more common.
  - In positive selection, T cells in the thymus gland that bind moderately to major histocompatibility complexes (MHC class I and class II molecules) receive survival signals to distinguish between self-proteins and nonself-proteins.
  - However, T cells, whose T cell receptors (TCRs) bind too strongly to histocompatibility complexes (MHC class I and class II), and will likely be self-reactive, which are killed in the process of negative selection.
- After positive and negative selection, host is left with three types of mature T cells: CD4+ helper T cells, CD8+ cytotoxic T cells, and CD4+ regulatory T (Treg) cells. T cells directly eliminate infected host cells by activating other immune cells, producing cytokines and regulating immune response.
- CD4+ helper T cells express CD4+ receptors, and help in the activation of CD8+ cytotoxic T cells, B cells and other immune cells. CD8+ cytotoxic T cells express CD8+ receptors, which remove virus infected cells and cancer stem cells. CD4+ regulatory T cells (Treg cells) express CD4+ and another receptor called CD25, which can distinguish between self- and nonself-molecules, hence reduce the risk of development of autoimmune diseases.
- Naïve CD4+ helper T cells that arise in the bone marrow and mature in thymus gland, which preferentially migrate to secondary lymphoid tissues such as lymph nodes and spleen, where naïve T cells become activated by antigens and differentiate into effector T cells. Activated CD4+ helper T cells preferentially migrate to inflamed tissue, where these cells provide defense against microbial infections. Memory CD4+ helper T cells preferentially migrate to inflamed tissue and mucosal tissue.
- When naïve T cell encounters a recognizable antigen-presenting cell (APC), the naïve cell receives extracellular stimulatory signals to undergo maturation. There are three types of signals, which include: TCR, BCR and cytokine signals. If a T cell receives all these three signals, it will undergo maturation to become an effector T cell. If a T cell only receives one of the signals (TCR or BCR), it will become useless. Effector T cells have relatively short life span and carry out functions to regulate adaptive cell-mediated immune response.

CD4+ Helper T Cells

CD4+ helper T cells possess receptors that bind to peptides displayed by the body's class II major histocompatibility complex (MHC) molecules. CD4+ helper T cells synthesize different cytokines that play diverse roles in inducing immune response.

- Activated CD4+ helper T cells secrete cytokines that recruit macrophages and CD8+ cytotoxic T cells to the injured tissue site.
- After interacting with the appropriate MHC II receptors, CD4+ helper T cells become type 1 (Th1) and type 2 (Th2) helper cells that regulate immune system. Th1 cell activates CD8+ cytotoxic T cells. Th2 cell synthesizes cytokines, which stimulate B cells to form antibody forming plasma cells.

CD8+ Cytotoxic T Cells

CD8+ cytotoxic T cells are effector cells that destroy virus-infected cells, cancer stem cells and tissue grafts through a process called **apoptosis**, while sparing

neighboring healthy cells. Apoptosis occurs when a cell's internal organelles are destroyed causing them to die from the inside out. CD8+ cytotoxic T cells express CD8+ glycoprotein on the cell surfaces that bind to protein fragments displayed by the body's major histocompatibility complex (MHC) class I molecules. CD8+ cytotoxic T cells participate in cell-mediated immunity that can reject tissue grafts. CD8+ cytotoxic T cells can induce apoptosis and cell death by process involving release of perforins forming holes in cell membrane and granzymes causing enzymatic breakdown.

CD4+, CD25+, FOXP3+ Regulatory T (Treg) Cells

CD4+, CD25+, FOXP3+ regulatory T (Treg) cells are specialized subtypes of T cells that act to suppress immune system, thereby maintaining homeostasis and self-tolerance. It has been observed that regulatory T cells are able to inhibit T cell proliferation and pro-inflammatory cytokines production and play a critical role in preventing autoimmunity.

γ and δ T Cells

γ and δ T cells are a unique T cell subpopulation that are rarely present in secondary lymphoid organs, but are present in skin, intestine and lungs. γ and δ T cells synthesize cytokines that make key contributions to antitumor immune responses in these tissues. Four subsets of human γ and δ T cells have been identified based on expression of CD45 and CD27 cell surface markers: naïve, effector, memory and terminally differentiated cells.

Memory T Cells

Memory T cells are antigen-specific T cells that persist long-term after an infection has been eliminated, which are rapidly converted into numerous effector T cells upon subsequent exposure to specific familiar invading antigen, thus providing a quick response to past infection. Memory T cells primarily reside in peripheral blood. However, vast majority of memory T cells reside in lymphoid tissues, intestine, skin and lungs.

B Cells

B cells play a central role in adaptive humoral immune response. B cell arises from common lymphoid progenitor that expresses recombination activating genes such as RAG1 and RAG2. There are four main B cell types through development stages of pro-B cell, pre-B cell: transitional/immature B cells, naïve cells, plasma cells and memory B cells.

- Early B cell development is antigen-independent phase, characterized by the ordered rearrangement of IgH and IgL chain loci, and Ig proteins themselves play critical role in regulation of B cell development.
- In the antigen-dependent phase, after formation and maturation in the bone marrow, the naïve B cells move into the lymphatic system to circulate throughout the body. Each B cell has numerous distinctive surface antigen-specific receptors that are inherent to the host's DNA. Naïve cell expresses membrane-bound antibodies on its cell surface.
- In the lymphatic system, when naïve B cell encounters an antigen that matches membrane-bound antibodies on their cell surface leading to maturation and division to become either a memory B cell or an effector B cell, which is called an antibody secreting plasma cell. Antibodies can bind to antigens directly.
- Memory B cells express the same membrane-bound antibody on their surface as the original naïve B cell, or the parent B cell. Plasma cells lack membrane-bound antibody on their surface. Plasma cells secrete antibodies, which provide protection against pathogens. Antibodies may directly neutralize pathogens.
- When the activated B cell divides and differentiates, both plasma cells and memory B cells are produced. B cells produce antigen-specific antibodies that may directly neutralize viral particles or that can act indirectly triggering other effector mechanisms such as phagocytosis, activation of complement system including antibody-dependent cellular cytotoxicity (ADCC), or blocking secreted virulence factors. Immunoglobulins can prevent tumorigenesis by attacking oncogenic viruses and cancer stem cells.
- B cells can have antitumor activities through the recognition of tumor specific antigen and antibody production, antigen-presenting cell (APC) or direct killing of cancer stem cells.
- Different subsets of memory B cells and plasma cells can be identified based on expression of immunoglobulin subtypes (IgA, IgG, IgD, IgM, IgE).
- CD40 expressed on B cells bind to CD40L (CD40 ligand) of T cells. T cells perform a number of specific cellular responses such as assisting B cells and killing foreign cells such as Mycobacterium tubercle bacilli. Interaction of B cells and T cells participates in cell-mediated immunity.
- B cell also expresses a specialized receptor, called **B cell receptor (BCR)**, which plays key role in binding, internalization and processing of antigen leading to initiate signaling pathways and synthesis of cytokines.

Immature/Transitional B Cells in Bone Marrow

Like T cells, immature/transitional B cells are formed from the multipotent hematopoietic stem cells (HSCs)

in the bone marrow and follow a pathway through lymphoid stem cell and lymphoblast. Transitional/immature B cells become mature in the bone marrow. B cell maturation occurs through positive selection for B cells with normal functional receptors.

- Negative selection is then used to eliminate self-reacting B cells that minimizes the risk of auto-immunity. Negative selection of self-reacting B cells can involve elimination by apoptosis, or modification of the antigenic receptors so B cells are no longer self-reactive (i.e. induction of anergy in the B cell).
- Immature B cells that pass the negative selection in the bone marrow travel to the spleen for the final stages of maturation. There, immature/transitional B cells become naïve mature B cells (i.e. mature B cells that have not yet been activated). B cells are activated, when their B cell receptor (BCR) binds to either soluble or membrane bound antigen and trigger downstream signaling cascade.
- One important difference between **TCRs** and **BCRs** is the way they can interact with antigen isotopes. TCRs can only react with antigenic epitopes that are presented within the antigen-binding cleft of MHC class I molecules or MHC II class II molecules. On the contrary, BCRs react with free antigenic epitopes displayed on the surface of intact pathogens without presentation with MHC class molecules.
- Another important difference is that TCRs only recognize antigenic protein epitopes, whereas BCRs can recognize antigen epitopes associated with different molecular classes (e.g. proteins, poly-saccharides and lipopolysaccharide).

Naïve B Cells

Differentiation of immature/transitional B cells to naïve mature B cells is dependent on strong BCR signaling. A naïve B cell is a mature B cell that has not been exposed to antigen and not yet been activated in the peripheral (secondary) lymphoid organs/tissues.

- Naïve B cells circulate through peripheral blood and lymphatic system and enter peripheral lymphoid organs/tissues (spleen, tonsils, lymph nodes, Peyer's patches and mucosal tissues) close to the T cell zone.
- Naïve B cells express high levels of IgD and CD23, intermediate levels of IgM and CD21. Once exposed to an antigen, the naïve B cell either becomes a memory B cell or a plasma cell that secretes antigen-specific antibodies.
- Memory B cells do not secrete antibody until activated by specific antigen. If a naïve B cells have not encountered an antigen, they again enter circulation and undergo apoptosis in the absence of BCR survival signal.

Activated B Cells

If naïve B cells encounter antigen and receive help from CD4+ helper T cells, activated B cells remain in the B cell zone of peripheral (secondary) lymphoid organs and initiate a germinal center response.

- B cells interact with other immune cells such as CD4+ helper T cells that secrete cytokines. Soluble antigens bind to the B cell receptor (BCR) complex and get internalized and degraded into peptides.
- Peptide MHC class complexes bind to T cell receptor (TCR) on CD4+ helper T cells that stimulate cytokines release essential for differentiation into antibody secreting plasma cells.

Plasma Cells

Plasma cells develop from B cells in bone marrow, which synthesize large amounts of immunoglobulins (e.g. IgG, IgA, IgM, IgD and IgE) in response to encounter with antigen. Plasma cells play a significant role in the adaptive immune response. B cells and plasma cells are the main cells responsible for humoral immunity.

- Blimp-1, IRF4, and XBP1 transcription factors are known to be essential for the differentiation of mature B cells into plasmablasts and then mature plasma cells.
- Plasmablasts are most immature cells of the plasma cell lineage that can proliferate and secrete small quantity of immunoglobulins. Mature plasma cells lack proliferative ability but secrete large quantity of immunoglobulins.
- Plasma cells have basophilic abundant cytoplasm and an eccentric nucleus with heterochromatin in a characteristic 'cartwheel appearance'. Plasma cells contain cytoplasmic inclusions called Russell bodies.

Memory B Cells

Memory B cells are able to persist in quiescent state over decades in the bloodstream and germinal centers of the secondary lymphoid organs (most abundant in spleen).

- Memory B cells maintain memory for a given antigen without the need for constant antigenic stimulation. Memory B cells are primed to react quickly and vigorously on subsequent stimulation by the cognate antigen. B cells in the marginal zone express CD27 marker.
- IgG+ and IgM+ memory B cells have a distinct function. IgG+ memory B cells predominantly differentiate into plasma cells, whereas IgM+ memory B cells predominantly enter the marginal zone of lymphoid tissue and secrete IgM and class-switched subsets.

Natural Killer Cells

Natural killer cells (NK cells) are derived from bone marrow, differentiate and mature in bone marrow, lymph nodes, spleen, tonsils and thymus gland and appear as large lymphocytes with numerous cytoplasmic granules in the peripheral blood.

- Natural killer cells do not display antigen specificity. Their cytotoxicity is neither MHC restricted nor antibody dependent. Immunophenotyping shows CD16+ (Fc receptor for IgG) and CD56+.
- Natural killer cells are 'first line of defense' against viruses, fungi and cancer stem cells. Fas ligand (FasL) expressed on the NK cells binds to fas protein expressed on the surface of carcinoma cells, fungi.
- Natural killer cells can kill target cells by two major mechanisms: death receptor and granule-dependent (perforin and granzymes) pathways. In both cases, the target cell dies as a result of the activation of a battery of cytotoxic proteases within the target cell, called caspases.
- Functional activity of natural killer cells is regulated by balance between signals from activating NKG2D family and inhibitory receptors. NKG2D receptors recognize surface molecules induced by various kinds of stress like infection and DNA damage.
- Natural killer cells inhibitory receptors recognize major compatibility complex (MHC) class I molecules expressed on all healthy cells, that prevent NK cells from killing normal cells. Virus infected cells and cancer stem cells enhance expression of ligands for activating receptors and reduces expression of class I MHC molecules which favors cytotoxicity by NK cells.

Macrophages

Monocytes constitute 2–8% of white blood cells, which are derived from a common precursor in the bone marrow. Monocytes migrate and differentiate into tissue macrophages. In the early development, as in fetal life, precursors in the yolk sac and fetal liver give rise to monocytes that seed tissues to generate specialized tissue resident macrophages.

- Tissue macrophages are distributed in the connective tissues. Half-life of blood monocytes is one day, while life span of tissue macrophage is several months or years. If the injurious agent is eliminated, macrophages eventually disappear either dying off or making their way into the lymphatic channels and lymph nodes.
- Macrophages are largest phagocytes (big eaters) that participate in specific-immune reactions. In addition, they process and present antigen (along with human leukocyte antigen (HLA) class II antigens) to CD4+ helper T cells leading to phagocytosis and degradation of injurious agent.
- Chemokines are the major chemical mediators of macrophage chemotaxis derived from mononuclear cells (macrophages, T cells), bacterial products, derived from injured tissue (e.g. break down products of collagen, fibronectin), growth factors (PDGF, TGF-α), fibrin peptides, and complement system cascade product (C5a).
- Macrophages possess receptors for IgG and C3b, which process antigen, enhance immune response, and participate in phagocytosis of injurious agent. Macrophages synthesize a variety of cytokines (IL-1 and TNF-α), acid hydrolases, neutral proteases, oxygen-derived free radicals and prostaglandins.
- TNF-α, IFN-γ and matrix proteins (fibronectin) activate macrophages, to become epithelioid cells with abundant cytoplasm resulting in formation of epithelioid cell granulomas and multinucleated giant cells in tuberculous and systemic fungal infections. TNF-α inhibitors can cause the breakdown of granulomas leading to dissemination of the disease.
- Macrophages express opsonin receptor (FcR1, CR1 and C1q), scavenger receptors, macrophage integrin receptors (MAC1 CD11 and CD18). FcR1, CR1, C1q receptors recognize microbes coated by IgG, C3 (classical and alternate pathways) and plasma proteins derived mannose-binding lectin (MBL). Scavenger receptors on macrophages bind to microbes and modified low-density lipoprotein (LDL).
- Macrophages enhance immune response by activation of endothelium (vascular response) and leukocytes (cellular response). Macrophages also participate in delayed hypersensitivity reactions, which may be capable of directly killing cancer stem cells.
- Reticuloendothelial cells distribution in various tissues are given in Table 4.20. Macrophage products and their actions are shown in Fig. 4.17. Macrophage surface structures are given in Table 4.21. Sources of chemokines and their role in chemotaxis of macrophages are given in Table 4.22. Macrophage products and their actions are given in Table 4.23. Complement receptor on macrophage is shown in Fig. 4.18.

Antigen-presenting Cells

Antigen-presenting cells (APCs) include dendritic cells in lymphoid tissue, Langerhans' cells in skin and macrophages. APCs express large quantities of cell surface HLA class II antigens. APCs capture antigens for display to B cells and T cells. Dendritic cells initiate T cell response. Follicular dendritic cells display antigens to B cells and initiate humoral immune

response. Macrophages perform in the effector phase of cell-mediated immunity. APCs have poor phagocytic function. APCs may be either professional or non-professional types.

Table 4.20 Reticuloendothelial cells distribution in various tissues

Tissue	Reticuloendothelial cells
Blood	Monocytes
Connective tissue	Macrophages
Lung	Alveolar macrophages
Peritoneum	Peritoneal macrophages
Kidney	Mesangial cells
Liver	Kupffer's cells
Lymph nodes	Sinus histiocytes
Spleen	Littoral cells
Placenta	Hofbauer cells
Skin	Melanophages
Brain	Microglial cells
Synovium	Synovial macrophages (type A synoviocytes)
Bone	Osteoclasts
Adipose tissue	Lipophage
Specialized histiocytes	▪ Epithelioid cells ▪ Histiocytic giant cells ▪ Langhan's giant cells ▪ Foreign body giant cells ▪ Touton giant cells

- Professional APCs express MHC II molecules and interact with native T cells. Examples of professional APCs are macrophages, dendritic cells, Langerhans' cells of skin certain activated epithelial cells.
- Non-professional APCs do not express MHC II molecules; hence APCs do not interact with native T cells, and are stimulated by interferon. Examples of nonprofessional APCs are fibroblasts, endothelial cells, thyroid follicular cells, glial cells and β cells of pancreas.

Table 4.21 Macrophage surface structures

Characteristics	Macrophage Surface Molecules
Antigenic presentation	CD40
Facilitated uptake	▪ Fc receptor for IgG ▪ Complement receptor for C3b (CR1)
Adhesion molecules	▪ LFA3 ▪ ICAM 1
Bacterial adhesion	▪ Lipopolysaccharide (LPS) receptor ▪ Toll-like receptor (CD14) ▪ Mannose receptor ▪ Scavenger receptor ▪ Glycan receptor
Cell activation	▪ IFN-γ receptor ▪ TNF-α receptor
Co-stimulators	▪ B7 family of molecules ▪ MHC I molecule ▪ MHC II molecule

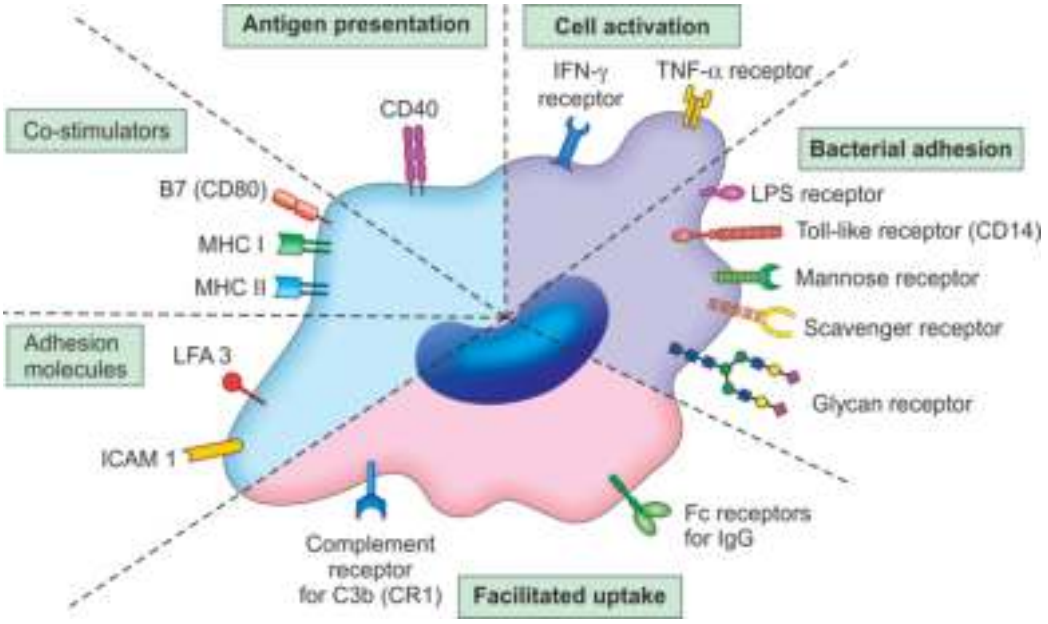


Fig. 4.17: Macrophage surface structures mediate cell function. Bacteria and antigens either bind directly to receptors or bind through antibody or complement receptors (opsonization) and can be phagocytosed; the cell is activated and presents antigen to T cells. The dendritic cell shares many of these characteristics.

Table 4.22 Sources of chemokines and their role in chemotaxis of macrophages

Source of Chemokines	Examples of Chemokines
Blood cells	Macrophage chemotactic protein 1 (MCP-1)
Bacteria	Bacterial products
Injured tissue	Breakdown products of collagen fibers and fibronectin
Growth factors	PDGF, TGF-α
Fibrinopeptides	Fibrin degradation products (FDPs)
Complement	C5a

- Professional and nonprofessional APCs are given in Table 4.24. APCs expressing class II MHC molecules and presenting antigens to CD4+ helper T cells are given in Table 4.25.

Dendritic Cells

Dendritic cells have dendritic cytoplasmic processes and distributed throughout the tissues with reticulo-endothelial system, which cells express large quantities of cell surface MHC class II antigens.

- The most efficient 'antigen-presenting cells' (APCs) are dendritic cells in lymph nodes, which process and present the antigen to lymphocytes resulting in clonal expansion of lymphocytes. Dendritic cells initiate adaptive immune system. In contrast to macrophages, dendritic cells are poorly phagocytic; however, like macrophages, dendritic cells are antigen-presenting cells (APCs) to lymphocytes.
- Dendritic cells originate from common precursor cell of the myeloid lineage in the bone marrow and further differentiates into subsets: classical dendritic cells and plasmacytoid dendritic cells. Inflammatory

Table 4.23 Macrophage products and their actions

Inflammatory Mediators	Actions	
Cytokines		
IL-1, TNF-α	Chemotaxis of inflammatory cells	
IL-6	Synthesis of acute phase reactant by liver	
IL-2	Autocrine action	
Chemokines		
IL-8, MCP-1	Chemotaxis of inflammatory cells and paracrine action	
Lysosomal enzymes		
Neutral hydrolases	Toxic to extracellular matrix	
Acid hydrolase, serine protease	Hydrolyzing the muramic acid-N-acetylglucosamine bond of bacterial glycopeptides leading to killing of microbes	
Metalloproteinases (MMPs)*	▪ Degradation of type 3 collagen fibers	▪ Deposition of type 1 collagen fibers leading to increased tensile strength
Cell-derived arachidonic metabolites		
Prostaglandins	▪ Platelet aggregation ▪ Vasodilatation ▪ Increasing vascular permeability with formation of inflammatory exudate	▪ Modulation of phagocytic activity of leukocytes ▪ Pain ▪ Fever
Leukotrienes	▪ Vasoconstriction ▪ Increased vascular permeability	▪ Bronchospasm
Plasminogen activator		
Plasminogen activator	▪ Cleaves plasminogen to form plasmin, that degrades fibrin strands resulting in dissolution of blood clot ▪ Activation of coagulation system	
Reactive oxygen and nitrogen species		
Superoxide, highly reactive hydroxyl molecule and hypochlorous acid (bleach)	Toxic to microbes and host cells	
Growth factors, fibrogenic and angiogenic factors		
PDGF, FGF, and TGF-β	▪ Fibroblast proliferation ▪ Collagen deposition	▪ Angiogenesis

**Metalloproteinases (MMPs) also known as collagenases synthesized by macrophages, neutrophils, fibroblasts, synovial cells and some epithelial cells.*

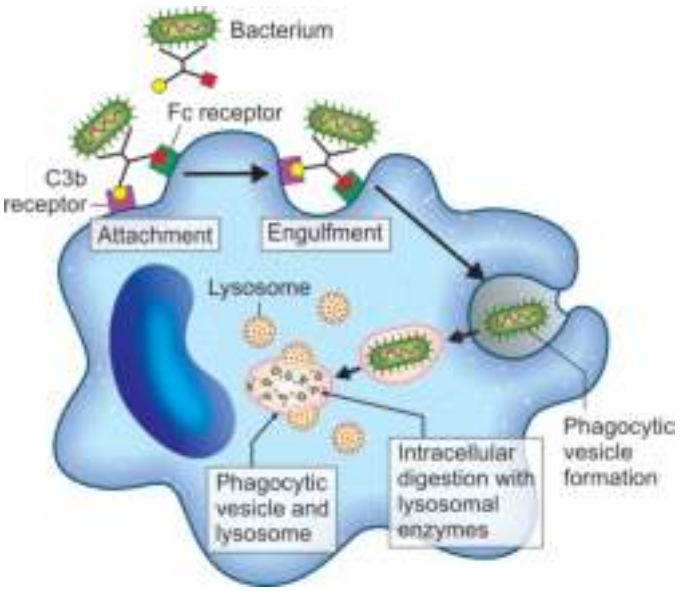


Fig. 4.18: Complement receptor on macrophage. One function of the complement is to aid phagocytosis. Complement proteins attract phagocytes to engulf a foreign organism. The organism is enclosed in a phagocytic vesicle, which then merges with a lysosome. Lysosomal enzymes digest the organism.

dendritic cells may arise from monocytes in inflamed tissues.

- Classical dendritic cells are capturing protein antigens of microbes that enter through epithelia and presenting the antigens to CD4+ helper T cells that induce adaptive immunity. Classical dendritic cells synthesize inflammatory cytokine IL-12.
- Plasmacytoid dendritic cells produce the antiviral interferon (IFN) cytokine in response to viruses and may capture blood-borne microbes and transmit these antigens to the spleen for predation to T cells.

Langerhans' Cells of Skin

Like dendritic cells, Langerhans' cells of the skin express MHC class II antigens. Electron microscopic examination, Langerhans' cells contain 'Birbeck granules' and 'tennis racket-shaped cytoplasmic structures.

Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are innate counterparts of T cells derived from common lymphoid progenitor cells. ILCs do not express the diversified antigen

Table 4.24 Professional and nonprofessional antigen-presenting cells (APCs)

Characteristics	Professional Antigen-presenting Cells	Nonprofessional Antigen-presenting Cells
Examples of antigen-presenting cells	▪ Dendritic cells ▪ Langerhans' cells of skin ▪ Macrophages ▪ Certain activated epithelial cells	▪ Fibroblasts and endothelial cells ▪ Thyroid follicular cells ▪ Glial cells ▪ β Cells of pancreas
MHC II expression	Present	Absent
Interaction with native T cells	Yes	No
Cytokines required or not for stimulation	Cytokines not required	Cytokines (interferon) required

Table 4.25 Antigen-presenting cells (APCs) expressing class II MHC molecules and presenting antigens to CD4+ helper T cells

Antigen-presenting Cells	Functions
Monocytes	▪ Derived from bone marrow and present in blood ▪ Precursors to macrophage-lineage and cytokines release
Tissue macrophages	▪ Possess Fc and C3 receptors ▪ Initiate inflammatory response ▪ Possess antibacterial, antiviral and antitumor properties
Langerhans' cells	▪ Reside in spleen, lymph nodes and other organs. These are activated by interferon-γ and tumor necrosis factor ▪ Transport antigen to lymph nodes
Dendritic cells	▪ Present in lymph nodes and tissues ▪ Efficient antigen-presenting cells
Microglial cells	▪ Present in central nervous system ▪ Cytokines synthesis
Kupffer's cells	▪ Present in liver ▪ Filter particles (viruses) from blood

Monocytes, tissue macrophages, Langerhans' cells, dendritic cells, microglial cells, Kupffer's cells express class II MHC molecules and present antigens to CD4+ T cells.

receptors expressed on T cells and B cells. ILCs are present in gastrointestinal tract, respiratory system as well as oral cavity.

- Three groups of innate lymphoid cells include cytotoxic ILC, innate lymphoid tissue inducer cells and noncytolytic ILC, which have been defined based on shared expression of surface markers, transcription factors and effector cytokine production.
- In response to pathogenic tissue damage, innate lymphoid cells (ILCs) contribute to tissue immune response (i.e. homeostasis, pathogen clearance) via the secretion of effector signaling cytokines and the regulation of both innate and adaptive immune cells. ILCs are especially present in mucosal barriers, where they are exposed to allergens, pathogens and commensal microbes.

LYMPHOID TISSUES/ORGANS

The lymphoid organs of immune system are divided into primary (central) and secondary (peripheral) lymphoid systems. The primary lymphoid organs are the thymus and bone marrow, where immune T and B cells differentiate and mature respectively, and then migrate to secondary (peripheral) lymphoid organs, lymph nodes, spleen, mucosa-associated lymphoid tissue (MALT), skin-associated lymphoid tissue (SALT) and gut-associated lymphoid tissue (GALT) in Peyer's patches in which immature cells interact with each other and process antigens. Primary and secondary lymphoid organs are given in Table 4.26.

Pathology Pearls: Development of Lymphoid Organs

- Thymus gland is formed due to migration of cells of pharyngeal pouches into the chest.
- B cell and T cell precursors originate early in the embryonic life in the yolk sac and eventually migrate to the bone marrow and thymus gland.
- Precursor B cells and T cells arise from pluripotent hematopoietic stem cells (HSCs). B cells start maturation in bone marrow and complete maturation in spleen.
- Precursor T cell migrates to thymus gland for maturation.
- T cells and B cells interact with each other and with accessory cells and antigens in secondary lymphoid organs.
- Bone marrow contains B cells (90%) and T cells (10%). Lymph nodes contain B cells (40%) and T cells (60%). Spleen contains B cells (45%) and T cells (55%).

Primary Lymphoid Organs

Bone marrow and thymus gland are primary (central) lymphoid organs. Thymus gland nearly fills the region over the midline of the upper thoracic region in newborn babies. It progressively becomes small in size in adults.

Table 4.26 Primary and secondary lymphoid organs

Primary (Central) Lymphoid Organs
▪ Bone marrow (site for origin of B cells and T cells from pluripotent hematopoietic stem cell and maturation and migration of B cells to secondary lymphoid organs)
▪ Thymus gland (site of maturation of T cells)
Secondary (Peripheral) Lymphoid Organs
▪ Lymph nodes
▪ Spleen (site for complete maturation of B cells)
▪ Mucosa-associated lymphoid tissue (MALT)
▪ Skin-associated lymphoid tissue (SALT)
▪ Gut-associated lymphoid tissue (GALT) in Peyer's patches

T cells and B cells interact with each other and with accessory cells and antigens in the secondary lymphoid organs.

- Children are born without a thymus gland suffer from DiGeorge syndrome characterized by severe immunodeficiency disorder. During embryonic and fetal development, bone marrow pluripotent hematopoietic stem cells (HSCs) produce B cells and T cells. Precursor T cells and B cells undergo further development or maturation at different sites.
- B cells start maturation in the stromal cells of bone marrow, and then migrate for complete maturation in the spleen. Mature B cells produce antibodies and T cells synthesize cytokines that mediate and coordinate both cellular and humoral immune responses.
- Immature T cells migrate in the thymus gland (cortex and medulla) for differentiation and maturation.

Secondary Lymphoid Organs

After maturation of B cells in bone marrow and T cells in thymus gland, mature lymphocytes reach the secondary (peripheral) lymphoid organs via circulation. Secondary lymphoid organs comprise lymph node, spleen, tonsils, mucosa of appendix and 'Peyer's patches' of small intestine. Cellular and humoral immune responses occur in the secondary (peripheral) lymphoid organs and tissues; effector and memory cells are generated in peripheral lymphoid organs.

Lymph Nodes

Lymph nodes are peripheral lymphoid organs, which mount immune responses to antigens in intercellular fluid and lymph, absorbed either through the skin (superficial lymph nodes) or from internal viscera (deep lymph nodes). Each lymph node has its own arterial supply and venous drainage.

- Beneath the collagenous capsule is the subareolar sinus, which is lined by with phagocytic cells. Naïve

lymphocytes migrate from the blood circulation through vascular endothelial venules into lymph nodes, where these cells are activated by distinct antigens. Activated lymphocytes exit the lymph nodes via the afferent lymphatic system.

- The cortex of lymph node contains aggregates of B cells (primary follicles), most of which are stimulated to form secondary follicles.
- The paracortical region of lymph node contains mainly T cells, many of which are associated with the interdigitating cells (antigen-presenting cells).
- The medulla of lymph node contains both T cells and B cells as well as most of the plasma cells. These lymphoid cells are organized into cords of lymphoid tissue. Lymphocytes and antigens from surrounding tissue spaces or adjacent lymph nodes pass into the sinus via the afferent lymphatic system. Lymphocytes can leave the lymph node through the efferent lymphatic vessel.

Spleen

Spleen is a secondary lymphoid organ which also regulates the destruction of red blood cells, which consists of white pulp and red pulp. Spleen contributes to the maturation of red blood cells by pitting function, which has ability to remove solid particles from the cytoplasm of red blood cells without causing injury to the cell membrane. The spleen responds predominantly to blood-borne antigens. Blood enters the spleen via the trabecular arteries, which give rise to the many-branched central arteries. Some of trabecular arteries end in the white pulp, supplying the germinal centers and mantle zones, but most empty into or near the marginal zones.

- **White pulp:** The white pulp contains germinal centers and is surrounded by the marginal zone, which contains numerous macrophages, antigen-presenting cells (APCs), slowly recirculating B cells, T cells and natural killer cells around splenic arteriole. Dendritic cells present antigens to T cells, where T and B cells interact at the edges of white pulp lymphoid follicles, generating antibody-secreting plasma cells found mainly within the sinuses of red pulp.
- **Red pulp:** The red pulp contains capillaries venous sinuses separated by splenic cords. Blood enters the tissue via the trabecular arteries, which give rise to the many-branched central arteries. T cells are distributed in the periarteriolar sheaths of the spleen. Red pulp stores red blood cells.

Tonsils, MALT, GALT and SALT

Tonsils, appendix, mucosa-associated lymphoid tissue (**MALT**) and gut-associated lymphoid tissue (**GALT**) in Peyer's patches of small intestine are secondary lymphoid organs, which respond to antigens that have penetrated the surface mucosal barriers.

- **Tonsils:** The tonsils are small masses of secondary lymphoid organs located in the pharynx, which function similarly to other types of secondary lymphoid organs and also capture antigens from respiratory tract pathogens.
- **Mucosa-associated lymphoid tissue (MALT):** Lymphoid cells stimulated by antigen in Peyer's patches migrate via the regional lymph nodes and thoracic duct into the blood stream and then to lamina propria of the gut and probably other mucosal surfaces (lungs). Thus, lymphocytes stimulated on the mucosal surface may become distributed throughout the MALT.
  - Many commensal bacteria reside in the small intestine. The mucus-secreting epithelium provides an innate barrier to microbial invasion.
  - Bowel specialized epithelial cells promote transport of antigens from the lumen into the underlying tissues.
  - Dendritic cells, T cells and macrophages in the lamina propria provide innate and specific (adaptive) immune defense against invading microbes. Immunoglobulin A (IgA) is produced in large quantity in the mucosal tissues that is transported into the lumen, where it binds and neutralizes microbes.
- **Gut-associated lymphoid tissue (GALT):** GALT is a component of the mucosa-associated lymphoid tissue (MALT) which works in the immune system to protect the body from pathogens invasion in the gut.
- **Skin-associated lymphoid tissue (SALT):** SALT responds to antigens that have penetrated the skin barrier.

MAJOR HISTOCOMPATIBILITY COMPLEX

Human leukocyte antigen (HLA) system is a cluster of genes located on the short arm of chromosome 6, known as the 'major histocompatibility complex' (MHC) in human.

- MHC (HLA) system codes for proteins that differentiate between self and nonself cells. The biologic role of HLA system is clinically important in immune response and solid organ transplantation, where HLA typing and lymphocyte cross-matching of donor and recipient are now widely used to predict tissue compatibility and to prevent allograft rejection.
- Molecular HLA allele typing is routinely performed to provide MHC (HLA) class I and MHC (HLA) class II allele matching in unrelated donor hematopoietic stem cell (HSC).

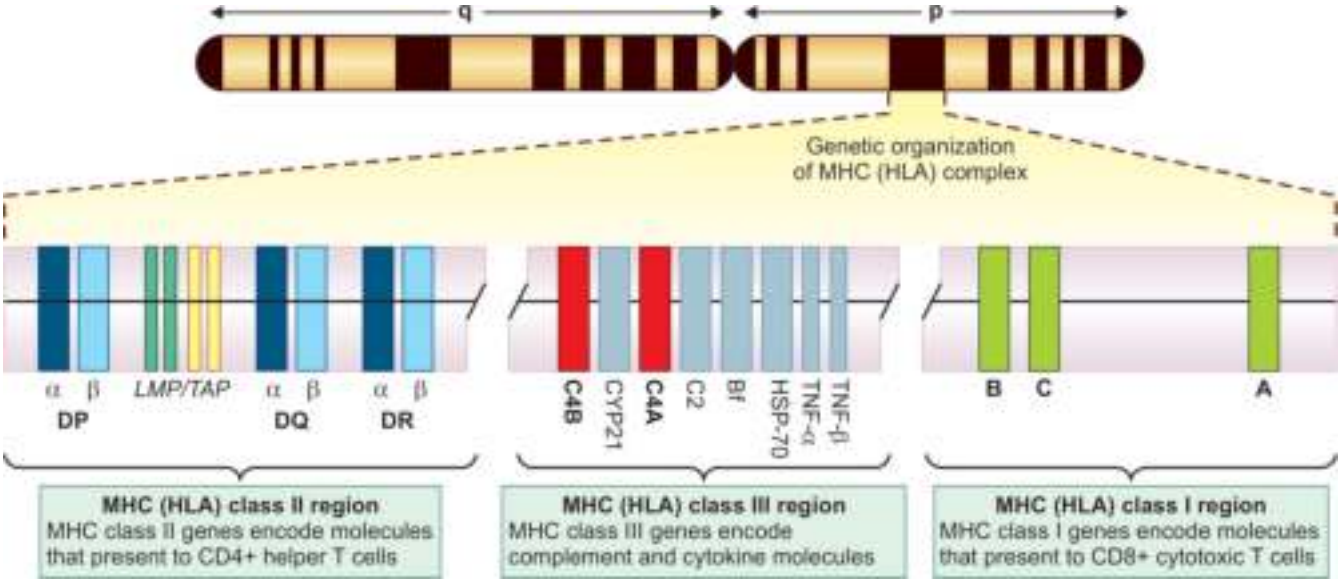


Fig. 4.19: Genetic organization of major histocompatibility complex (human-leukocyte antigen). Loci HLA-A, HLA-B and HLA-C are class I MHC genes encode molecules that present antigen to CD8+ cytotoxic T cells. Loci HLA-DP, HLA-DQ and HLA-DR are class II MHC genes encode molecules that present to CD+ helper T cells. LMP (large multifunctional protease) and TAP (transport associated with antigen presentation) genes encode LMP and TAP molecules that are involved in antigen processing.

- All nucleated human cells express MHC (HLA) class I molecules, whereas MHC (HLA) class II molecules are displayed primarily on macrophages, dendritic cells, Langerhans' cells, B cells, and some T cells. Two major classes of MHC (HLA) antigens are separated on the basis of structure and tissue distribution. MHC class I molecules present antigen to CD8+ cytotoxic T cells. MHC class II molecules that present antigen to CD4+ helper T cells.
- Genetic organization of major histocompatibility complex (human-leukocyte antigen) is shown in Fig. 4.19. Comparison of major histocompatibility complex, i.e. HLA class I and HLA class II molecules is given in Table 4.27.

Major Classes of HLA Genes

The class I region on chromosome 6 contains the classical HLA-A, HLA-B and HLA-C genes that encode the heavy chains of class I molecules. The class II region on chromosome 6 consists a series of subregions, each containing A and B genes encoding α- and β-chains, respectively. HLA-DP, HLA-DQ and HLA-DR genes

Table 4.27 Comparison of major histocompatibility complex, i.e. HLA class I and HLA class II molecules

Characteristics	HLA Class I Molecule	HLA Class II Molecule
HLA molecules expression on various cells	Most nucleated human cells	▪ Macrophages ▪ Dendritic cells ▪ Langerhans' cells ▪ B cells ▪ CD4+ helper T cells
Loci	▪ HLA-A ▪ HLA-B ▪ HLA-C	▪ HLA-DP ▪ HLA-DQ ▪ HLA-DR ▪ HLA-DN ▪ HLA-DO
Location of peptide binding cleft	Present between α_1 and α_2	Present between α_1 and β_1
Functions	Present endogenous antigen to cytotoxic T cells involved in tissue/organ graft rejection and killing of virus-infected cells	Present endogenous antigen to CD4+ helper T cells, which synthesize cytokines
Diagnostic technique	Standard serologic techniques used for identification of HLA-A and HLA-B antigens with the aim to predict the likelihood of long-term graft survival	Standard serologic techniques or mixed lymphocyte reactions are used for identification of HLA-DP, HLA-DQ, and HLA-DR antigens

1. *GlyCAM 1 facilitates lymphocyte recirculation by providing a receptor for leukocyte attachment to high endothelial venules.*
2. *Both class I and class II MHC molecules contain peptide-binding clefts and invariant portions that bind CD8 (α_3 domain of class I) or CD4 (the β-domain class II).*

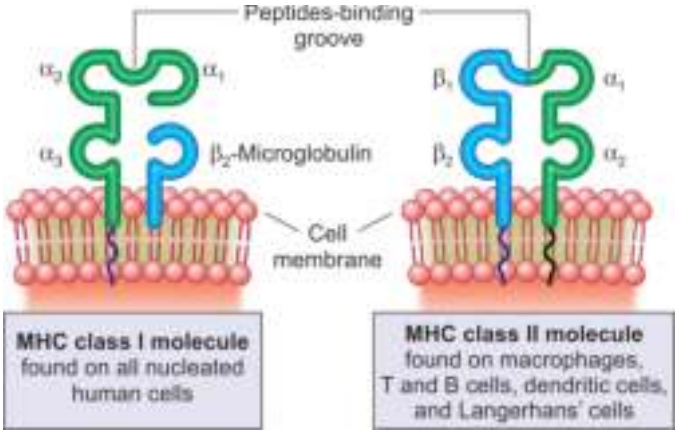


Fig. 4.20: Structure of MHC (HLA) class I and class II molecules. MHC class I and class II molecules play a pivotal role in the adaptive immune response. Both classes of MHC molecules share the task of presenting peptides on the cell surface for recognition by naïve CD4+ helper T cells and CD8+ cytotoxic T cells.

encode HLA class II molecules found on the surface of antigen-presenting cells (APCs) that present peptides to CD4+ helper T cells, class III region on chromosome 6 does not encode HLA molecules, but contains genes for complement system components (C2, C4, factor 4B), 21-hydroxylase, tumor necrosis factors (TNFs) and some others. Structure of MHC (HLA) class I and class II proteins is shown in Fig. 4.20.

MHC (HLA) Class I Molecules

The heavy chain of MHC class I molecules is encoded by genes at HLA-A, HLA-B, and HLA-C loci. HLA class I molecules are found on almost all nucleated human cells that present antigens from inside.

- MHC (HLA) class I molecules are heterodimers consisting of a 44-kilodalton polymorphic transmembrane glycoprotein-α and a 12-kilodalton nonpolymorphic molecule β_2-microglobulin. The later β_2-microglobulin molecule lacks a membrane component and is noncovalently associated with the larger heavy chain. Structural polymorphism occurs primarily in the extracellular domains of the α-chain.
- MHC (HLA) class I molecule is recognized by CD8+ cytotoxic T cells, which are involved in organ graft rejection and cell-mediated killing of virus-infected cells. Standard serologic techniques are used for identification of HLA-A and HLA-B antigens with the aim to predict the likelihood of long-term organ graft survival.

MHC (HLA) Class II Molecules

In humans, MHC (HLA) class II molecules are encoded by multiple different loci HLA-DP, HLA-DQ, HLA-DR, HLA-DN, and HLA-DM, identified by standard serologic techniques or by mixed lymphocyte reactions.

- MHC (HLA) class II molecules play an important role in immune system, which are essential in the defense against infection and important in organ transplantation medicine.
- MHC (HLA) class II molecules are chiefly found on immunocompetent professional antigen-presenting cells such as macrophages, dendritic cells, Langerhans' cells, B cells, and some T cells (CD4+ helper T cells). MHC (HLA) class II molecules consist of α- and β-chains, and are recognized by CD4+ helper T cells.
- Antigen-presenting cells (APCs) present antigen to CD4+ helper T cells, because of their ability to process endocytose antigens and partly possessing cell surface proteins that bind to T cell surfaces. CD4+ helper T cells become activated to synthesize an array of cytokines.
- Despite the essential function of HLA class II molecules in immune defense against pathogens, some alleles are frequently linked to autoimmune diseases.

Clinical Aspect of MHC (HLA) System

The array of HLA alleles on a homologue of our chromosome 6 is known as a haplotype. An individual inherits one HLA haplotype from each parent. Identical twins have the identical haplotype. Therefore, the preference of organ transplantation is performed as follows: identical twins > sibling > unrelated donor. The procedure carried out to match HLA proteins of donor and recipient is called tissue typing. When HLA types are matched properly, the survival of transplanted organs increases dramatically.

Organ Transplants

Autograft refers to grafting of own tissue from one region to another region (skin graft). Organ graft performed between identical twins is known as isograft. Organ graft performed between individuals of same species, but with different MHC (HLA) alleles, is known as allograft (allogenic graft). Success of the allograft depends upon matching and immunosuppressive drugs. Xenograft is referred to graft between animals of different species.

MHC (HLA) Class I and II Molecules Associated Diseases

MHC (HLA) class II molecules play an important role in immune defense against pathogens; however, some alleles are frequently linked to immune-mediated disorders.

- HLA-DR1 and HLA-DR4 predispose to rheumatoid arthritis, systemic lupus erythematosus and type 1 diabetes mellitus, whereas HLA-DR2 confers susceptibility to multiple sclerosis.
- Similarly, HLA-DQ2 and DQ8 are linked to celiac disease.

Table 4.28 MHC (HLA) class I and II molecules associated disorders

HLA Class	Diseases
MHC (HLA) class I molecules linked disorders	
B27 is now called B*2701–2725 according to revision by WHO in 2010	▪ Ankylosing spondylitis ▪ Psoriatic arthritis ▪ Reiter's syndrome
B8	▪ Graves' disease (hyperthyroidism) ▪ Myasthenia gravis
CW6	Psoriasis
B21	Behçet's disease
MHC (HLA) class II molecules linked disorders	
DR2	▪ Multiple sclerosis ▪ Narcolepsy ▪ Goodpasture's syndrome
DR3	▪ Type 1 diabetes mellitus ▪ Dermatitis herpetiformis ▪ Chronic active hepatitis ▪ Sjögren's syndrome ▪ Graves' disease
DR4	▪ Pemphigus vulgaris ▪ Rheumatoid arthritis ▪ Type 1 diabetes mellitus
DR8	Type 1 diabetes mellitus
DQ1	Pemphigus vulgaris
DQ2 and DQ8	▪ Celiac disease ▪ Type 1 diabetes mellitus

- HLA-B27 antigen is associated with almost 90% of cases of ankylosing spondylitis. HLA-B8 is linked to Graves' disease and myasthenia gravis. HLA-CW6 is associated with psoriasis.
- Specific HLA antigens are also associated with uveitis, and Reiter syndrome (urethritis, conjunctivitis, and arthritis), as well as with many other entities. MHC (HLA) class I and II molecules associated disorders are given in Table 4.28.

CYTOKINES

Cytokines are soluble proteins synthesized by lymphocytes (lymphokines), monocytes-macrophages (monokines), and NK cells, as well as other cell types. They act as effector molecules influencing the behavior of B cells, T cells, natural killer cells, monocytes, macrophages, hematopoietic stem cells, and many other cell types synthesize interleukins, growth factors, interferons and tumor necrosis factors involved in immune response. Role of cytokines in specific immune response is given in Table 4.29.

Table 4.29 Role of cytokines in specific immune response

Cytokine	Actions
Interleukins	
IL-6	Maturation of B and T cells
IL-15	Proliferation of T cells and B cells
IL-9	Proliferation of T cells
IL-12	Activates T cells and natural killer cells
IL-5	Maturation of B cells to plasma cells
IL-3	Acts as a growth factor for hematopoietic stem cells
IL-4	Promotes growth of B and T cells; enhances expression of HLA class II antigens
IL-7	Acts as a growth factor for bone marrow stem cells
Interferon	
IFN-γ	▪ Activates macrophages and T cells during chronic inflammation ▪ Enhances expression of HLA class II antigens ▪ Differentiation of T cells and B cells ▪ Increases the cytotoxicity of natural killer cells ▪ Activates neutrophils and stimulates diapedesis
Tumor necrosis factors	
TNF-α and TNF-β	▪ Stimulate T cell proliferation ▪ IL-2 synthesis ▪ Cytotoxic to some tumor cells

- **Interleukins' specific immune response:** IL-6 promotes maturation of B and T cells. IL-15 causes proliferation of T cells and B cells. IL-9 causes proliferation of T cells. IL-12 activates T cells and natural killer cells. IL-5 promotes maturation of B cells to plasma cells.
- **Interleukins as growth factors:** IL-3 acts as a growth factor for hematopoietic stem cells. IL-4 promotes growth of B and T cells; enhances expression of HLA class II antigens. IL-7 acts as a growth factor for bone marrow stem cells.
- **Interferon-γ (IFN-γ):** IFN-γ also activates macrophages and T cells during chronic inflammation, which enhances expression of HLA class II antigens. IFN-γ participates in differentiation of T cells and B cells, and increases the cytotoxicity of natural killer cells. IFN-γ activates neutrophils and stimulates diapedesis.
- **Tumor necrosis factors α and β (TNF-α and TNF-β):** TNF-α and TNF-β stimulate T cell proliferation and IL-2 production and destroy cancer stem cells.

T CELL- AND B CELL-MEDIATED IMMUNE RESPONSES

Immune cells play an important role in adaptive immune response, which includes T cells, B cells, monocytes-macrophages, Langerhans' cells of the skin, and dendritic cells of lymphoid tissue. Both B and T cells may respond to the same antigen, the way the signal is presented as fundamental.

DEVELOPMENT OF B CELLS AND T CELLS

Both B cells and T cells are derived from pluripotent hematopoietic stem cells (HSCs) in the bone marrow, which differentiate into two distinct lymphoid progenitor cells, then to precursor T cells and B cells. B cells develop in bone marrow mature in spleen. Immature T cells develop in bone marrow and migrate to thymus gland for maturation.

- During development, both B cells and T cells develop millions of genetically different clones through independent segregation, random genetic rearrangement and recombination. B cells have antibody (Ig) receptors and T cells have smaller unrelated glycoprotein molecule T cell receptors (TCR) called CD markers. Lymphocytes with self-reactive receptors are normally eliminated during development or functionally inactivated (known as anergy).
- After maturation naïve lymphocytes (i.e. T cells and B cells) migrate from bone marrow and thymus gland, to the peripheral lymphoid tissues/organs such as lymph nodes, spleen, mucosa-associated lymphoid tissue (MALT), gut-associated lymphoid tissue (GALT) in Peyer's patches and skin-associated lymphoid tissue (SALT) throughout the body, and serve as defense force against pathogens.
- Adaptive immune responses are initiated by antigen and antigen-presenting cells (APCs) in the secondary lymphoid tissues. Lymphocytes encounter and respond to antigen in the peripheral lymphoid tissues. Mucosal surfaces have specialized immune structures that orchestrate responses to environmental microbial encounters. Stages of B cells and T cells development in bone marrow and thymus gland are shown in Fig. 4.21.

FUNCTIONS OF B CELLS AND T CELLS

Lymphocytes constitute 20–40% of white cells, which include T cells (60–70%), B cells (10–20%) and natural killer cells (15%), identified by cell surface glycoproteins specific for both cell type and stage of differentiation by use of a panel of monoclonal antibodies. Lymphocytes play key roles in adaptive immune response.

- Lymphocytes consist of distinct classes with different functions and protein products. Subsets of T cells include CD4+ helper T cells, CD8+ cytotoxic T cells, and CD4+ regulatory T cells.

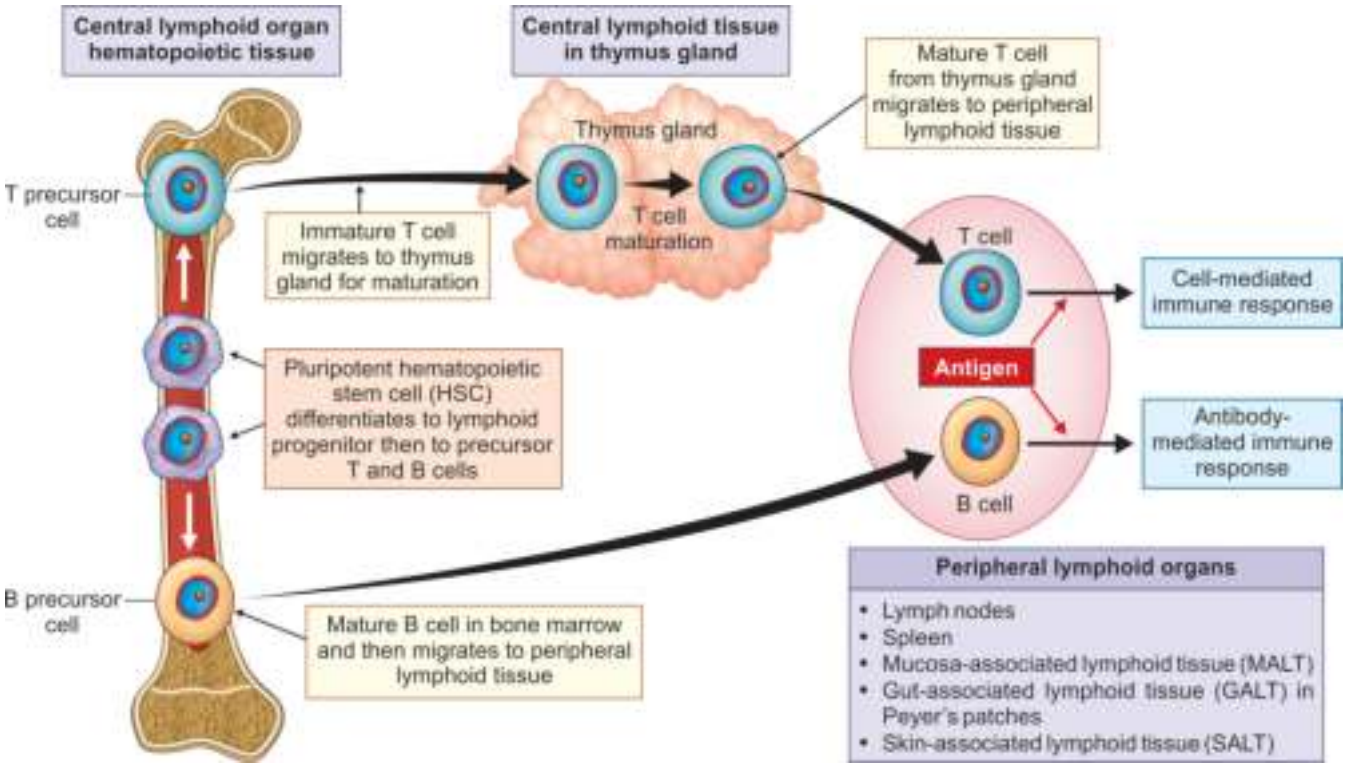


Fig. 4.21: Stages of B cells and T cells development in bone marrow and thymus gland. B cell and T cell are derived from pluripotent hematopoietic stem cells in the bone marrow. T cells migrate to thymus gland for maturation. (a) Stages of B cell development: B cell development starts with rearrangement of the heavy-chain locus in the bone marrow. Immature B cells migrate to the spleen for maturation. There are three subsets of mature B cells: follicular B2 cells, marginal zone B cells and B1 cells. Following exposure to antigens, B cells differentiate into antibody-producing plasma cells. Follicular B2 cells may remain plastic and can differentiate to B1 cells upon self-reactivity of B cell antigen receptor. (b) Differentiation of CD4+ helper T cell subsets: after activation by antigen-presenting cells, CD4+ T cells can differentiate into several T cell subsets: Th1, Th2, Th17 and CD4+ regulatory T cells. The differentiation of each T cell subset is regulated by different transcription factors.

- T cells orchestrate cell-mediated immunity and regulate B cell responses to most antigens. B cells can be transitional/immature, naïve B cells, plasma cells and memory B cells.
- Mature lymphocytes in peripheral lymphoid organs respond to diverse foreign antigens and recirculate in the blood and lymph. Some immature B cells leave the bone marrow and complete their maturation in the spleen. B cells and T cells recognize distinct type of antigens and differentiate into effector cells whose functions are to eliminate the antigens.
- Naïve CD4+ helper T cells engage MHC II molecules on antigen-presenting cells (APCs) and become activated. Clones of the activated CD4+ helper T cell, in turn activate B cells and CD8+ cytotoxic T cells. CD4+ regulatory T cells prevent autoimmune response.
- B cells develop into antibody secreting plasma cells to fight infection. Antibodies protect against extracellular pathogens and their toxic products. Natural killer cells (NK cells) attack any foreign cells including cancer cells.
- Inherited and acquired defects in the immune system result in increased susceptibility to infection. Understanding adaptive immune response is important for control of allergies, autoimmune diseases, and the rejection of transplanted organs. Vaccination is the most effective means of controlling infectious diseases.

CREATION OF ANTIGEN-BINDING T CELL RECEPTORS AND B CELL RECEPTORS

Each lymphocyte produces its own unique receptor, which is structurally organized so that it responds to a different antigen. There is small amount of DNA that encodes the production of B cell receptor (BCR) and T cell receptor (TCR). It is possible for a gene to produce BCR and TCR. Antibodies and T cell receptors are composed of constant and variable regions that provide different functions.

- Therefore, the millions of unique antigen-binding molecules on T cells and B cells are created by a process of gene rearrangement which occur in the primary lymphoid organs (bone marrow and thymus gland) in cells within the lymphoid lineage. B cells and T cells sense the presence of antigens in their environment.
- **Antigen receptor genes** are assembled by somatic gene rearrangements of incomplete receptor gene segments. The gene segments are referred to be as V (variable), D (diversity) and J (joining) and when they are randomly recombined by the action of the RAG genes (recombination activating genes), they are spliced together to create the RNA coding for the N-terminal amino acids in the variable domains of the BCR and TCR.
- Terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase present in the immature pre-B cells and pre-T cells. TdT randomly inserts N-nucleotides to the V (variable), D (diversity), and J (joining) gene segment during gene rearrangements, therefore TdT plays a vital role in the development of the adaptive immune system.
- The immunoglobulin heavy-chain (IgH) and T cell receptor β-chain genes rearrange first in B cell and T cell lineages respectively. Both loci require two recombination events to assemble functional genes; D (diversity) to J (joining) recombination occurs first followed by V (variable) to DJ (diversity and joining) recombination. BCR and TCR recognize antigens by fundamentally different mechanisms.
- Structure of TCR and BCR is shown in Fig. 4.22A and B. Immunotyping of surface markers of human T cells and B cells is shown in Fig. 4.23. V(D)J somatic recombination of the heavy chain immunoglobulin (IgH) and light chain immunoglobulin (IgL) gene segments is shown in Fig. 4.24.

Pathology Pearls: Key Features of Lymphocytes

T Cell Receptor (TCR) and B Cell Receptor (BCR)

- One important difference between TCRs and BCRs is the way they can interact with antigen isotopes.
- TCRs can only react with antigenic epitopes that are presented within the antigen-binding cleft of MHC class I or MHC class II molecules.
- On the contrary, BCRs react with free antigenic epitopes displayed on the surface of intact pathogens without presentation with MHC.
- TCR spans the membrane and projects variable binding regions into extracellular space to bind processed antigens via MHC molecules on antigen-presenting cells (APCs).
- BCR is embedded in the membranes of B cells and bind to a variety of antigens through their variable regions. The signal transduction region transfers the signal in the cell.

Cluster of Differentiation (CD) System

- Lymphocytes possess protein markers on their surfaces that are named using the CD system to delineate particular cell type or stage of differentiation.
- Subsets of lymphocytes are defined by the CD surface markers that the cells carry helper T cells express CD4. On the other hand, suppressor T cells express CD8.
- Markers for T cells include CD1, CD2, CD3, CD4, CD5, CD6, CD7 and CD8. Markers for B cells include CD19, CD20, CD21 and CD22.
- Normal ratio of CD4+ helper T cells to CD8+ cytotoxic T cells are 2:1. This ratio is altered to 0.5:1 or less in acquired immune deficiency syndrome. T cells participate in specific acquired immunity.

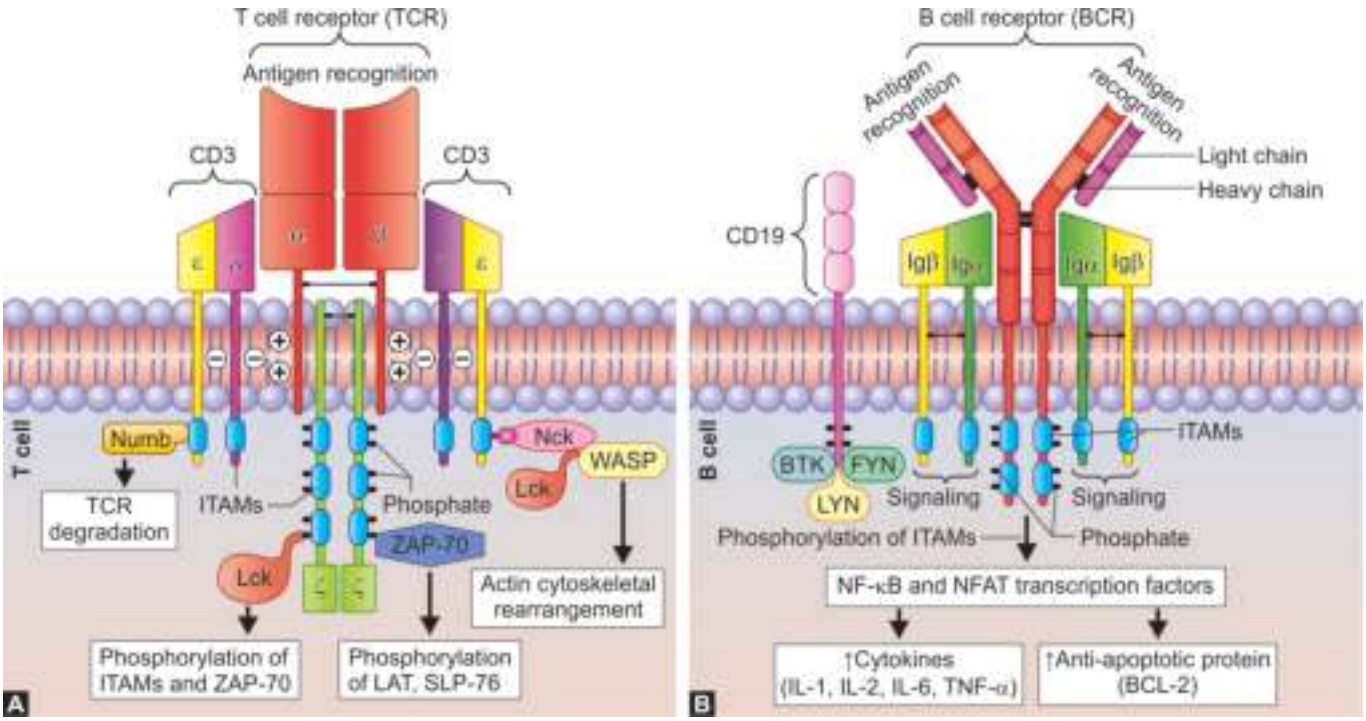


Fig. 4.22A and B: Structure of T cell receptor (TCR) and B cell receptor (BCR). Both TCR and BCR possess unique binding sites. TCR recognizes antigens when displayed to major compatibility complex (MHC). BCR detects and binds to soluble, free and unprocessed antigen. Both TCR and BCR are activated to initiate an immune response to specific binding of their receptors to antigens expressed of tumor cells and viruses.

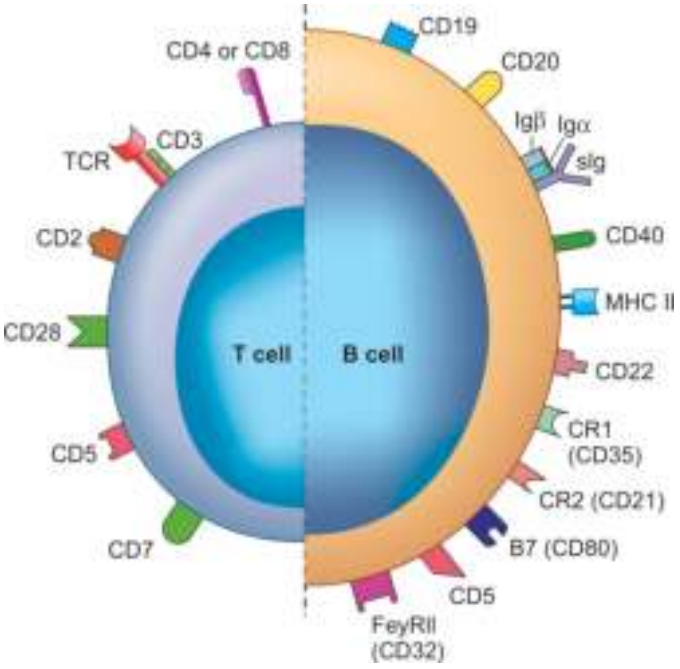


Fig. 4.23: Immunotyping of surface markers of human T cells and B cells. Expression of different markers allows the separation/differentiation of T cells and B cells express CD3, CD4, CD8, or CD25 markers. Immature B cells express CD19, CD20, CD34, CD38 and CD45R, but not IgM. Mature B cells express CD19 and IgM. Activated B cells express CD30, a regulator of apoptosis. Memory B cells express CD20 and CD40. Plasma cells lose CD19 but gain CD78.

Postulates of the Clonal Selection Hypothesis

T cells and B cells with self-respective receptors are normally eliminated during development or these lymphocytes are functionally inactivated. Each lymphocyte bears a single type of receptor with a unique specificity.

- Interaction between a foreign molecule and a lymphocyte receptor capable of binding that molecule with high affinity results in activation of lymphocyte.
- The differentiated effector cells derived from an activated lymphocyte will bear receptors of identical specificity to those of the parental cell from which that lymphocyte has been derived.
- Lymphocytes bearing receptors specific for ubiquitous self-molecules are deleted at an early stage in lymphoid cell development and are therefore absent from the repertoire of mature lymphocytes.

Clonal Selection in Lymphoid Organs

All T cells originate from pluripotent hematopoietic stem cells in the bone marrow and generate a large population of immature progenitors and migrate to thymus gland for maturation. The thymocytes progress from double negative thymocytes to become double positive thymocytes (CD4+ and CD8+) and finally to single positive (either CD4+ or CD8+).

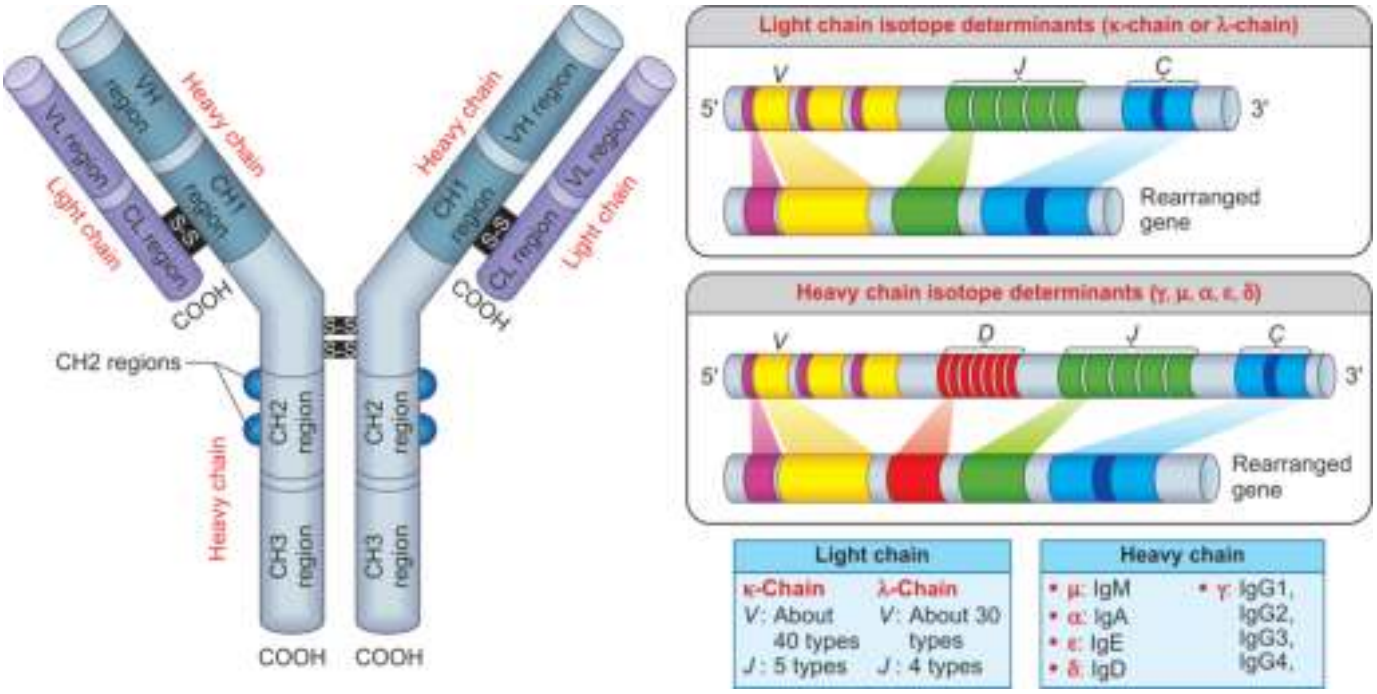


Fig. 4.24: V(D)J somatic recombination of the heavy chain immunoglobulin (IgH) and light chain immunoglobulin (IgL) gene segments. The immunoglobulin IgH locus is organized in gene segments: the variable (V), diversity (D) and joining (J) and constant (C) gene segment. The variable (V) region comprising the V, D and J gene segments is generated by random recombination of these sequences. As IgL lacks diversity (D) gene segment, similar rearrangement of variable (V) and joining (J) genes occurs in IgL.

- Clonal selection is used during negative selection to eliminate lymphocytes that may be able to bind with self-antigens.
- During T cell differentiation, the naïve T cell undergoes proliferation by clonal expansion and differentiation into memory T cells and effector T cells. Many subsets of CD4+ helper T cells are generated during T cell differentiation and perform different functions for the immune system.
- Clonal selection of T cells and B cells can occur by two mechanisms: antigen-independent and antigen-dependent. Overview of the antigen-dependent and antigen-independent clonal selection of lymphocytes diversity in lymphoid organs is shown in Fig. 4.25A and B.

Clonal Selection of Lymphocytes by Antigen-independent Mechanism

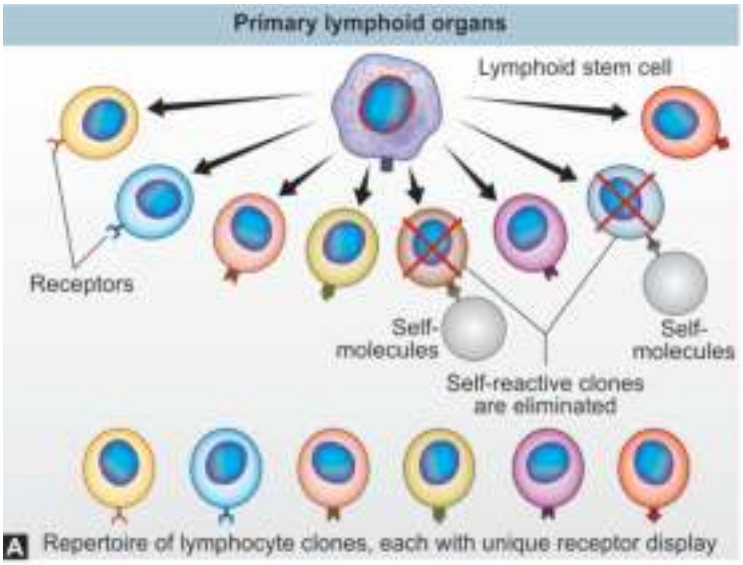
Hematopoietic stem cells (HSCs) in bone marrow participate in the production of lymphocytes. During development, hematopoietic stem cell undergoes rapid cell division to form numerous progenies.

- During this period of cell differentiation, random rearrangements of genes encoding cell surface protein receptors take place. Differentiation leads to formation of large array of genetically distinct cells known as **clones**. Each clone bears a different receptor. Each receptor is specific to react with only a single type of foreign antigen.
- During differentiation, lymphocyte clones harmful to self-molecules formed are eliminated from the pool of diversity known as immune tolerance.
- The specificity for a single molecule is programed into the lymphocyte, which is set for the life of a given clone. The net result is an enormous pool of mature but naïve lymphocytes, which are ready for further differentiation under the influence of certain immune stimuli.

Clonal Selection of Lymphocytes by Antigen-dependent Mechanism

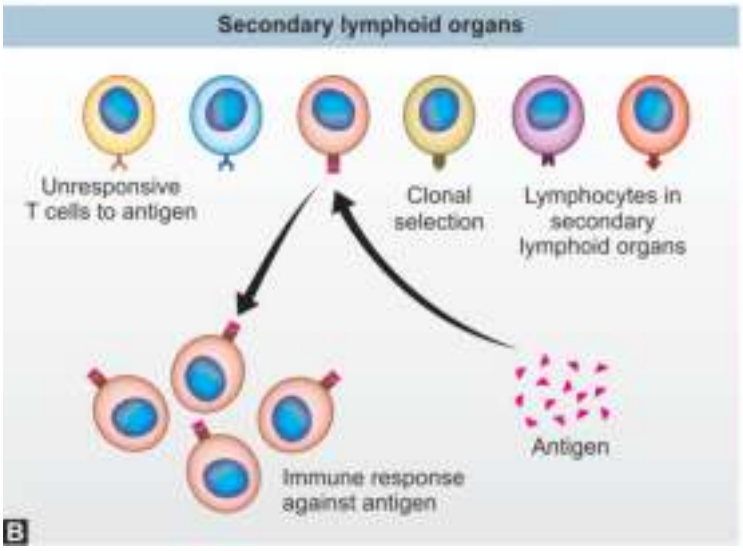
Lymphocytes come to populate the lymphoid organs, which will finally encounter antigens. These antigens activate the immune function. Entry of a specific antigen selects only the lymphocyte clones the carrier matching surface receptors, which triggers immune response, which varies according to the type of lymphocyte involved.

- B cell and T cell receptors function in identification, communication, and development. When receptors on B cells and T cells interact with antigenic determinant specific to it, the lymphocyte becomes activated and divides to form a clone of cells.
- B cells and T cells are derived from hematopoietic stem cell, and exhibit the same specificity for antigenic determinant. B cells and T cells are transformed to responder cells and memory cells.



Antigen-independent clonal selection of lymphocytes

- B cell and T cell originate in hematopoietic stem cells in bone marrow. B cell undergoes maturation and migrates to spleen for maturation. T cell migrates to thymus gland for maturation.
- During antigen-independent period of cell differentiation, random rearrangements of genes encoding cell surface protein receptors take place.
- Differentiation leads to formation of large array of genetically distinct cells known as clones.
- Each clone bears a different specific receptor, which reacts with only a single type of foreign antigen.
- During differentiation, lymphocyte clones harmful to self-molecules formed are eliminated from the pool of diversity known as immune tolerance.
- The specificity for a single molecule is programed into the lymphocyte. It is set for the life of a given clone. The net result is an enormous pool of mature but naïve lymphocytes, which are ready for further differentiation under the influence of certain immune stimuli.



Antigen-dependent clonal selection of lymphocytes

- Lymphocytes come to populate the lymphoid organs, which finally encounter antigens.
- Antigens trigger the immune function by clonal selection of lymphocyte matching surface receptors.
- Immune response triggered by antigens varies according to the type of lymphocyte involved.
- B cell and T cell receptors function in identification, communication, and cell development. When receptors on B cells and T cells interact with antigenic determinant specific to it, the lymphocyte becomes activated and divides to form a clone of cells.
- B cells and T cells are derived from a single parental cell, and exhibit the same specificity for antigenic determinant.
- B cells and T cells are transformed to responder cells and memory cells.
- The process by which diversity is generated in clonal selection. Immune tolerance or tolerance to self occurs during this time.

Fig. 4.25A and B: Overview of the antigen-dependent and antigen-independent clonal selection of lymphocytes diversity in lymphoid organs. T cell progenitors originating from pluripotent hematopoietic stem cell (HSC) migrate to thymus gland for maturation. During development, pluripotent hematopoietic stem cell undergoes differentiation and genetic rearrangement to produce immature progenitors of lymphoid lineage in primary lymphoid organs with many different antigen surface receptors. Those immature progenitors of lymphoid lineage that bind to antigens from the host own tissues are eliminated, while the rest immature lymphocytes mature into inactive lymphocytes.

- Diversity of T cells and B cells occurs by the process of clonal selection. Immune tolerance or tolerance to self occurs during this time.

EXPOSURE OF HOST TO ANTIGENS

Antigen is a substance capable of stimulating an adaptive immune response, specifically activating B cells and T cells. There are different types of antigens on the basis of origin: exogenous antigens (e.g. pollen, food allergen, aerosols), endogenous antigens (e.g. bacteria, viruses), and autoantigens (self-proteins such as nucleic acids can induce autoimmunity).

- An antigen that is not yet processed by an antigen-processing cell (APC) is known as **native antigen**. Each antigen has several antigenic determinants or epitopes. An immunoglobulin has at least two binding sites that can bind to specific antigens. Antigen combines with the immunoglobulin according to the lock and key mechanism.
- Antigen-specific activation of lymphocytes involves the specific receptors on B cells and T cells, which differ in the process of the recognition of antigens. B cell receptor (BCR) detects and binds soluble antigens that are present freely, whilst T cell receptor (TCR) only recognizes antigens when displayed on major histocompatibility complex (MHC), i.e. MHC (HLA) class molecules. Both B cell receptor (BCR) and T cell receptor (TCR) repertoires are generated through identical processes of V(D)J recombination,

exonuclease trimming of germline genes and random addition of nontemplate encoded nucleotides.

CO-OPERATION IN IMMUNE CELLS AGAINST ANTIGENS

Immune cells participating in specific immune response comprise T cells, B cells, Langerhans' cells of the skin, dendritic cells of lymphoid tissue and monocytes/ macrophages, Both B and T cells may respond to the same antigen, the way the signal is represented.

- B cells can recognize the antigen irrespective of the form antigen is presented. B cells are capable to bind free antigen in solution, antigen present on the membrane of cells and antigens insolubilized in various ways.
- In contrast, T cells can only bind antigen present on the membrane of cells. CD4+ helper T cells bind to

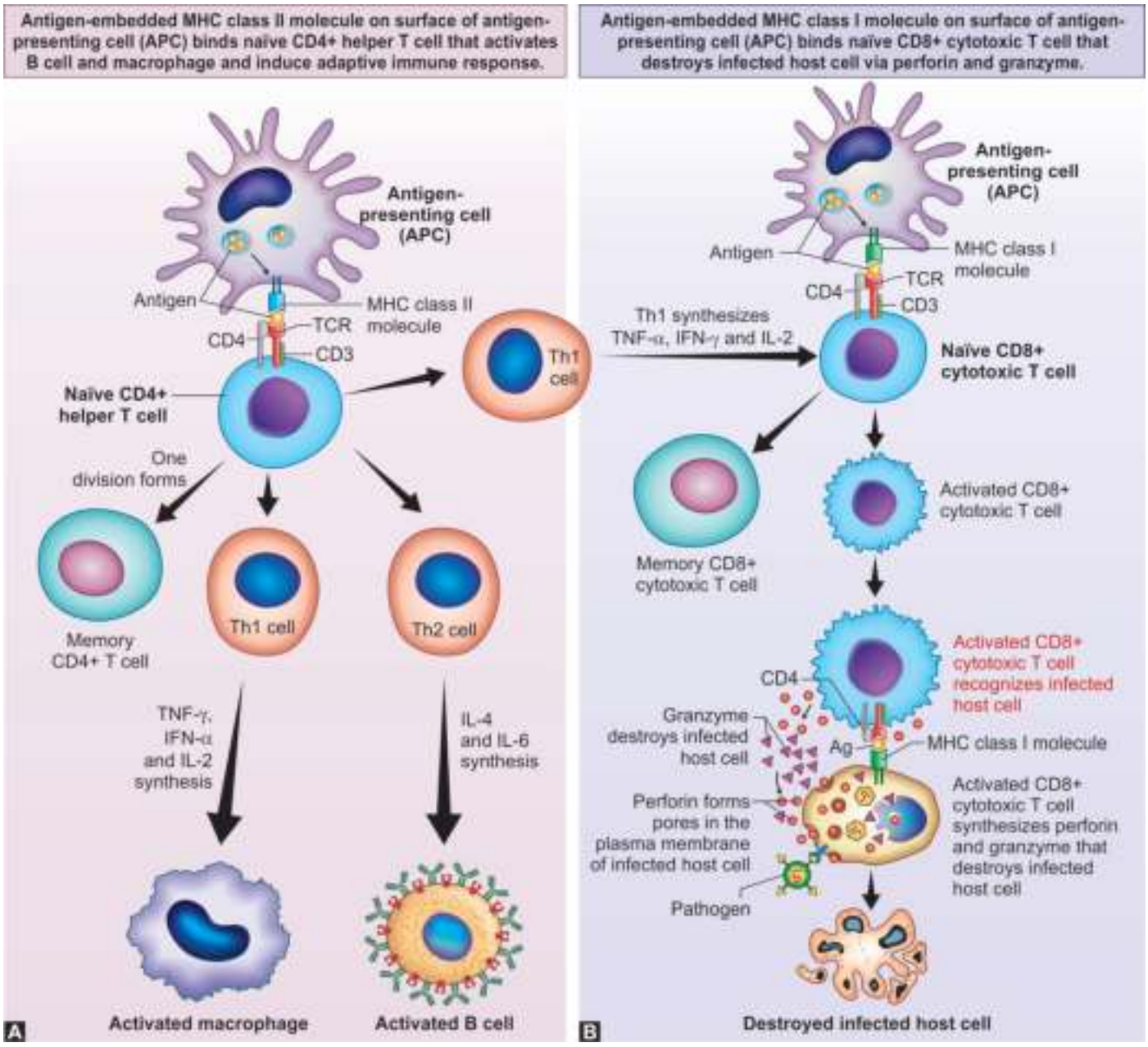


Fig. 4.26: Antigen-embedded different MHC class molecules on surface of antigen-presenting cells and their binding to naïve CD4+ helper T cells or CD8+ cytotoxic T cells. (A) When naïve CD4+ T cell interacts with antigen-presenting cell (APC), both cells release cytokines. In response to cytokines the cloned activated helper T cells produce different cytokines that activate B cells and naïve CD8+ cytotoxic T cells, which become CD8+ cytotoxic T cells. (B) When CD8+ cytotoxic T cells interact with the MHC class I molecule–epitope complex on an infected cell, CD8+ cytotoxic T cells synthesize perforins and granzymes, which kill infected host cells. Perforins induce pores in the plasma membrane and granzymes enter the cell and break down proteins causing lysis of infected cell.

antigen that has been processed by antigen-presenting cells (with the exception of superantigens). Antigen is presented to macrophages with the help of second signal provided by a glycoprotein on the surface of the antigen-presenting cell (APC) that is coded for a gene sequence within major histocompatibility complex (MHC).

ANTIGEN PROCESSING AND PRESENTATION BY ANTIGEN-PRESENTING CELLS

With the exception of superantigens, T cells recognize antigen that has been processed in the same way within antigen-presenting cells (APCs).

- Antigens enter the major fluid compartments. Fluid movement from the intravascular compartment to interstitial and intracellular compartments occur in the capillaries. Antigen-presenting cells (APCs) (dendritic cells and macrophages) process antigen and present to either CD4+ helper T cells or CD8+ cytotoxic T cells depending on type of antigen.
- Antigen derived from the cytoplasm (e.g. viral proteins) is processed by MHC class I molecules to CD8+ cytotoxic T cells. Antigen-presenting cells (dendritic cells and macrophages) process exogenous antigen make small fragments of antigen by MHC class II molecules then to CD4+ helper T cells.
- Some antigens may directly stimulate B cells to form antibody secreting plasma cells and memory B cells. Memory B cells have long life span over 40 years that is regulated by the BCL-2 gene, which is present only in the memory B cells. Antigen-embedded different MHC class molecules on surface of antigen-presenting cells and their binding to naïve CD4+ helper T cells or CD8+ cytotoxic T cells are shown in Fig. 4.26A and B.

Antigen Presentation by Antigen-presenting Cells with MHC Class II Molecules

Exogenous antigen (foreign proteins peptide) is endocytosed by antigen-presenting cells (APCs). The α- and β-chains of MHC class II molecule assemble in the rough endoplasmic reticulum of cell. Invariant (inv) protein synthesized by endoplasmic reticulum directs assembled MHC class II molecules in the endosome, where it binds with endocytosed antigen (foreign protein peptide).

- Assembling of α- and β-chains MHC class II molecule complexed with a membrane bound invariant (inv) protein dictate the folding of MHC class II molecule and prevent premature binding of proteins to MHC class II molecule before the latter reach the location of the intracellular processed antigen. In the endosome, there occurs limited proteolysis of antigen (foreign protein peptide) and lysis of invariant (inv) protein.
- Then MHC class II molecule is transported along with foreign protein peptide and presented to antigen-presenting cells (APCs). Processing and presentation of antigen byAPCs in association with MHC class II molecule are shown in Fig. 4.27.

Antigen Presentation by Antigen-presenting Cells with MHC Class I Molecules

Viral coded proteins presented on the surface of cells expressing MHC class I molecule undergo proteolysis with the formation of peptides, some of which are recognized by T cell receptors (TCRs). Some viral proteins are degraded by proteasome leading to release of viral peptides.

- The viral peptides transported to endoplasmic reticulum of the infected cell bind with MHC class I heavy chains followed by complexing with β_2-macroglobulin.
- MHC class I molecule and viral peptides are transported via Golgi apparatus. Then viral particles are presented on infected surface in association with

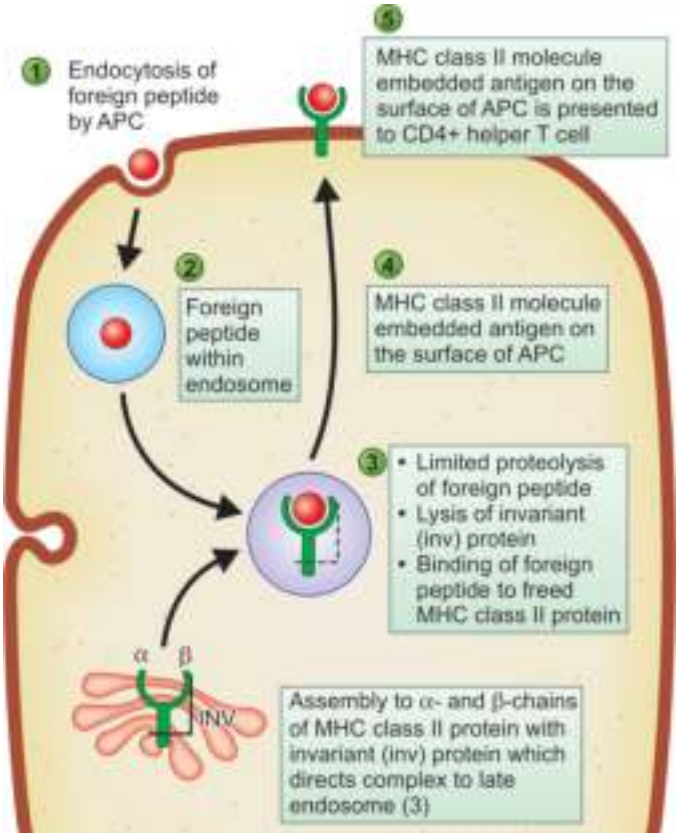


Fig. 4.27: Processing and presentation of antigen by antigen-presenting cells (APCs) in association with MHC class II molecule to CD4+ helper T cells. Antigen-presenting cells (APCs) with MHC class II molecule is essential for the activation of CD4+ helper T cells. APCs ingest pathogens by the process of phagocytosis, degrades them in the phagolysosome, process protein antigens, select the most antigenic/immunodominant epitopes with MHC class II molecule for presentation to CD4+ helper T cells.

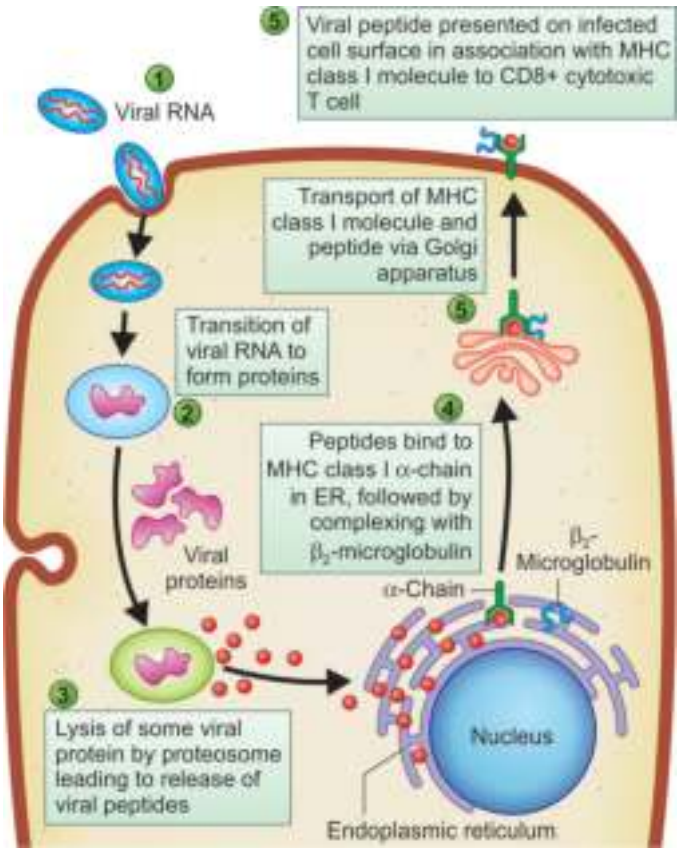


Fig. 4.28: Processing and presentation of antigen by antigen-presenting cells (APCs) in association with MHC class I molecule to CD8+ cytotoxic T cells. MHC class I molecule carries peptides of foreign protein into the cell surface. Once embedded in the surface, a signal is transmitted to CD8+ cytotoxic T cells that foreign protein inside the cell.

MHC class I molecule. Processing and presentation of antigen-presenting cells (APCs) of antigen in association with MHC class I molecule are shown in Fig. 4.28.

T CELL AND CELL-MEDIATED ADAPTIVE IMMUNE RESPONSE

B cells and T cells are the major components of adaptive immune system. Pluripotent hematopoietic stem cell (HSC) differentiates to lymphoid progenitor cell then to precursor T cells and B cells in the bone marrow.

- B cell develops in the bone marrow and matures in spleen and then migrates to peripheral lymphoid tissue. Once produced from hematopoietic pluripotent stem cells in the bone marrow, T progenitor cells migrate to the thymus gland and go through a series of developmental stages that ensures both function and tolerance before T cells leave and become functional components of the adaptive immune response.
- In thymus gland, thymic epithelial cells present MHC class molecules to untrained thymocytes (double negative for CD4 and CD8 receptors). Thymocytes that successfully bind with MHC class molecules continue to develop (positive selection), while those thymocytes fail to bind to MHC class molecules undergo apoptosis (negative selection). Thymocytes that survive by positive selection become double positive (CD4+ and CD8+ receptors). The surviving thymocytes move to the thymic medulla, where they encounter antigen-presenting cells (APCs). These APCs present self-antigens from other body cells. Thymocytes that bind with self-antigens undergo apoptosis, which indicates that thymocytes will attack body cells. This process is called negative selection. Cell signals cause self-tolerant thymocytes to express either the CD4 or CD8 receptor. Those cells express CD4 receptor becomes CD4+ helper T cells, while those cells express the CD8 receptors become CD8+ cytotoxic T cells.
- Mature T cell from thymus gland migrates to peripheral lymphoid tissue such as lymph nodes, spleen, mucosa-associated lymphoid tissue (MALT), gut-associated lymphoid tissue (GALT) in Peyer's patches and skin-associated lymphoid tissue (SALT).
- Mature T cells become activated by recognizing processed foreign antigen in association with a self-MHC class molecule and start dividing rapidly by mitosis. This proliferation of T cells is known as clonal selection, which is essential to make the adaptive immune response strong enough to effectively eliminate a pathogen. Clonal selection is the process of antigen binding only to those T cells that have receptors specific to the antigen. Overview of adaptive cellular and humoral immune response is shown in Figs 4.29 and 4.30.

CD4+ HELPER T CELL AND CELL-MEDIATED ADAPTIVE IMMUNE RESPONSE

Upon uptake, conventional antigen is processed into short peptides by antigen-presenting cell (APC), and then presented MHC class II molecule to naïve CD4+ helper T cells. Cytokines synthesized by CD4+ helper T cells play diverse roles in the immune response.

- Antigen-presenting cells (APCs) synthesize IL-12 that activates CD4+ helper T cells leading to differentiation into CD4+ T helper type 1 (Th1) cell. IL-4 synthesized by naïve CD4 helper T cell results in differentiation into CD4+ T helper type 2 (Th2) cell. Transforming growth factor β, IL-6, IL-21 and IL-23 lead to activation of CD4+ T helper type 17 (Th17) cell. Activated CD4+ helper T cells also differentiate into CD4+ T helper type 17, CD4+ regulatory T cells (Tregs), memory

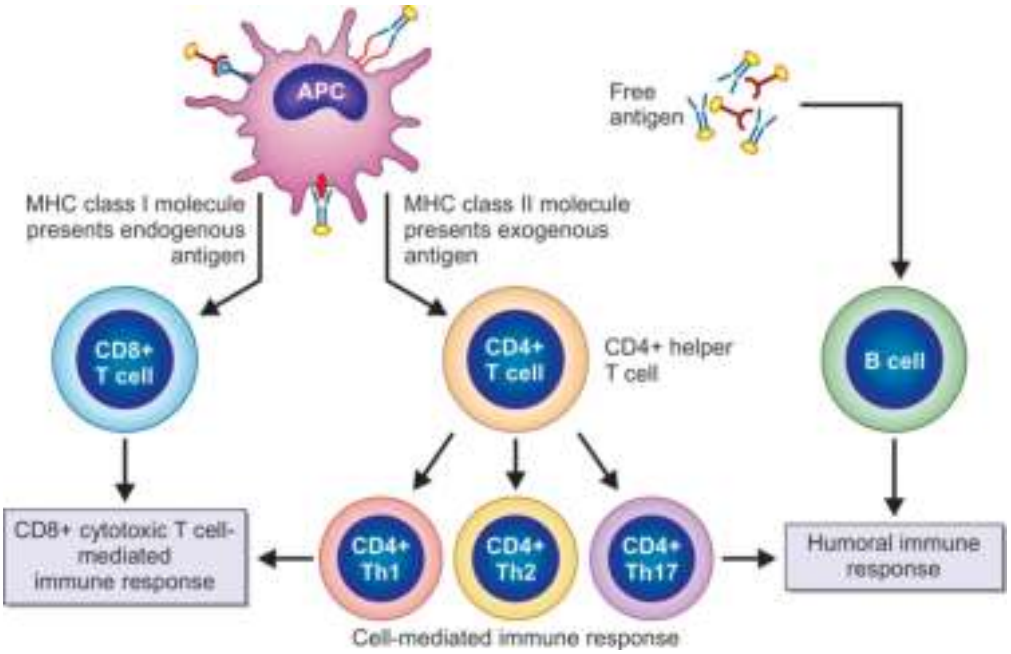


Fig. 4.29: Overview of adaptive cellular and humoral immune response. Activation of CD4+ helper T cells by antigen, leads to their differentiation into subsets of helper T cells (Th1, Th2, Th17, Treg cells) and memory T cells assisting other CD8+ cytotoxic T cells that eliminate foreign cells. T cells also synthesize cytokines that regulate cell-mediated immune response. B cell participates in antibody-mediated immune response.

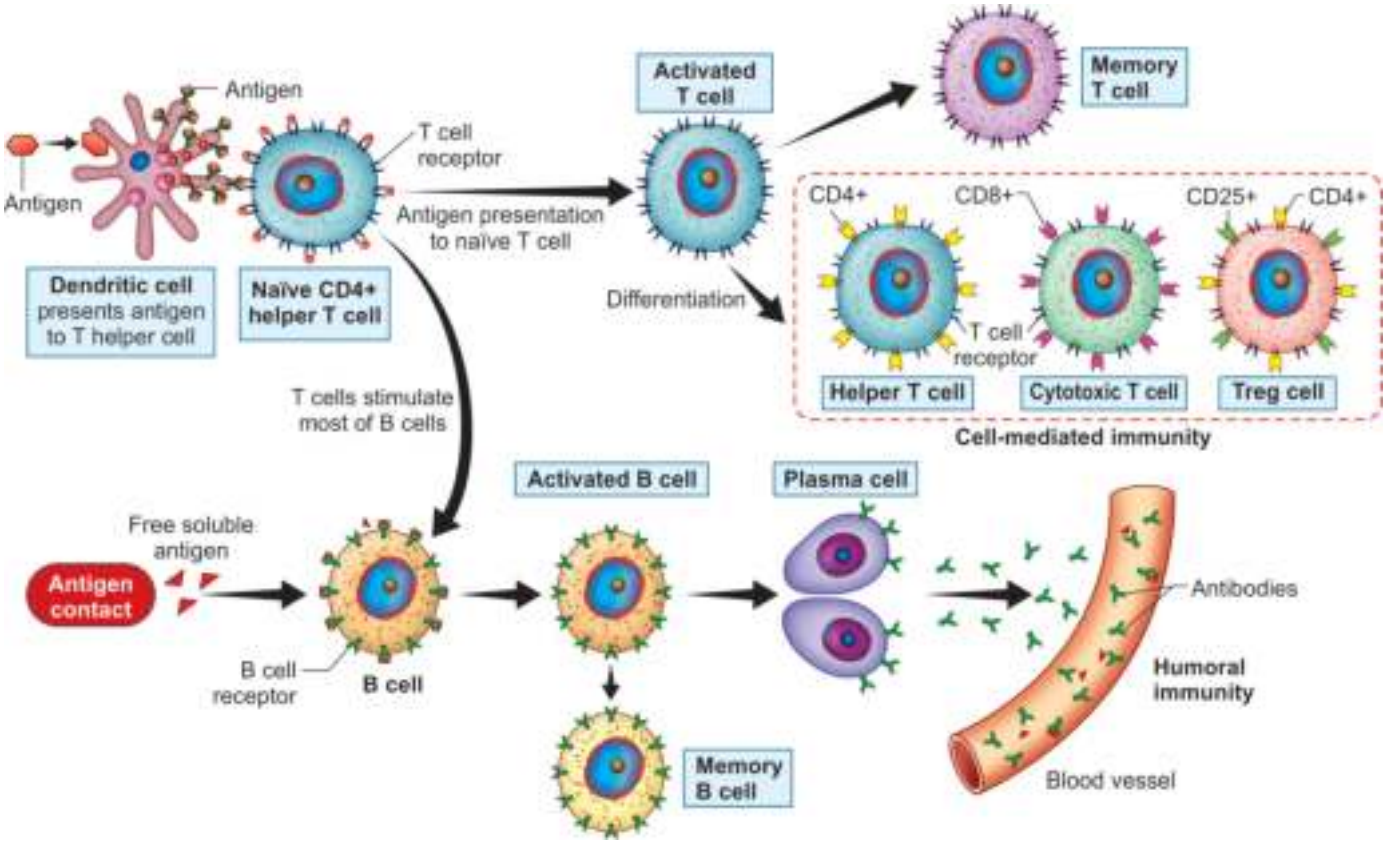


Fig. 4.30: Overview of cellular and humoral adaptive immune system. An adaptive immune system is based on clonally diverse T cells and B cells that can recognize specific pathogens and provide protective immunological memory against subsequent encounter to same pathogen in vertebral species. T cell is involved in cell-mediated immunity and B cell carry out humoral immunity. Antigen-presenting T cell and naïve CD4+ helper T cell leads to clonal proliferation and differentiation of activated CD4+ helper T cells to effector T cells and memory T cells. CD4+ helper T cells also stimulate B cells, which differentiate to effector B cells, antibody secreting plasma cells and memory B cells.

T cells. Differentiation of each T cell subset is regulated by different transcription factors.

- CD4+ helper T cells assist CD8+ cytotoxic T cells that eliminate foreign infected cells or cancer stem cells. CD4+ helper T cells activate macrophages.
- CD4+ helper T cells also activate B cells, which undergo cell division resulting in formation of many plasma cells, which synthesize five classes of immunoglobulins circulating in tissue fluids (blood and lymph) providing humoral immunity.
- Immunoglobulins differ in size and function, which include IgG, IgA, IgM, IgD and IgE. Antibody opsonizes microbes for phagocytosis both directly via Fc receptors and indirectly via complement system activation. The Fab (fragment antigen-binding) part of the antibody binds specific antigen on the microbe and varies from one antibody to another. Some of plasma cells become memory plasma cells that recognize the same antigen in the future. CD4+ helper T cell differentiates into subsets is shown in Fig. 4.31.

Naïve CD4+ Helper T Cell Activation and Differentiation

Antigen-presenting cells (macrophages or dendritic cells) are found in large number in lymphoid tissues, which detect invading foreign antigens (microbes) and present them to CD4+ helper T cells, which recognize the antigen and initiate the specific immune response. Interaction between macrophages and CD4+ helper T cells is shown in Fig. 4.32. Activation of CD4+ helper T cell by antigen-presenting cell (APC) is shown in Fig. 4.33. APCs and CD4+ T cell interaction inducing adaptive immune response are given in Fig. 4.34.

- Naïve CD4+ helper T cells engage MHC II molecule on APCs, where naïve CD4+ helper T cells are activated by antigen to proliferate and differentiate into effector T cells and memory T cells. Some of effector T cells and memory T cells migrate into the peripheral tissue at the site of infection. Superantigens present in Staphylococci, Group A Streptococci) and Epstein-Barr virus can also activate CD4+ helper T cells by binding to MHC class II molecule on antigen-presenting cells without being processed by APCs.
- When naïve CD4+ helper T cells engage MHC class II molecule on APCs, both naïve CD4+ helper T cell and APC release cytokines such as TNF-α, IL-1 and IL-6. Clones of the activated CD4+ helper T cell, in turn, activate B cells and naïve CD8+ cytotoxic T cells, which become activated CD8+ cytotoxic T cells.
- On stimulation of CD4+ helper T cells differentiate to Th1, Th2, Th17 and CD4+ regulatory T cell (Treg

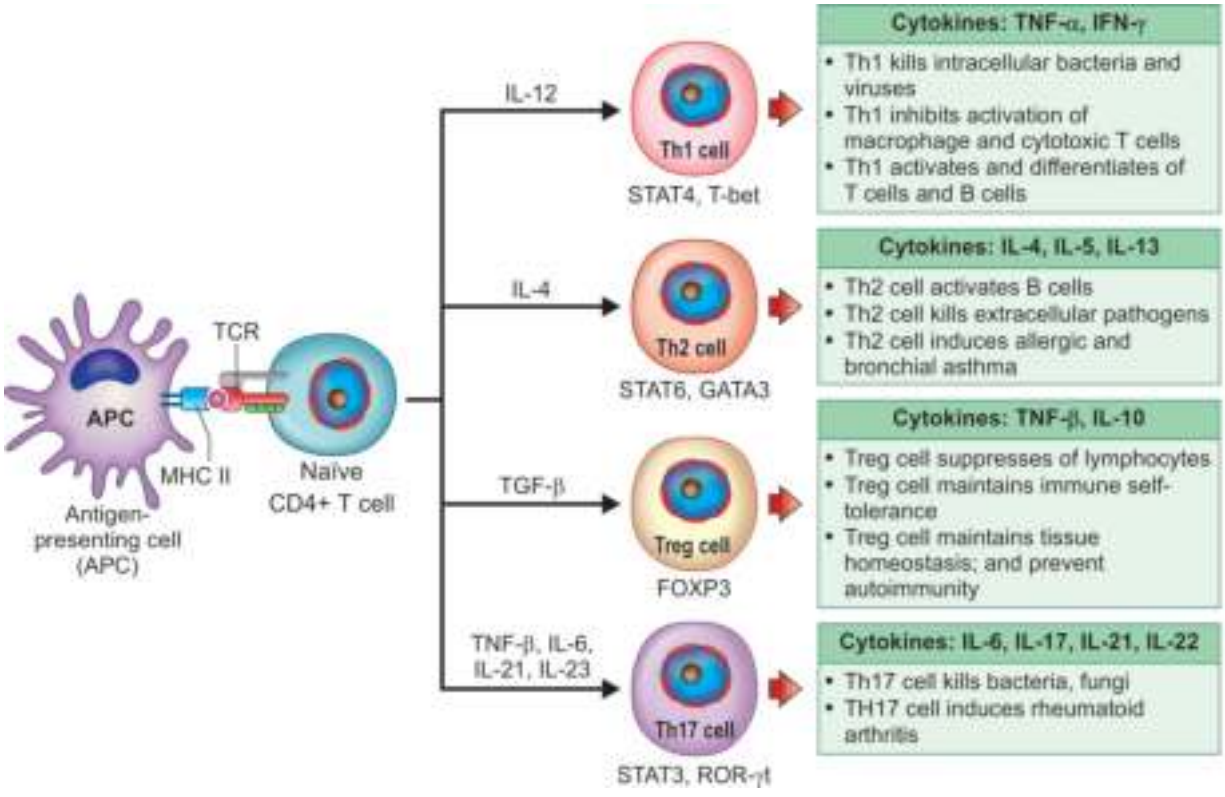


Fig. 4.31: Differentiation of CD+ helper T cell subsets/after activation by antigen-presenting cells, CD4+ helper T cell can differentiate into several subsets: T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) and CD4+ regulatory T (Treg) cells. The differentiation of each helper T cell subset is regulated by different transcription factors.

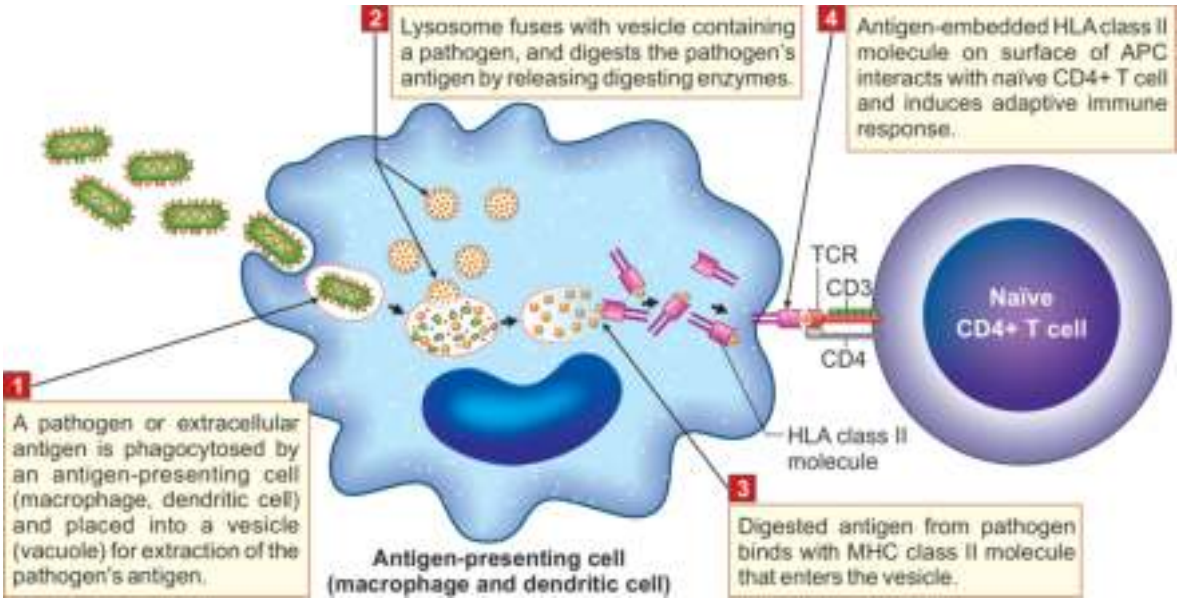


Fig. 4.32: Interaction between macrophages and CD4+ helper T cells. A macrophage takes up antigen, process and presents it with the MHC class II protein. This is recognized by the T cell receptor (TCR) of a specific population of CD4+ helper T cells and CD8+ cytotoxic T cells. CD4+ helper T cell is the key coordinator of adaptive immune response. CD4+ helper T cells play important role in adaptive immunity by activating B cells to form immunoglobulins secreting plasma cells. CD4+ helper T cell activate macrophages that secrete various cytokines. CD4+ helper T cells activate CD8+ cytotoxic T cells that recognize MHC class I molecule on cells that are not part of the immune system and destroy them too. Memory T cells are retained for future use.

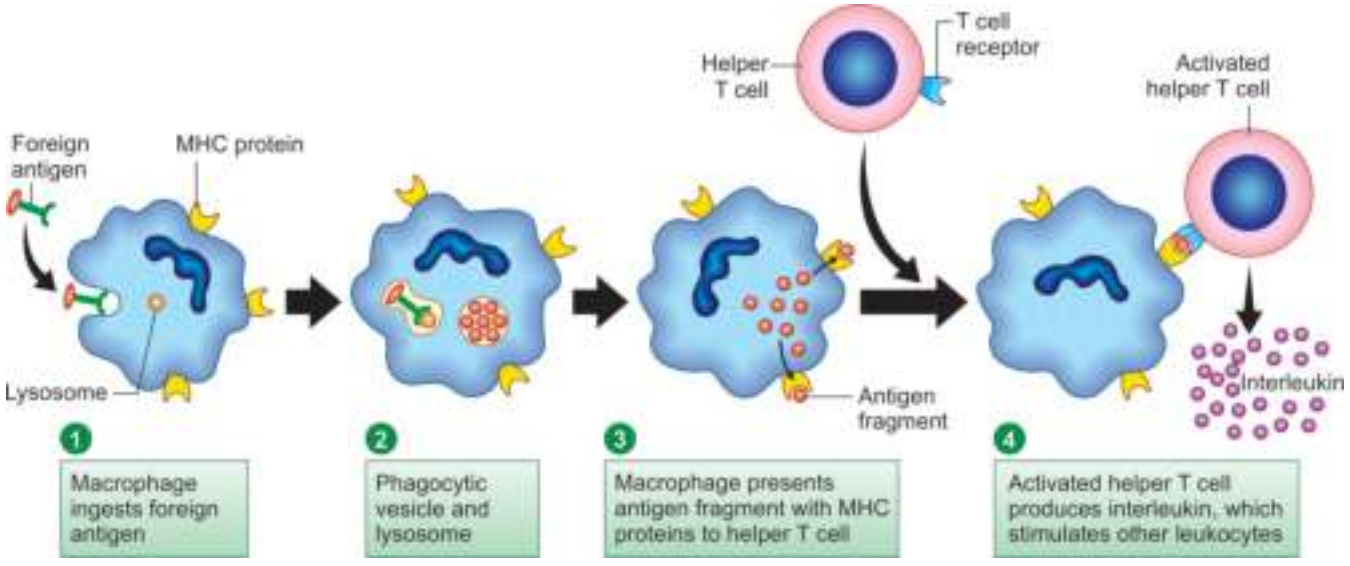


Fig. 4.33: Activation of CD4+ helper T cell by antigen-presenting cell (APC). An antigen-presenting cell (APC), a macrophage displays digested foreign antigen on its surface along with self-major histocompatibility complex (MHC) antigen. A helper T cell is activated by contact with this complex and synthesizes stimulatory interleukins.

cell) subsets, which synthesize cytokines: INF-α and IFN-γ (Th1 cell), IL-4, IL-5 and IL-13 (Th2 cell), IL-6, IL-17 and IL-21, IL-22 (Th17 cell) and TGF-β and IL-10 (Treg cell). CD4+ helper T cell type 1 (Th1) is involved with elimination of intracellular pathogens and is associated with organ-specific autoimmunity. CD4+ helper T cell type 2 (Th2) mounts immune response to extracellular parasites, including helminths, and plays major role in induction and persistence of bronchial asthma as well as other allergic diseases.

- IL-12 is critical cytokine that initiates the downstream signaling pathway to develop Th1 cells. IL-12 is synthesized in large amounts by APCs through the pattern recognition receptors (PRRs). IL-12, in turn induces natural killer cells (NK cells) to produce IFN-γ. IFN-γ is essential for the activation of mononuclear

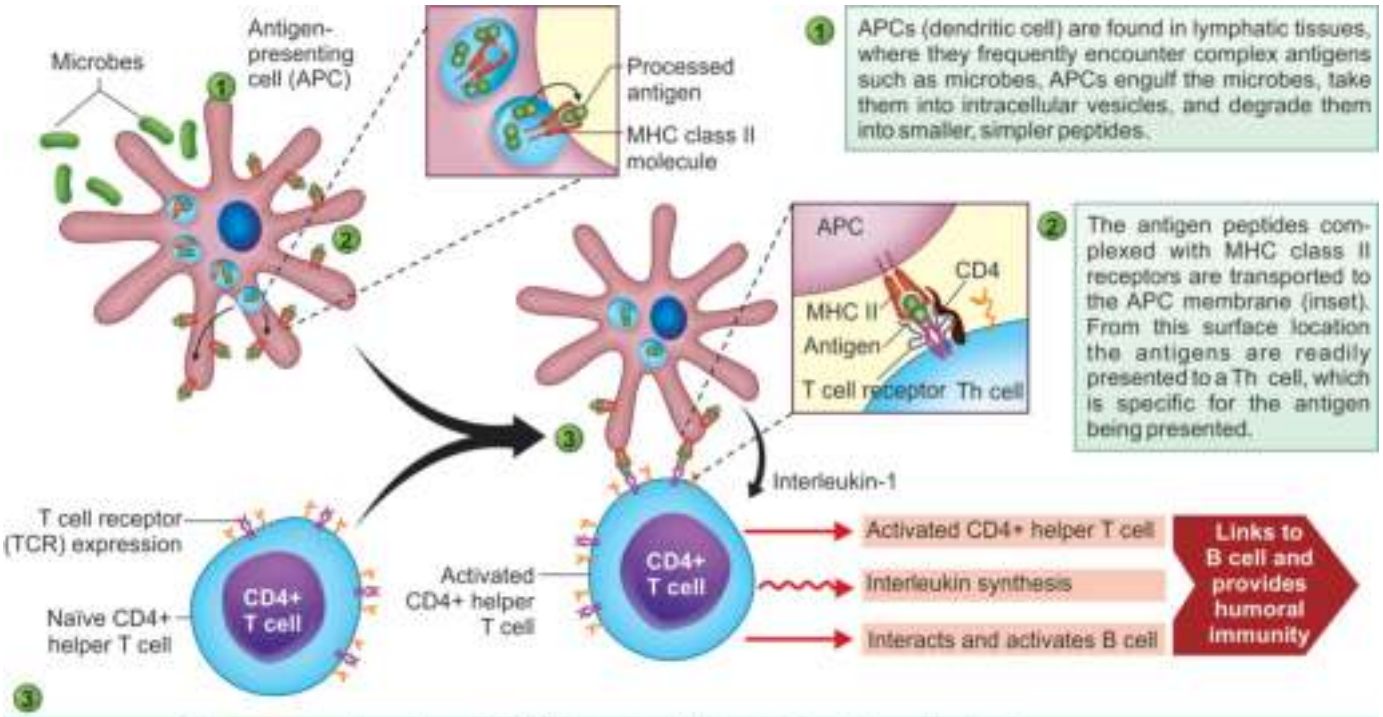


Fig. 4.34: Antigen-presenting cells (APCs) and CD4+ helper T cell interaction inducing adaptive immune response. Naïve CD4+ helper T cells engage MHC class II molecules on antigen-presenting cells (APCs) and become activated. Activated CD4+ helper T cells differentiate into subsets (Th1, Th2, Th17 and Treg cells). Activated CD4+ helper T cells, in turn activate B cells to form antibody secreting plasma cells. Activated CD4+ helper T cells activate CD8+ cytotoxic T cells, which destroy pathogen.

phagocytes, including macrophages, microglial cells, thereby resulting in enhanced phagocytic activity.

- Amplification of T cell response is mediated by growth factor IL-2 within hours of activation of T cells following recognition of antigen by T cell receptor.
- Significance of IL-2 synthesis following tissue rejection is underlined by the effectiveness of drug cyclosporine A, which is widely used to prevent rejection of allogenic transplants. Cyclosporine A blocks the transcription of IL-2 gene, which normally follows T cell activation.

Pathology Pearls: Superantigens Activate Naïve CD4+ Helper T Cell

- Superantigens are present in Staphylococci, Group A Streptococci) and Epstein-Barr virus, that can also activate naïve CD4+ helper T cell by binding to MHC class II molecule on antigen-presenting cells without being processed by APCs, and activate T cells via T cell receptor (TCR).
- Interaction of superantigens and TCR leads to massive influx of potent chemical mediators that can cause blood vessel damage, toxic shock syndrome and multiple organs failure. It is also suggested that superantigens may be implicated in rheumatoid arthritis.
- Whole T cell subpopulations can be activated by superantigens independently of antigen specificity.

CD8+ CYTOTOXIC T CELLS AND CELL-MEDIATED CYTOTOXICITY

Armed CD8+ cytotoxic T cells are essential in host defense against pathogens that reside in the cytosol, the commonest of which are the **viruses**. CD8+ cytotoxic T cells can kill any cell harboring such pathogens by recognizing foreign peptides that are transported to the cell surface bound to MHC class I molecule. CD8+ cytotoxic T cells can kill CSCs and reject tissue grafts. CD8+ cytotoxic T cells kill virus infected host cells by releasing two types of preformed cytokines protein: pore-forming perforin and cytotoxic granzymes.

- **Perforins** are proteins that can punch holes in the membranes of target cells. First the perforins cause ions to leak out of target cells and create a passageway for granzymes to enter. **Granzymes** enter and break down proteins causing death of virus infected cell and CSCs through a process called **apoptosis**. These properties allow CD8+ cytotoxic T cell to attack and destroy any cell that is infected with a cytosolic pathogen.
- A membrane-bound molecule, Fas ligand (FasL) expressed by CD8+ cytotoxic T cells, is also capable of inducing apoptosis by binding to FasR expressed by some target cells. CD8+ cytotoxic T cells also secrete interferon-γ (IFN-γ), which is inhibitor of MHC class I molecule expression and macrophage activation. CD8+ cytotoxic T cells kill virus infected target cells with great precision, sparing adjacent normal cells. This precision is critical in minimizing tissue damage while allowing the elimination of infected cells.

CD4+, CD25+, FOXP3+ REGULATORY T CELLS

CD4+, CD25+, FOXP3+ regulatory T cells (Tregs) are specialized subpopulation of T cells that act to suppress immune response, thereby maintaining homeostasis and involved in self-immunologic tolerance. CD4+ regulatory T cells limit the activation of other CD4+ helper T cells and prevent autoimmune response.

NATURAL KILLER CELLS KILL VIRUSES AND CANCER STEM CELLS

Natural killer cells (NK cells) are derived from large granular lymphocytes. Immunophenotyping of natural killer cells include CD16, CD56 and CD57. NK cells attack viruses and cancer cells.

- NK cells can kill target cells by two major mechanisms: the death receptor and granule-dependent pathways. In both cases, the target cell dies as a result of the activation of a battery of cytotoxic proteases within the target cell, called caspases.
- In the granule-dependent pathway, several proteolytic enzymes called **perforins** and **granzymes** are delivered to the target cells, promoting the activation of a family of death inducing proteases called caspases.
  - Perforin is a pore-forming protein in the plasma membrane of target cell.
  - Granzyme induces cell death mediated CD8+ cytotoxic T cells and natural killer cells.

B CELLS AND HUMORAL ADAPTIVE IMMUNE RESPONSE

B cells have an essential role in host defense via the production of the antibody-mediated immune response to microorganisms. Persons lacking B cells fail to produce antibodies and are prone to serious infectious diseases.

- Major players of humoral immune response are B cells, which develop in the bone marrow and undergo maturation in the spleen. Just like T cells, B cells also learn not to react to body's own antigens, which do not react to self-molecules, hence are eliminated or ignored.
- Similar to T cells, B cells are formed in billions of variations, each carrying a unique surface receptor protein, called B cell receptor (BCR), which are actually membrane-bound immunoglobulins. B cell receptor contains two chains: heavy chains and light chains. The existence of BCR variations means that the body already has all the antibodies, it can possibly synthesize right type of immunoglobulin from the start. For resource management purposes, it makes sense not to produce all of antibodies in large quantity.
- Antigen-binding site on immunoglobulin is made of variable (V), diversity (D), joining (J) protein segments encoded by genes of same name: heavy chains (all three segments) and light chains (V, J segments).
- The majority of mature B cells, namely the follicular B cells circulate and enter into secondary lymphoid tissues/organs, the same locations as mature T cells, where B cells expect encounter with the pathogens. T cells and B cells are usually separated into defined T cell and B cell zones within secondary lymphoid tissues/organs.
- Upon antigen recognition by the membrane-bound receptors, B cells proliferate to increase their numbers and differentiate to secrete their specific antibody as one of the five immunoglobulin classes: IgG, IgA, IgM, IgD and IgE.
- Activated B cells undergo first rounds of proliferation and differentiation, giving rise to the first batch of plasma cells producing mainly IgM and small quantity of IgD antibodies. Some of activated B cells committed to become memory B cells.
- Second batch of plasma cells produces better antibodies and live longer than the first batch. These plasma cells synthesize antibodies of different classes (predominantly IgG) which neutralize the pathogen in many different ways. Upon re-exposure to the pathogen, memory B cells mount a much faster immune response. Plasma cells are formed within hours, producing huge amounts of the best possible antibody within days, destroying the pathogen so quickly that no signs of illnesses are noticeable.

Pathology Pearls: Stages of B Cell Development

- Both B cells and T cells are derived from pluripotent hematopoietic stem cell (HSC) in the bone marrow, which differentiates to lymphoid progenitor cell then to precursor B cells and T cells.
- B cell starts maturation in bone marrow and complete maturation in spleen through various developmental stages: pro-B cell, pre-B cell, immature B cell, naïve B cell and mature B cell.
- Pro-B cells undergo the rearrangement of immunoglobulin heavy chains. Pre-B cells express pre-B cell receptor (BCR) for successful production of a complete heavy chain.
- Signals through the pro-B cell to pre-B cell stage facilitate the survival and proliferation of pre-B cells.
- Pre-B receptor signaling inhibits further heavy locus rearrangement and enforces allelic exclusion.
- The functional rearrangement of immunoglobulin (Ig) light chain genes results in the expression of cell surface IgM of immature B cells.
- Immature B cells arriving in the spleen turnover rapidly and require cytokines and positive signals through B cell receptor for maturation and long-term survival.
- Immature B cells that express autoreactive BCR with a high-affinity change their specificity or immature B cells are deleted (central tolerance) before they leave the bone marrow.
- Receptor editing generates a different light chain and changes the specificity of the BCR.
- B cells that recognize self-antigens in the bone marrow or that fail to generate non-self-reactive BCR might undergo death by apoptosis.
- Mature B cells that recognize self-antigens in peripheral lymphoid tissues in the absence of signals from CD4+ helper T cells become unresponsive (anergy) or undergo death by apoptosis (peripheral tolerance).
- In the follicles of secondary lymphoid organs, naïve B cells recognize rapid proliferation, forming a germinal center.
- Activated B cells in germinal centers differentiate into long-lived antibody secreting plasma cells or memory B cells. B-1 cells are innate lymphocyte subset of B cell lymphocytes that arise early in development.

B CELL ACTIVATION AND DIFFERENTIATION

Mature naïve B cells form a primary follicle in the cortical region of lymph node. When the B cell encounters and binds an antigen, it changes its structure and functions. Interaction of antigen and B cell leads to proliferation of B cell, generating several clones resulting in formation of germinal center. The lymphoid follicle is now called a secondary lymphoid follicle.

- Series of events occur following antigen binding that lead to B cell activation. Ig-α, Ig-β and CD19 are intracellular side chains of the B cell receptors that cluster when two B cell receptors are cross-linked to an antigen.
- BTK, FYN and LYN proteins phosphorylate tyrosine kinase residues on immunoreceptor tyrosine bases activation motif (ITAM) units that lead to synthesis of transcription factors like nuclear factor κB-light chain enhancer of activated B cells (NF-κB), nuclear factor of activated T cells (NFAT cells). NF-κB and NFAT transcription factors increase gene expression coding for cytokines (IL-1, IL-2, IL-6, TNF-α) and upregulation of BCL-2 antiapoptotic cell surface markers.
- Complement fragment C3d can bind an antigen and then can be bound by molecule CD21 complement receptor type 2 (CR2) on a B cell. B cell can also be activated, when B cell has a receptor that is bound to an antigen.
- Activated B cell differentiates into plasma cells that secrete their specific antibody as one of the five immunoglobulin classes: IgG, IgA, IgM, IgD and IgE. Activation of B cells and immunoglobulins secreting plasma cells is shown in Fig. 4.35.

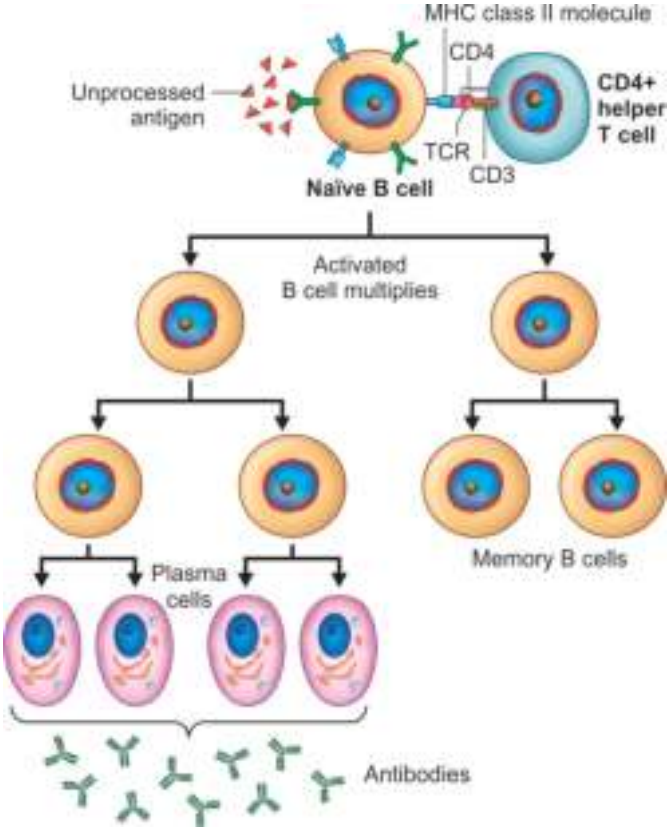


Fig. 4.35: Activation of B cells and immunoglobulins secreting plasma cells. Before a B cell can be activated by CD4+ helper T cell, the B cell must bind to receptor, endocytose and process the same antigen. B cell uses MHC class II molecule to present the processed antigen to CD4+ helper T cell. T cell receptor binds to the MHC class II molecule/antigen complex. There is costimulation of the B cell by CD4+ helper T cell. There is costimulation by cytokines released by CD4+ helper T cell. B cell undergoes proliferation resulting in formation of B memory cells and antibody secreting plasma cells.

- B cell presents an antigen on major histocompatibility complex (MHC) class II molecule to a CD4+ helper T cell. On activation, CD4+ helper T cell expresses CD4L on its surface, which binds CD40 on the B cell. CD40L and CD40 binding leads to expression of a cytokine receptor on B cell and causes T cell to release cytokines IL-4, IL-5 and IFN-γ. The type of cytokine determines what type of antibody the B cell will produce by activation-induced cytidine deaminase. IL-4 and IL-5 cytokines participate in synthesis of IgE. IFN-γ participates in synthesis of IgG.
- CD4+ helper T cell transmits additional signals in the form of interleukins and B cell growth factors. The linked receptors and the chemical stimuli serve to activate B cell. Such activated signals increase in cell metabolism, resulting in cell enlargement, proliferation and differentiation. Immunophenotyping of B cells include CD19, CD20, CD21 and CD22.
- Activated B cell undergoes numerous mitotic divisions, which expand the clone of B cells bearing this specificity and produce memory B cells and antibody secreting plasma cells. Memory B cells are persistent, long-term memory B cells that can react with the antigen on future exposures.
- The plasma cells are short lived, active secretory cells that synthesize and release antibodies. These antibodies (here is IgM) have same specificity as the immunoglobulin (Ig) receptor and circulate in the fluid compartments of the body, where they react with the same antigens and microbes.
- Immunoglobulin is a large Y-shaped protein molecule consisting of four polypeptide chains—two identical light chains and two identical heavy chains bound by disulfide bonds. Each chain of immunoglobulin consists of a variable region (V) and a constant region (C). The variable regions of light and heavy chains with ends form the antigen-binding fragments (Fabs) that bind with a unique specificity to an antigen. The crystalline fragment (Fc) determines the location and function of the antibody molecule. Different immunoglobulin classes engage different mechanisms to neutralize antigen.

SYNTHESIS OF IMMUNOGLOBULIN

Immunoglobulins are synthesized by three mechanisms: (a) T cell-dependent B cell activation and immunoglobulin synthesis, (b) T cell-independent B cell activation and immunoglobulin synthesis, and (c) collaboration of antigen-presenting cells (**APCs**), B cells and T cells and immunoglobulins synthesis. CD4+ helper T cell-dependent and -independent B cell activation and immunoglobulin synthesis are shown in Fig. 4.36A and B.

- In T cell-dependent B cell activation and immunoglobulin synthesis, B cell recognizes and internalizes an antigen and presents it to CD4+ helper T cell that is specific to the same antigen. CD4+ helper T cell interacts with the antigen presented by B cell, which activates and stimulates T cell to induce the production of cytokines, that activate the B cells. The activation of B cell triggers proliferation and differentiation into B cells and plasma cells that produce different classes of antibodies as a result of class switching. Memory B cells are also produced.
- In T cell-independent B cell activation and immunoglobulin synthesis, antigens have repeated epitopes that can induce B cell recognition and activation without involvement of CD4+ helper T cells. A second signal, such as interaction of toll-like receptors (TLRs) with pathogen-associated molecular patterns (PAMPs), is also required for activation of B cell.

CD4+ Helper T Cell-dependent B Cell Activation and Immunoglobulin Synthesis

Costimulatory signaling molecules are needed for B cell activation. Once the antigen is expressed in the membrane of B cells, both B cells and CD4+ helper T cells come in contact with each other that leads to release of cytokines especially IL-1 and IL-6. The collaboration between B cells and CD4+ helper T cells is essential for the initiation of B cell growth and differentiation into immunoglobulins-secreting plasma cells. Immunoglobulin production initiates with IgM. If antigenic stimulus continues, the constant region of IgM is changed to IgG, IgE or IgA but the variable region that binds to the antigen will remain.

CD4+ Helper T Cell-independent B Cell Activation and Immunoglobulin Synthesis

B cells can produce immunoglobulins by antigenic stimulus without the collaboration of CD4+ helper T cells and antigen-presenting cells (APCs). These antigens are present in some bacteria in the form of polysaccharides or lipopolysaccharides but never proteins, which are large polymeric molecules with repeating antigenic determinants. Bacterial antigens can react directly with B cell receptor (BCR) by cross-linking the cell surface immunoglobulin stimulating several BCRs at the same time, and so antigenic stimulus initiates immunoglobulin production especially IgM but not any other immunoglobulin. Once activated the B cell proliferates into antibody secreting plasma cells.

Immunoglobulin Synthesis Through Collaboration of APCs, B Cells and T Cells

Antigens can also bind to antigen-presenting cells (e.g. macrophages and dendritic cells), which express

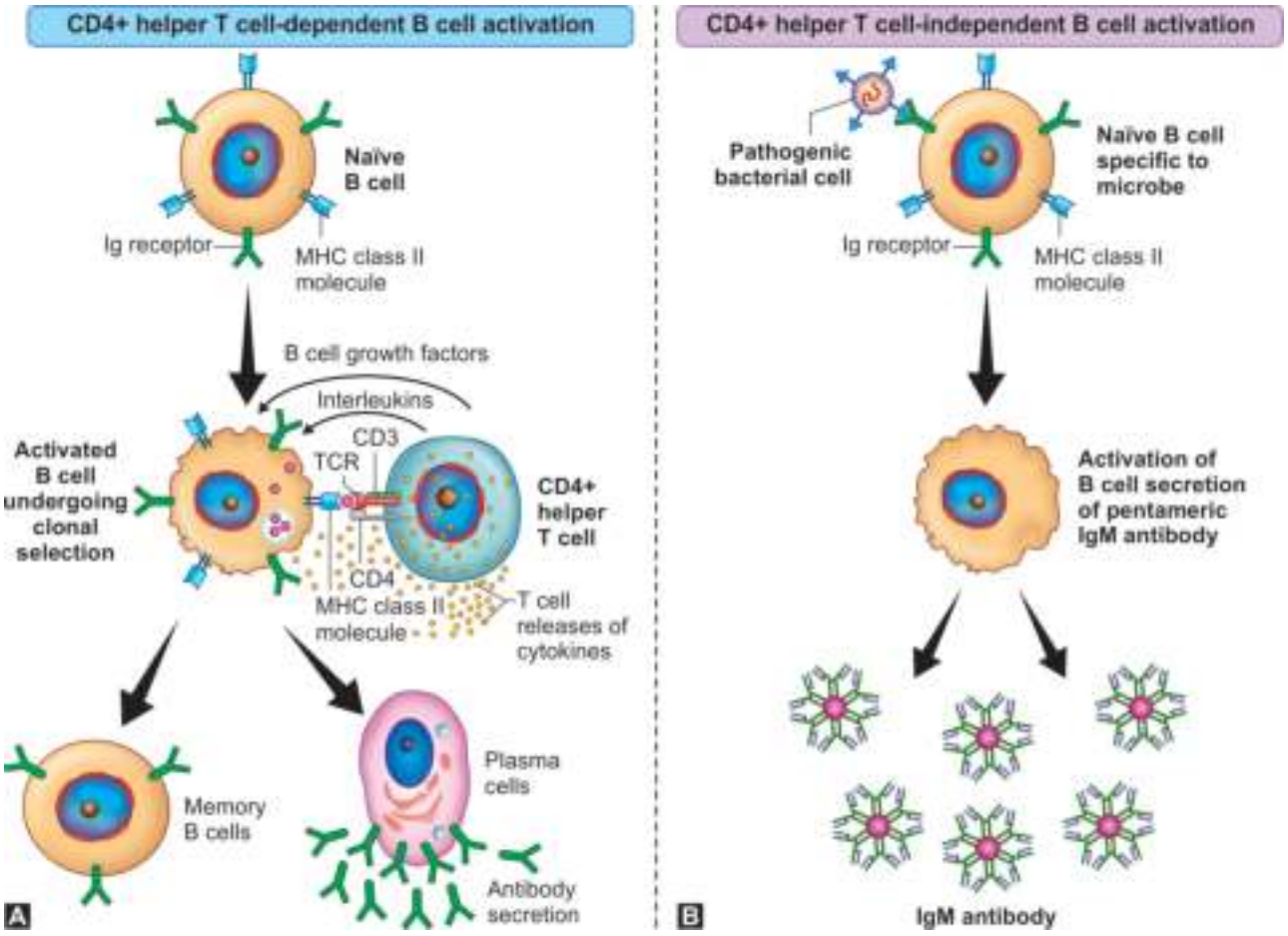


Fig. 4.36A and B: Sequence of events in T cell activation and differentiation into different types of T cells. (A) CD4+ helper T cell-dependent B cell activation: after initially binding an antigen to the B cell receptor (BCR), the B cell internalizes the antigen and presents it on MHC class II. The CD4+ helper T cell recognizes the MHC class II molecule–antigen complex and activates the B cell. As a result, memory B cells and plasma cells are made. (B) CD4+ helper T cell-independent B cell activation: naïve B cell specific to pathogenic bacteria gets activated that results in IgM synthesis.

MHC class II molecule in their membranes. Macrophages and dendritic cells present antigen (epitopes) to the CD4+ helper T cells.

- Antigen is phagocytosed inside a vesicle and transported to the lysosomes, where antigen will be degraded to fragments by different lysosomal enzymes. Fragments of the degraded antigen will bind to MHC (HLA) class II molecule and are transported to the macrophage membrane, where antigenic fragment will be recognized by CD4+ helper T cells. It should be noted that T cell receptor (TCR) cannot recognize antigen, which is not associated with HLA class II molecule.
- Activation of CD4+ helper T cells requires the performance of antigen-presenting cells (APCs) and MHC (HLA) class II molecule. When antigen-presenting cells (APC) and CD4+ helper T cells interact, both cells are activated that synthesize cytokines, which lead to stimulation and clonal proliferation of CD4+ helper T cells. Stimulation of CD4+ helper T cells is mediated by MHC class II molecule and the different epitopes of the same antigen and enhanced by the release of IL-4.
- At this stage, CD4+ helper T cells can stimulate B cells for immunoglobulin production. Some of the B cells are transformed into plasma cells which secrete immunoglobulins, and some other B cells remain as memory B cells. Memory B cells have long life span, i.e. regulated by the BCL-2 gene, which is present only in the memory B cells.

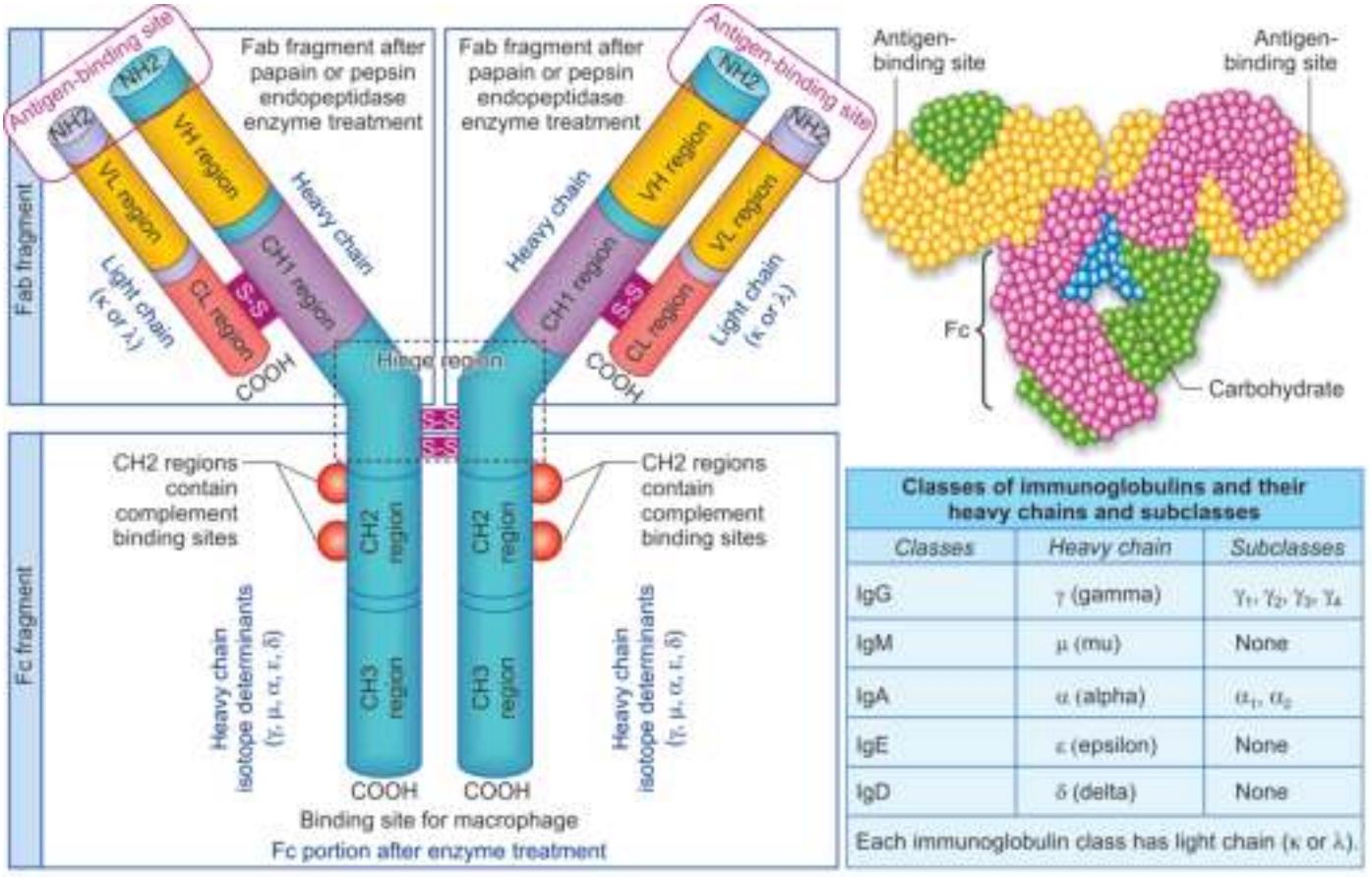
CLASSES OF IMMUNOGLOBULIN

CD4+ helper T cells stimulate B cell differentiation and antibody secreting plasma cells. Plasma cells produce five classes of immunoglobulins (antibodies), which differ in size and function. These include IgG, IgA, IgM, IgD and IgE.

- Depending on the class of immunoglobulin involved, their operations include: agglutination and lysis (IgM), opsonization of pathogens for phagocytosis both directly via Fc receptors and indirectly via

complement system activation by classical pathway, blocking entry the microorganisms from respiratory tract, gastrointestinal tract, eyes and urinary tract (IgA), killing of virus infected cells via antibody-dependent cell cytotoxicity (ADCC) and neutralization of bacterial toxins.

- The Fab (antigen-binding fragment) of antibody binds to specific antigen on the pathogen. The Fc (fragment crystallizable) is identical in all immunoglobulins of the same class/subclass that activates complement system leading to chemotaxis of phagocytes to the site of infection and increased capillary permeability in order to facilitate immunoglobulins passage from the blood circulation to the tissues.
- IgG is present throughout the tissue fluids, which provides long-term immunity. IgA provides protection in body secretions. IgM predominates in plasma. IgD is expressed on B cells as an antigen receptor. IgE binds to tissue cells and induce inflammation. Basic structure of immunoglobulin molecule classes is shown in Fig. 4.37. Characteristics of immunoglobulins' classes are shown in Fig. 4.38. Characteristics of immunoglobulins' classes and their functions are given in Table 4.30.



Classes of immunoglobulins and their heavy chains and subclasses

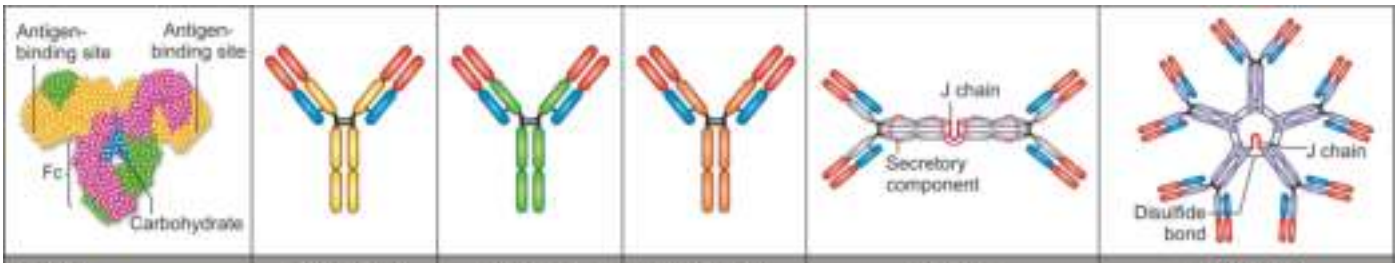
Classes	*Heavy chain*	*Subclasses*
IgG	γ (gamma)	γ_1, γ_2, γ_3, γ_4
IgM	μ (mu)	None
IgA	α (alpha)	α_1, α_2
IgE	ε (epsilon)	None
IgD	δ (delta)	None

Each immunoglobulin class has light chain (κ or λ).

Immunoglobulin structure

1. Immunoglobulin consists of 2 heavy chains and 2 light chains joined together by disulfide bonds.
2. Each heavy chain consists of a variable region (VH) and constant region encompassing functional domains named CH1, CH2 and CH3. Each light chain has a variable region (VL) and a constant region (VC).
3. The carboxy (–COOH) end of both the heavy chains and light chains is highly conserved, the amino acid sequence is very similar for all immunoglobulins of the same class: IgG, IgM, IgA, IgE or IgD.
4. Both the heavy chains and light chains at the amino (–NH2) end of proteins are highly variable, which allows for the development of specific antigen-binding sites.
5. Enzyme will cleave the immunoglobulin molecule at several points in the hinge region, resulting in three fragments: one Fc fraction crystallizable and two Fab fragments (antigen-binding fraction). Hinge region is a flexible amino acid stretch in the central portion of the heavy chains of the IgG or IgA immunoglobulin classes, which link these two chains by disulfide bonds.
6. Papain cleaves the heavy chain CH2 domains above the disulfide bond that connect heavy chains and yields three different fragments. Pepsin endopeptidase enzyme cleaves IgG heavy chains at a point below the disulfide bond that links two heavy chains. The resulting fragment consists of two Fab fragments linked together by heavy chain disulfide bonds.

Fig. 4.37: Basic structure of immunoglobulin molecule. Immunoglobulin contains two identical heavy chains and two identical light chains. Bonded together by disulphide bonds, it forms the Y-shaped molecule.



Forms	Monomer	Monomer	Monomer	Dimer	Pentamer
Heavy chain Ig	**IgG**	**IgD**	**IgE**	**IgA**	**IgM**
Heavy chain isotype	Gamma (γ)	Delta (δ)	Epsilon (ε)	Alpha (α)	Mu (μ)
J chain	–	–	–	Present	Present
Secretory component	–	–	–	Present	–
Number of antigen-binding sites	2	2	2	4	10
Molecular weight (daltons)	1,50,000	1,80,000	2,00,000	3,85,000	9,00,000
Antigen-binding	Present	Present	Present	Present	Present
Antibody-dependent cell cytotoxicity	Present	–	–	–	–
Opsonization	Present	–	–	–	–
Placental transport	Present	–	–	–	–
Classical complement pathway	Present	–	–	–	Present
Mature B cell receptor	–	Present	–	–	Present
Memory B cell receptor	Present	–	Present	Present	–
Triggers mast cell degranulation	–	–	Present	–	–

Fig. 4.38: Characteristics of immunoglobulin classes. IgG is most abundant immunoglobulin in serum and extracellular fluids, which combats pathogens and toxins within body tissues. IgG crosses placenta. IgA is most abundant immunoglobulin in mucous membranes, which protects external surfaces of the body. IgM is the first immunoglobulin produced during an immune response, which is present both in fluids and secretions. IgD immunoglobulin is found on the surface of B cell plasma membrane. IgA and IgM have an additional component referred to as the secretory component, which is required to transport across the epithelial cell barrier. IgE mediates immediate hypersensitivity reactions.

Table 4.30 Characteristics of immunoglobulins' classes and their functions

Characteristics	IgG	IgD	IgE	IgA	IgM
Composition	Monomer	Monomer	Monomer	Dimer, monomer, secretory	Pentamer
Heavy chains	Gamma (γ)	Delta (δ)	Epsilon (ε)	Alpha (α)	Mu (μ)
Subclasses	IgG1, IgG2, IgG3, IgG4	Not applicable	Not applicable	IgA1, IgA2	Not applicable
Number of antigen binding sites	2	2	2	2 or 4	10
Molecular weight (daltons)	150,000	180,000	200,000	385,000	900,000

Contd...

Table 4.30 Characteristics of immunoglobulins' classes and their functions (*Contd...*)

Characteristics	IgG	IgD	IgE	IgA	IgM
Present in	Blood, lymph and intestine	Surface of receptor on B cells	Basophils and mast cells	Glandular secretions (sweat, tears, saliva, mucus of gut, and digestive juices)	Blood and lymph
Total antibody % in serum	80%	0,001%	0.002%	13%	6%
Average life span in serum (days)	23	3	2.5	6	5
Crosses placenta	Yes	No	No	No	No
Fixation of complement or not	Yes	No	No	No	Yes
Binding of immunoglobulin to various cells	Phagocytes	Not applicable	Mast cells and basophils	Epithelial cells	Not applicable
Biological functions	Long-term immunity memory antibody neutralizing toxins by activating complement and viruses	Receptor on B cells for antigen recognition	Antibody against allergy and parasitic infections	Secretory antibody on mucous membrane giving local protection against pathogens	First antibody to be secreted synthesized in response to antigen and serving as B cell receptor

Pathology Pearls: Structure of Immunoglobulin

Heavy Chains and Light Chains in Immunoglobulin

- Immunoglobulin contains two identical heavy chains and two identical light chains, where the light chains can consist of either a κ- or λ-chain.
- The hinge region is a flexible amino acid stretch in the central part of the heavy chains of the IgG and IgA classes, which links these two heavy chains and light chains by disulphide bonds.
- Heavy chain isotopes include monomer (IgG), dimer (IgA) and pentamer (IgM). J chain connects monomers to form dimers and pentamers. J chains are synthesized by B cells and plasma cells. Secretory piece might be attached to a dimer is it passes through epithelial cells commonly found in saliva and tears.
- Papain digests IgG into two Fab fragments, each of which can bind antigen and a single Fc segment.

Variable and Constant Regions in Immunoglobulin

- Heavy and light chains of immunoglobulins can be separated functionally into variable (V) domains that bind antigens and constant (C) domains that specify effector functions such as activation of complement system or binding to Fc receptors.
- Constant regions contain the same amino acid sequences, while variable regions have different amino acid sequences.
- Variable domains are created by means of a complex series of gene rearrangement events, and can then be subjected to somatic hypermutation after exposure to antigen to allow affinity maturation.

Framework and Hypervariable Regions of Immunoglobulin

- Variable region of immunoglobulin is composed of two parts: the framework and hypervariable region. The framework region is structurally similar. But hypervariable regions are extremely diverse.
- Hypervariable regions allow for the synthesis of variety of antibodies, and in turn allow the immune system to recognize and protect against a wide variety of antigens.

IgG Immunoglobulin

IgG immunoglobulin has a molecular weight of 150,000 daltons with two antigen-binding sites, which makes up 75% of antibodies that is present throughout the tissue fluids and bloodstream.

- IgG has four subclasses: IgG1, IgG2, IgG3 and IgG4, which differ in constant regions. IgG serves as opsonin and activates classical complement system pathway. IgG has two binding sites, which activates complement system.
- Of all the immunoglobulins, only IgG can cross placental barrier, which provides immunity to fetus. IgG provides long-term immunity, which is predominant antibody of the secondary response.
- Decreased levels of IgG can be manifested in primary or secondary IgG deficiencies. Significant increase in IgG levels occur in the settings of inflammatory diseases (hepatitis, rubella, and infectious mononucleosis), collagen disorders (rheumatoid

Table 4.31 Properties of human IgG subclasses

Property	IgG1	IgG2	IgG3	IgG4
Normal serum concentration (mg/dl)	540 mg/dl	210 mg/dl	58 mg/dl	60 mg/dl
Half-life in days	21	20	7	21
Fc binding capacity on phagocytes	+	Absent	+	+/–
Activation of classical complement pathway	++	+	+++	–
Capacity to cross placenta	+++	+	++	+/–
Antibody activity	Protein antigens (e.g. diphtheria and tetanus)	Protein antigens (e.g. Pneumococcus, *Haemophilus influenzae*)	Protein antigens (e.g. diphtheria and tetanus)	Protein antigens (e.g. Pneumococcus, *Haemophilus influenzae*)

arthritis and systemic lupus erythematosus) and hematologic disorders (polyclonal gammopathy, monoclonal gammopathy, monocytic leukemia and Hodgkin disease). Properties of human IgG subclasses are given in Table 4.31.

IgE Immunoglobulin

IgE immunoglobulin has molecular weight of 200,000 daltons with two antigen-binding sites. IgE is present in small amount and largely bound to mast cells and to basophils in tissues. Subsequent exposure to antigen leads to release of histamine resulting in type 1 hypersensitivity reactions. IgE production is induced by IL-4. IgE immunoglobulin mediates immediate hypersensitivity reactions that are responsible for symptoms of hay fever, bronchial asthma, hives and anaphylactic shock. IgE binds to Fc receptors on the membranes of blood basophils and tissue mast cells. Cross-linkage of Fc receptor bound IgE molecule by antigen induces degranulation of basophils and mast cells. A variety of pharmacologically active chemical mediators present in the granules are released giving rise to clinical manifestations.

IgD Immunoglobulin

IgD immunoglobulin is monomer with molecular weight of 180,000 daltons and present in small amount in blood. IgD is expressed on B cells as an antigen receptor, which participates in activation of B cells. IgD is found alongside IgM antibody, that signals maturation of B cells.

IgA Immunoglobulin

IgA immunoglobulin has molecular weight of 385,000 daltons with four antigen-binding sites, which has two subclasses: IgA1 and IgA2, which differ in constant region. IgA provides protection in body secretions and bloodstream.

- IgA serves as opsonins for neutrophils, eosinophils and some macrophages. It is major class of antibody in the mucous membranes in gastrointestinal tract, respiratory tract, saliva and tears. IgA does not activate complement system and its half-life is six days.
- Decreased levels of IgA can be manifested in primary or secondary IgG deficiencies, which can occur in the settings of infectious diseases (tuberculosis and actinomyces), collagen disorders (rheumatoid arthritis and systemic lupus erythematosus), hematologic disorders (polyclonal gammopathy, monoclonal gammopathy) and liver diseases (chronic active hepatitis and Laënnec's disease).

IgM Immunoglobulin

IgM is the first class of antibody to be formed by B cell in response to an initial encounter with an antigen (e.g. primary immune response), which has molecular weight of 900,000 daltons with 10 antigen-binding sites predominantly present in plasma.

- IgM penetrates poorly into the tissues on account of larger size, which works against carbohydrate and lipid antigens.
- IgM neutralizes microorganisms especially viruses in intravascular compartments aided by its 10 binding sites.
- IgM activates complement system and lyses microorganisms or remove antigen–antibody complexes by complement receptors on phagocytic cells.

Pathology Pearls: Free Immunoglobulin Functions

Free immunoglobulin functions have binding sites that affix tightly to an antigen and hold it in place for agglutination of bacteria and viruses, opsonization of bacteria to facilitate their subsequent phagocytosis by phagocytes (neutrophils and macrophages), complement fixation (activation of complement system), agglutination of foreign cells, and neutralization (block attachment) of viruses and toxins released by bacteria. Summary of the

actions of free immunoglobulins is shown in Fig. 4.39A to F. Specific immunoglobulins used in diagnosis are given in Table 4.32.

Opsonization

Antigen (microbe) is covered with antibodies that enhances its ingestion and lysis by phagocytic cells.

Neutralization

IgG inactivates viruses by binding to their surface and neutralize toxins by blocking their active sites.

Agglutination

- Antibodies cause antigens (microbes) to clump together. IgM is more effective than IgG (bivalent).
- Agglutination of red blood cells (hemagglutination) is used to determine ABO blood types and to detect influenza and measles viruses.

Complement System Activation

Both immunoglobulins (IgG and IgM) trigger the complement system which results in cell lysis and inflammation.

Antibody-dependent Cell Cytotoxicity (ADCC)

- Antibody coated target cell is recognized by Fc receptors of lymphoid cells.
- Binding occurs between lymphoid and antibody coated target cells leads to lysis of target cells by natural killer cells. This mechanism is known as antibody-dependent cytotoxicity.

Table 4.32 Specific immunoglobulins used in diagnosis

Feature	IgG	IgM	IgA
Agglutination	+	+++	Negative
Complement fixation	+	+++	+
Time of appearance after first exposure to antigen	3–7 days	2–5 days	3–7 days
Time to reach peak titer (days)	7–21 days	5–14 days	7–21 days

PRIMARY AND SECONDARY IMMUNE RESPONSES

The first exposure to a pathogen or antigen to the immune system produces a primary immune response with a gradual increase in antibody titer low intensity. Subsequent exposure to the same pathogen or antigen elicits a highly intensified secondary immune response or anamnestic response due to the presence of memory of the first encounter. Primary and secondary immune responses are carried out with the help of T cells and B cells. Primary and secondary immune responses are shown in Fig. 4.40. Differences between primary and secondary immune responses are given in Table 4.33.

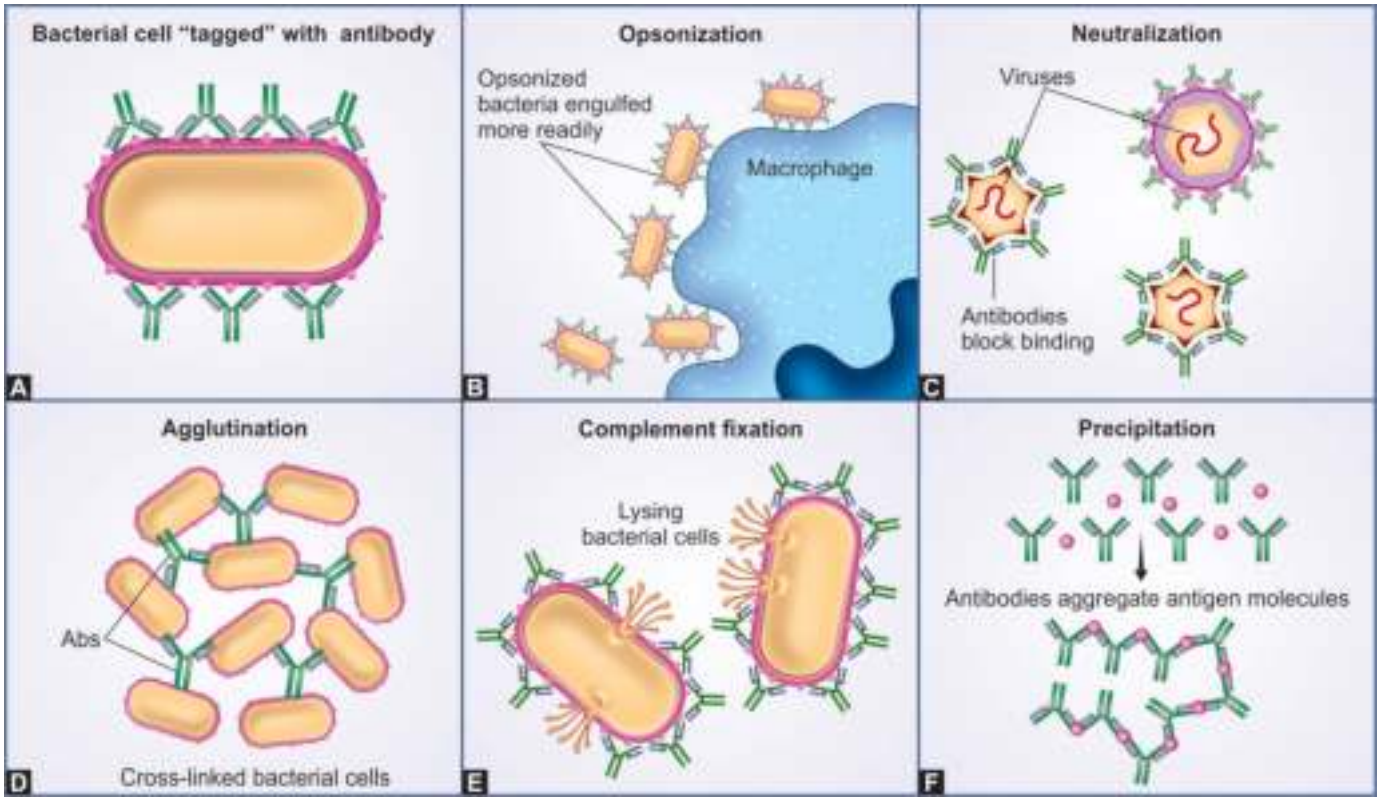


Fig. 4.39A to F: Functions of immunoglobulins. Immunoglobulins are glycoprotein molecules produced by plasma cells. Immunoglobulins act by recognizing and binding to specific antigens, such as bacteria or viruses, and aiding in their destruction by opsonization, neutralization, agglutination, and complement activation.

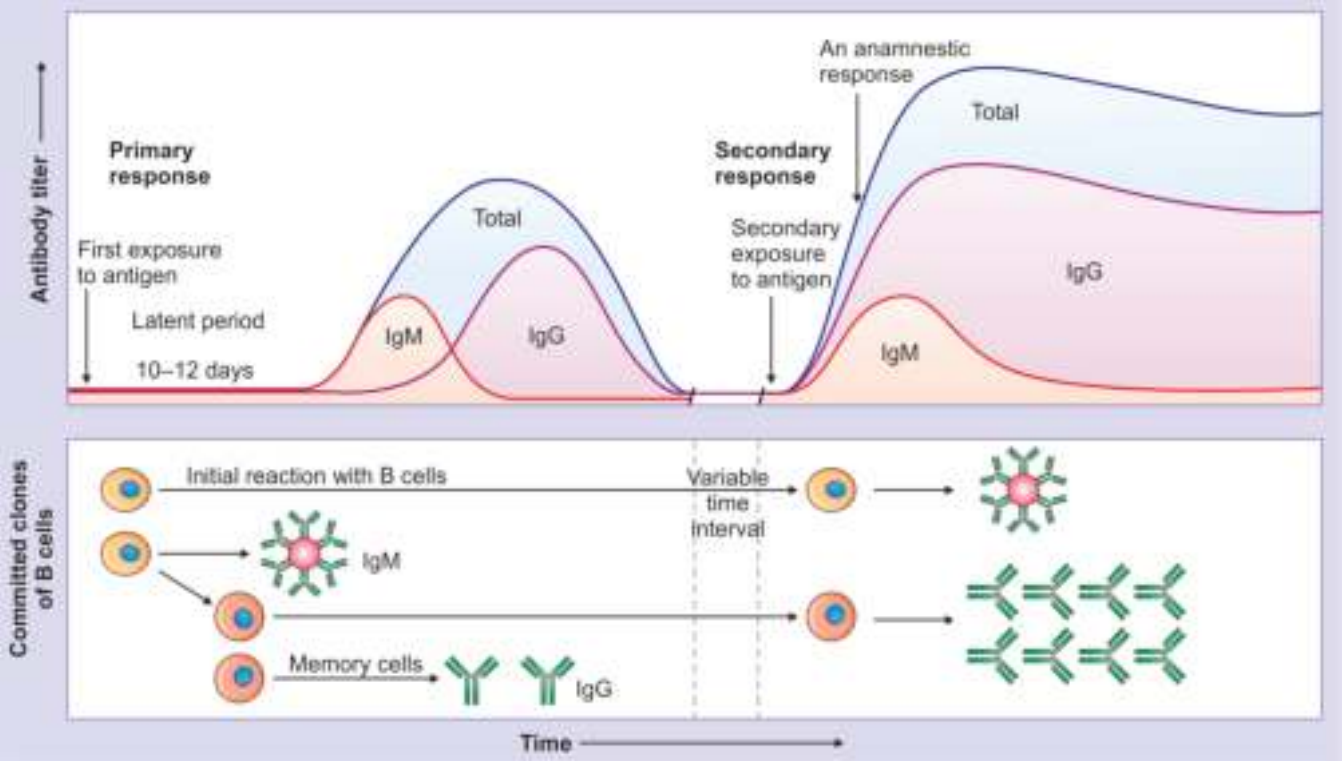


Fig. 4.40. Primary and secondary immune responses. The pattern of antibody titer and subclasses as monitored during initial and subsequent exposure (top). A view of the B cell responses that account for this pattern (bottom). It shows clonal selection by production of memory B cells and the predominant antibody class occurring during first and second contact with antigen. Note that residual B memory cells remaining from the primary response are ready to act immediately, which produces the rapid rise of antibody levels in the secondary period.

Table 4.33 Differences between primary immune response and secondary immune response

Primary Immune Response	Secondary Immune Response
First exposure to antigen results in synthesis of IgM	Second exposure to same antigen results in synthesis of IgG
Animal takes longer duration to respond	Animal takes shorter duration to respond
Response declines rapidly	Response lasts for longer duration
Immune response feeble	Immune response strong

Primary Immune Response

When an antigen is presented to the immune system for the first time, a primary immune response is produced in the lymph nodes and spleen. Memory B cells and T cells are produced during the primary immune response, which is characterized by slow immune response in which characterized by slow short-lasting immune response, IgM is predominating over IgG. Memory cells will remember each epitope's structure of antigen for future infections.

Secondary Immune Response

When an individual has been in contact with any antigen, and has produced memory B cells, if the same antigen enters host again, a secondary immune response is produced in the bone marrow followed by the lymph nodes and spleen. Secondary immune response is faster, more effective and long-lasting. During secondary immune response IgM levels are same as in primary immune response. But IgG levels are much higher and remain longer. Other immunoglobulins are also produced, such as IgA and IgE in the secondary immune response.

Pathology Pearls: Decline in Immune Responses and Immunologic Memory

- CD4+ regulatory T cells (Treg cells) help to end an immune response. After elimination of infectious microbe, majority of activated CD4+ helper T cells undergo apoptosis thus returning the immune system to its basal state called homeostasis.
- Decline of immune response occurs due to cessation of essential stimuli by infectious microbe. Activation of lymphocytes results in generation of long-lived memory cells which may survive for years after the injurious microbe.
- Immunological memory is the ability of the immune system recognize an antigen that has previously encountered and initiate quick and robust immune response.

SELECTED MONOCLONAL ANTIBODY-BASED DRUGS

Monoclonal antibodies (MABs) are pure forms of immunoglobulins produced in hybridoma cells, which may be tailored to high specifications and have numerous applications in medicine and research.

- Monoclonal antibodies can be hybridized with plant or bacterial toxin to form immunotoxin complexes that attach to a target cell and poison it. The most exciting prospect of this therapy is that it can target a specialized cancer stem cells and spare normal cells.
- Monoclonal antibody-based drugs are currently employed in the treatment of breast carcinoma, colorectal carcinoma, lung carcinoma, non-Hodgkin's lymphoma (NHL) and acute myelogenous leukemia. Selected monoclonal antibody-based drugs used in various disorders are given in Table 4.34.

Table 4.34 Selected monoclonal antibody-based drugs used in various disorders

Chemical Name of Drugs	Used in Treatment of Disorder
Transtuzumab	Breast carcinoma
Rituximab	Non-Hodgkin's lymphoma
Bevacizumab	Colorectal carcinoma and lung carcinoma
Ganitumab	Acute myelogenous leukemia
Omalizumab	Bronchial asthma
Infliximab	Crohn's disease
Palivizumab	Respiratory syncytial virus

At the end of a generic name, –mab indicates that the drug is a monoclonal antibody.

VACCINES AND IMMUNIZATION

Immunization is a process by which a person becomes protected against a specific disease through vaccination without becoming infected, which is based on the property of 'memory' of the immune system. **Edward Jenner** showed resistance to the specific disease because of presence of memory cells in cases of smallpox, which increases herd immunity, protection provided by mass immunity in a population.

- Vaccine is administered through needle injections or oral route to stimulate the body's immune response against diseases. Boosters (additional doses) are often required. Vaccination is performed for therapeutic purpose. Vaccination of patients at high-risk group requires specific vaccinations is given in Table 4.35.
- Active immunization is synonymous with vaccination and provides an antigenic stimulus that does not cause disease but can produce long-lasting, protective immunity.
- In passive immunization, preformed serum globulins and specific immune globulins pooled from donated serum are administered to achieve quick immune response in patients at risk for prevention of infection. Antiserum and antitoxins from animals are occasionally used. Antitoxin is administered against snake venom.
- Genetic engineering vaccine preparation techniques include cloning of antigens, recombinant attenuated microbes, and DNA based vaccines. Recombinant DNA technology has allowed the production of antigenic polypeptides of pathogen in bacteria or yeast on large scale, e.g. hepatitis B vaccine, which is prepared from yeast.

Table 4.35 Vaccination of patients at high-risk group require specific vaccinations

Chronic Medical Conditions related to Heart, Respiratory System, Kidneys and Liver	
▪ Pneumococcal polysaccharide vaccine	▪ Influenza vaccine
Immunosuppression State	
▪ Pneumococcal polysaccharide vaccine	▪ Influenza vaccine
Splenectomy or Spleen Dysfunction	
▪ *Haemophilus influenzae* type B vaccine (Hib vaccine) ▪ Pneumococcal polysaccharide vaccine	▪ Influenza vaccine ▪ Meningococcal C vaccine, then A, C W135, Y one month later
Hemodialysis of Patients	
Hepatitis B vaccine (HBV)	
Hemophilic Patients	
▪ Hepatitis B vaccine	▪ Hepatitis A vaccine
Injecting Drug Addicts	
▪ Hepatitis B vaccine	▪ Hepatitis A vaccine
Homosexual Men	
▪ Hepatitis B vaccine (HBV)	▪ Hepatitis A vaccine (HAV)

Pathology Pearls: Vaccination

- Live attenuated vaccines should not be used in immunocompromised patients.
- An attenuated vaccine contains microbes that replicate in host tissues but do not usually cause disease, e.g. polio live vaccine. Disease can occur in immunocompromised individuals (e.g. AIDS).

- An inactivated killed vaccine contains microbes that are unable to replicate, hence safe to use in all patients (e.g. polio, HAV and cholera vaccine).
- A **toxin** such as tetanus toxin is active and immunogenic and causes disease. A toxoid (e.g. tetanus toxoid) used in vaccination is inactive but is still immunogenic. Formalin is used for inactivation (diphtheria, pertussis and tetanus).
- Immunity to the polymer of ribose and ribitol-5-phosphate (PRP) capsule of *Haemophilus influenzae* is T cell-independent and the immune response is poor.
- The polymer of ribose and ribitol-5-phosphate (PRP) conjugated to tetanus toxoid is converted to a T cell-dependent antigen and is immunogenic as a conjugate vaccine (*Haemophilus influenzae* type B vaccine (Hib vaccine).

VACCINES

Vaccines help and protect children and adolescents from serious, often fatal illnesses.

- First generation vaccines are prepared by use of live and weakened or killed pathogens. Examples are smallpox vaccine and polio vaccine.
- Second generation vaccines are prepared by use of defined protein antigens or recombinant protein antigens such as hepatitis B surface antigen.
- Third generation vaccines are prepared from DNA that codes for specific proteins from a pathogen.

VACCINES PREPARATION

Vaccine is a preparation of antigenic proteins of pathogens or inactivated/weakened pathogens is introduced into the body. The antibodies produced against these antigens would neutralize the pathogen during actual infection.

- The vaccines also generate memory B cells and T cells that recognize the pathogen quickly on subsequent exposure.
- Louis Pasteur worked on cholera vaccine and later developed a vaccine against rabies. Other vaccines developed include diphtheria, tetanus, pertussis (whooping cough), tuberculosis (BCG) and hepatitis B vaccine.
- There is no effective vaccine in newly emergent diseases such as HIV/AIDS and malaria. Immunotherapeutic vaccines for malignant tumors—critically are needed as therapies.
- Strategies in vaccine design are shown in Fig. 4.41A to C. Comparison of attenuated vaccine, inactivated vaccine and DNA vaccine is shown in Table 4.36.

Pathology Pearls: Types of Vaccines

Live-attenuated Vaccines

Live-attenuated vaccines are prepared from weakened attenuated cells or viruses that are able to reproduce but have lost virulence.

Killed or Inactivated Vaccines

Killed vaccines are prepared by inactivation of viruses that do not reproduce disease but are antigenic.

Toxoid Vaccines

Toxoid vaccines are prepared by acellular or subunit components of microbes such as surface antigens or neutralized toxins.

DNA Vaccines

- There is new approach to immunization and immunotherapy by injecting piece of DNA containing the gene for the antigen of interest.
- There is generation of desired immune response (CD4+ helper T cells, CD8+ cytotoxic T cells, and B cells).
- Global use of DNA vaccines needs population safety.

DNA VACCINE

Vaccines against infectious agents excel at inducing antibodies and provide immune protection against most viruses and bacteria. Exceptions are intracellular organisms such as *Mycobacterium tuberculosis*, malarial parasite, *Leishmania donovani*, and HIV-protection depends more on cell-mediated immunity. There is new approach to immunization and immunotherapy by injecting piece of DNA containing the gene for the antigen of interest. There is generation of desired immune response (CD4+ helper T cells, CD8+ cytotoxic T cells, and B cells). Global use of DNA vaccines needs population safety.

Manufacture of DNA Vaccine

Direct introduction of a plasmid DNA encoding an antigenic protein is expressed within cells of the host. Naked DNA is simply DNA sequences inserted into bacterial plasmids (simple, extrachromosomal rings of DNA found in bacterial cells) and injected into the host. It is neither a chemical formulation nor a viral coat or envelope structure surrounds it. Preparation of DNA vaccine is shown in Fig. 4.42A to E. Currently approved bacterial vaccines are given in Table 4.37. Currently approved viral vaccines are given in Table 4.38.

- **Isolation of DNA:** DNA sequence coding for a specific protein is isolated from the genome of the infectious organism.

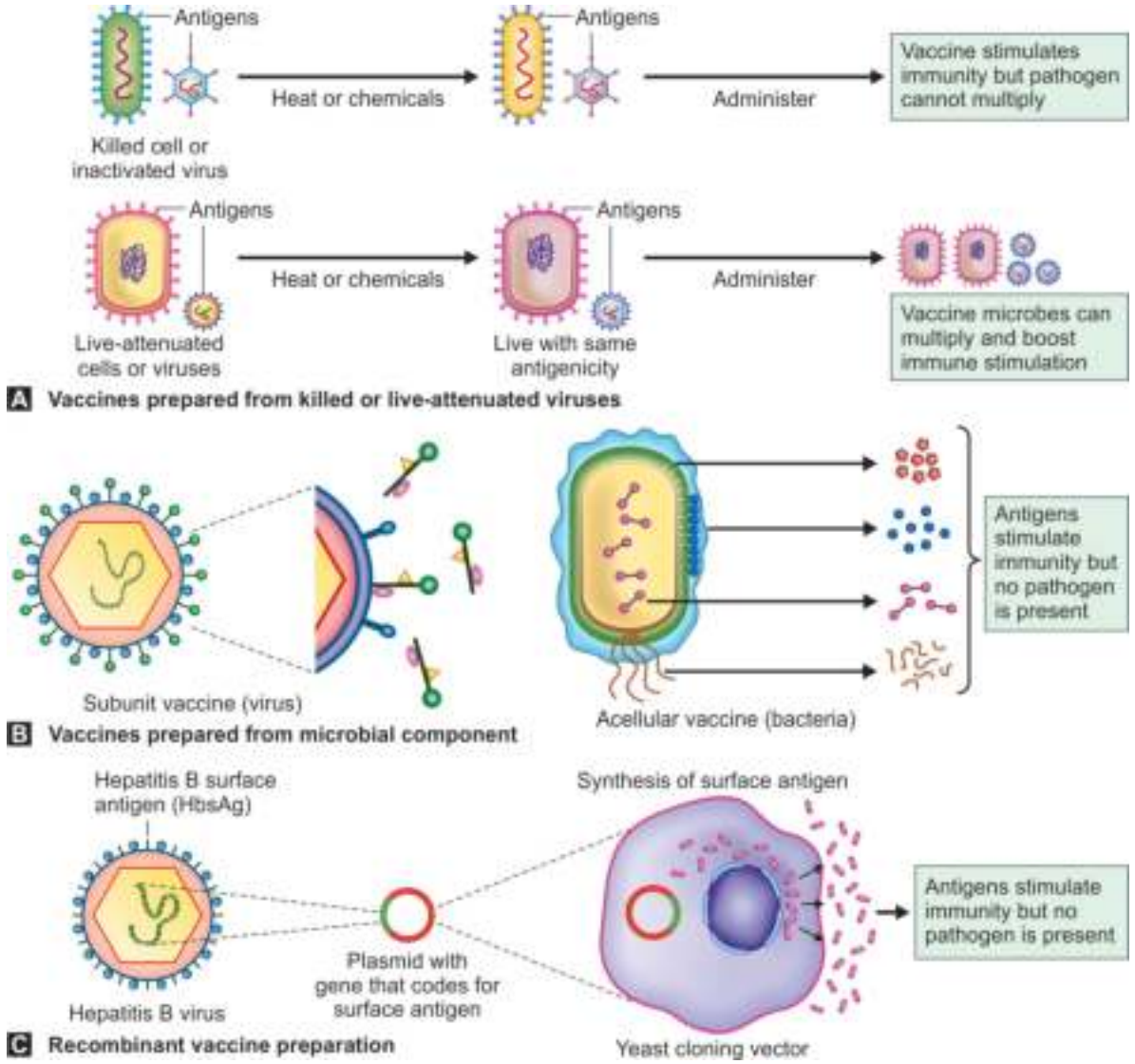


Fig. 4.41: Strategies in vaccine design. (A) Whole cell or viruses are killed or attenuated, (B) a cellular or subunit vaccines are prepared by disrupting the microbes to release various molecules or cell parts that may be isolated and purified, (C) recombinant vaccines are prepared by isolating a gene for the antigen from the pathogen (e.g. hepatitis B virus) and splicing into a plasmid. Insertion of the recombinant plasmid into a cloning host (yeast) leads to increased synthesis of viral surface antigen for vaccine preparation.

Table 4.36 Comparison of attenuated vaccine, inactivated vaccine and DNA vaccine

Characteristics	Attenuated Vaccine	Inactivated (Killed) Vaccine	DNA Vaccine
Production	Virulent human pathogen cultured through different hosts	Inactivation of virulent pathogen by chemicals, irradiation with γ-rays	Easily manufactured and purified
Booster dose requirement	Generally requiring only, a single booster	Requiring multiple boosters	Single injection of DNA vaccine sufficient
Relative stability	Less stable	More stable	Highly stable
Type of immune response	Humoral and cell-mediated immune response	Mainly humoral response	Humoral and cell-mediated immune response
Reversion tendency	May revert to virulent form	Cannot revert to virulent form	Cannot revert to virulent form

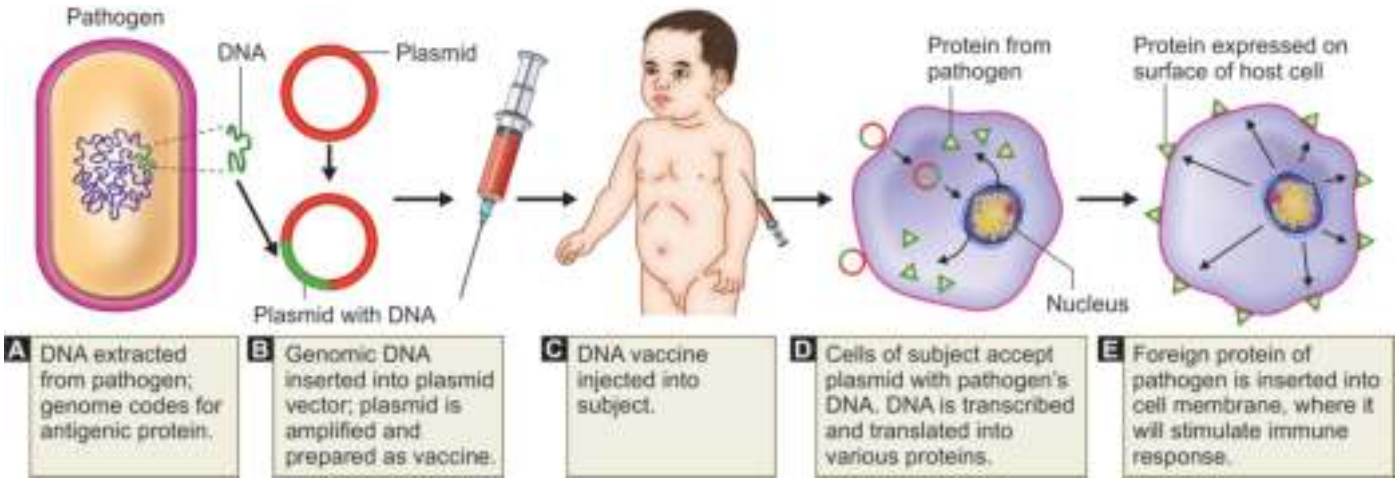


Fig. 4.42A to E: DNA vaccine preparation. DNA that codes for the protein is extracted from the pathogen genome. Extracted genomic DNA of pathogen is inserted into plasmid vector for amplification of DNA and preparation of vaccine. DNA vaccine is injected into the subject. Subject cells accept plasmid with extracted pathogen DNA. DNA is transcribed and transplanted into various proteins. Foreign protein of pathogen is inserted into cell membrane and stimulate immune response.

Table 4.37 Currently approved bacterial vaccines

Obtained from Bacteria	Routes of Administration	Recommended Usage
Vaccines containing killed whole bacteria		
Cholera	Subcutaneous route	Travelers
Plague	Subcutaneous route	Travelers
Vaccines containing live-attenuated bacteria		
Tuberculosis (BCG)	Intradermal route	High-risk persons
Typhoid	Oral route	Travelers
Acellular vaccines containing capsular polysaccharide		
Anthrax	Subcutaneous route	Military personals
Meningococci (meningitis)	Subcutaneous route	High risk infants and military personnel
Haemophilus influenzae (meningitis)	Intramuscular route	Infants and children
Pneumococci (pneumonia)	Intramuscular route/subcutaneous route	Immunocompromised persons
Pertussis containing recombinant protein	Intramuscular route	Newborn and children
Toxoid vaccines (formaldehyde-inactivated bacterial exotoxins)		
Diphtheria	Intramuscular route	Children
Tetanus	Intramuscular route	Children
Pertussis	Intramuscular route	Children
Botulism	Intramuscular route	Only in exposed persons working in laboratories

- **Eukaryotic promoter and terminator:** Eukaryotic promoter and terminator are added. Bacteria and viruses have different promoters for transcription than do eukaryotes. So, a eukaryotic promoter must be added so that a human cell can begin transcription. A terminator is also necessary—exact sequence for the protein is copied and nothing more.
- **Integration of DNA into plasmid vector:** Gene encoding antigenic protein of interest is cloned into a bacterial plasmid and engineered to augment the expression of inserted antigenic protein in mammalian cells. DNA is integrated into a plasmid vector.
- **Plasmid transformed into bacteria:** Plasmid is transformed into bacteria such as *E. coli*. Plasmid then becomes part of the bacteria's DNA, which has two major units: (a) Plasmid backbone contains origin of replication (ori) that delivers adjuvant, mitogenic activity and cloning sites for insertion of genes of interest. Antibiotic resistance gene confers antibiotic selected growth in *E. coli*. (b) Transcription unit is

Table 4.38 Currently approved viral vaccines

Obtained from Viruses	Routes of Administration	Recommended Usage
Vaccines prepared from inactivated virus		
Poliomyelitis (Salk)	Intramuscular route	Children (administered as first choice)
Rabies	Intramuscular route	Victims of animal bites
Influenza	Intramuscular route	High risk persons
Japanese encephalitis	Subcutaneous route	Endemic areas and laboratory persons
Hepatitis A	Intramuscular route	Travelers
Vaccines prepared from live-attenuated virus		
Adenovirus	Oral route	Military persons
Polio (Sabin)	Oral route	Children
Measles (rubeola)	Subcutaneous route	Children
Mumps	Subcutaneous route	Children
Rubella	Subcutaneous route	Children
Chickenpox (varicella)	Subcutaneous route	Children
Rotavirus (Rota Teq)	Oral route	Infants at risk
Smallpox (live vaccinia virus, not attenuated variola)	Multiple punctures	Since 2003, vaccine administered for voluntary health workers
Yellow fever	Subcutaneous route	Travelers, military persons
Influenza	Inhalation route	Same for inactivated
Subunit viral vaccines		
Hepatitis B*	Intramuscular route	Children from birth and health workers at risk
Influenza	Intramuscular route	Same for inactivated
Human papillomavirus*	Intramuscular route	Prevention of HPV infection
Recombinant vaccines		
Hepatitis B*	Intramuscular route	Used more than subunit but subunit for same groups
Pertussis	Intramuscular route	Newborn and children

**Hepatitis B and human papillomavirus vaccines are also prepared by recombinant methods.*

viral promoter/enhancer sequences such as antigen cDNA and termination–polyadenylation sequences.

- **Bacterial growth:** Many more copies of the plasmid containing the infectious organism's DNA must be produced.
- **Purification of plasmid DNA from bacteria:** Plasmid DNA is purified from the bacteria. All the other bacterial DNA and debris are separated from the plasmid vector. This plasmid can now be used to produce the infectious organism's proteins inside a person.

RNA VACCINE

RNA vaccine consists of copy of a molecule called messenger RNA (mRNA) that codes for a disease-specific antigenic protein produce an immune response. RNA vaccines are faster, cheaper and safe. Production of RNA vaccines is laboratory based and the process could be standardized and scaled allowing quick immune responses to large outbreaks and epidemics. A major advantage of RNA vaccines is that RNA can be produced in the laboratory from DNA template using a readily available materials such as chicken, eggs or other mammalian cells.

IMMUNODEFICIENCY DISEASES

Immunodeficiency diseases occur when one or more components system such as lymphocytes (T cell, B cell or combined T cell and B cell), phagocytes (neutrophils or macrophages), and complement system is defective. Immunodeficiency diseases can be primary or secondary.

- **Primary immunodeficiency** diseases are caused by genetic mutation that adversely affects the immune system.
- **Secondary immunodeficiency** diseases occur due to loss of previous functional immune system as a consequence of acquired immunodeficiency syndrome (AIDS), immunosuppressive drugs, starvation, malnutrition, infectious agents, chemotherapy, radiation, hematopoietic tumors and transplants. Patient develops numerous opportunistic infections and malignancies in AIDS.
- In normal health, CD8+ cytotoxic T cells, natural killer cells (NK cells), and macrophages recognize and destroy cancer cells by means of immune surveillance. Failure of immune system results in survival of cancer stem cells. Genetic alteration transforms normal cells to cancer stem cells due to activation of oncogenes or mutation of tumor suppressor genes, irradiation and viral infections.

PRIMARY IMMUNODEFICIENCY DISEASES

Primary immunodeficiency diseases occur due to congenital defects in B cell or T cell or combined (B and T cells). Combined defects in B cells and T cells lead to severe combined immunodeficiency disease (SCID). Patient lacks adaptive immune response associated with fatal outcome by two years of age without replacement of bone marrow or gene therapy.

- Defects in humoral immunity often cause with recurrent sinopulmonary infections with encapsulated bacteria (*Haemophilus influenzae*), parasitic infections (Giardia), chronic diarrhea and failure to thrive.
- Defects in cellular immunity lead to opportunistic infections (*Pneumocystis jirovecii*), disseminated viral infections (CMV, Epstein-Barr virus), failure to thrive, diarrhea, persistent and oral thrush.
- Defects in phagocytosis present with recurrent abscesses, oral ulcers, and severe pneumonias (catalase positive *Staphylococcus aureus*). Nitroblue tetrazolium (NBT) test is done to screen patients with chronic granulomatous disease.
- Phagocytic defects cause chronic granulomatous disease (NADPH oxidase defect), Chédiak-Higashi syndrome (abnormal phagosome formation) and leukocyte adhesion deficiency (absence of leukocyte adhesion molecules).
- Defects in complement proteins cause hereditary angioedema (C1-INH deficiency), systemic lupus erythematosus (C2 and C4 deficiency), paroxysmal nocturnal hemoglobinuria (PIG-A gene mutation encoding phosphatidylinositol-linked membrane proteins) and recurrent bacterial meningitis due to defective membrane attack complex (C5b–C9 deficiency).
- Primary immunodeficiency diseases involving lymphocytes are shown in Fig. 4.43. Classification of immunodeficiency diseases is given in Table 4.39. Primary immunodeficiency diseases are given in Table 4.40.

Pathology Pearls: Warning Signs of Immune Deficiency

- Recurrent infections of ear (otitis media), sinuses and lungs (pneumonias) within one year.
- History of abscesses in skin and deep organs
- History of little response to antibiotics
- History of persistent oral thrush (Candida infections), characterized by white, adherent, painless, discrete or confluent patches in the mouth, tongue or esophagus
- History of failure to thrive
- Family history of early childhood deaths
- Patient needs intravenous administration of antibiotics

B CELL DEFECTS: PRIMARY IMMUNODEFICIENCY DISEASES

Primary immunodeficiency diseases due to B cell deficiency include X-linked agammaglobulinemia (Bruton agammaglobulinemia), transient hypogammaglobulinemia of infancy, common variable unclassifiable immunodeficiency, selective IgA deficiency, selective IgM deficiency, selective IgG subclasses deficiency, immunodeficiency with hyper-IgM syndrome, secondary B cell immunodeficiency associated with drug, protein-losing states and X-linked lymphoproliferative disorder.

X-Linked Agammaglobulinemia (Bruton Agammaglobulinemia)

X-linked agammaglobulinemia (Bruton agammaglobulinemia) is characterized by complete lack of immunoglobulin due to defect in B cell maturation, in which naïve B cells cannot mature to plasma cells due to mutation of Bruton tyrosine kinase (BTK) gene.

Pathogenesis

In normal health, BTK gene located on the long arm of the X chromosome, encodes protein that participates in maturation of pre-B cells to B cells transforming into antibody secreting plasma cells. Mutation of BTK gene results in impaired maturation of pre-B cells to B cells. There is an absence of both mature B cells in peripheral blood and plasma cells in lymphoid tissues. Patient has absence of plasma cells leading to failure of synthesis of immunoglobulins. Cell immunity is not affected. Serum analysis reveals absence of immunoglobulins.

Clinical Features

X-linked agammaglobulinemia affecting male infants of 5–8 months of age, usually does not manifest

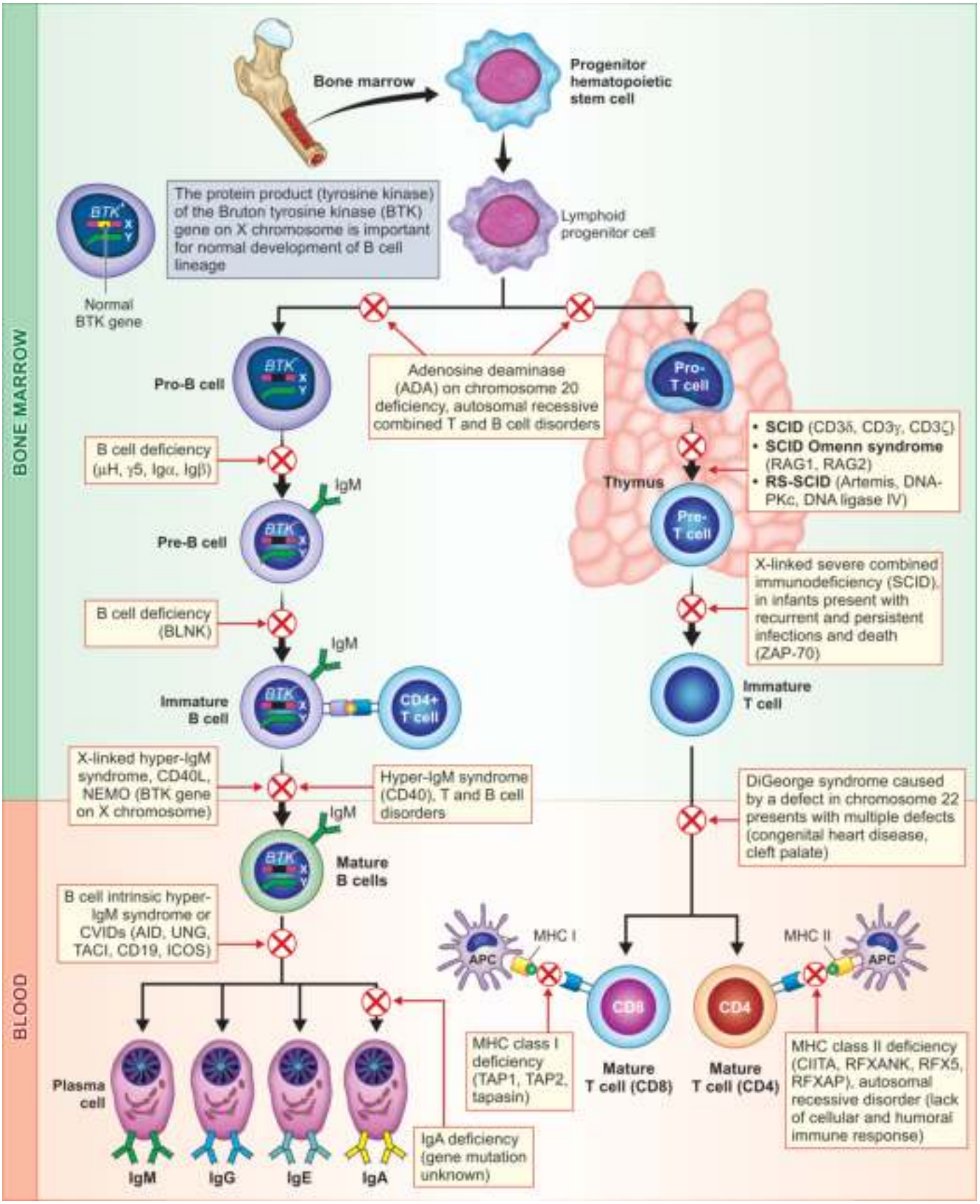


Fig. 4.43: Primary immunodeficiency disorders involving lymphocytes. Primary immunodeficiency diseases are genetically determined immunodeficiencies with immune and nonimmune defects resulting in severe infections that recur more frequently.

Table 4.39 Classification of immunodeficiency diseases

B Cell Defects: Primary Immunodeficiency Diseases
X-linked agammaglobulinemia of Bruton (lack of B cells with deficient antibody production associated with recurrent life-threatening bacterial infections)
Transient hypogammaglobulinemia of infancy
Common variable unclassifiable immunodeficiency (block in CD4+ helper T cell [Th17] differentiation, hypoglobulinemia, defective IgG, IgA; variable IgM associated with recurrent bacterial infections)
Selective IgA deficiency (MHC linked disorder, defect in class switch associated with mild infections of respiratory and gastrointestinal tract)
Selective IgM deficiency (defect in class switch associated with recurrent pyogenic infections)
Selective IgG subclasses deficiency
X-linked hyper-IgM syndrome (defective CD40 ligand, susceptibility to bacteria, *Pneumocystis jirovecii*, *Cryptosporidium parvum* infections)
Secondary B cell immunodeficiency associated with drug, protein-losing states
X-linked lymphoproliferative disorder [SAP (SH2D1A)] mutation, inability to control B cell growth and susceptible to EBV-driven B cell tumors, fatal infectious mononucleosis)
T Cell Defects: Primary Immunodeficiency Diseases
DiGeorge syndrome (congenital thymic hypoplasia associated with defective development of T cells in thymus gland linked to recurrent life-threatening fungal and viral infections)
T cell deficiency associated with purine nucleoside phosphorylase deficiency
T cell deficiency associated with absent membrane glycoprotein
T cell deficiency associated with absent class I or class II MHC antigens or both (base lymphocyte syndrome)
Chronic mucocutaneous candidiasis with or without endocrinopathy
Combined T Cell and B Cell Defects: Primary Immunodeficiency Diseases
Severe combined immunodeficiency diseases (RAG1/RAG2 deficiency, ADA deficiency, PNP deficiency, autosomal recessive-linked, sporadic associated with recurrent life-threatening infections)
Wiskott-Aldrich syndrome (immunodeficiency with eczema and thrombocytopenia, X-linked disorder, defective WASP gene, impaired T cell activation responses, susceptible to encapsulated extracellular bacteria, HSV, EBV infections)
Immunodeficiency with thymoma
Immunodeficiency with ataxia-telangiectasia (autosomal dominant disorder, defective cell-cycle kinase domain of ATM, T cells reduced, low IgA and IgE and susceptible to respiratory tract infections)
Immunodeficiency with short-limbed dwarfism
Immunodeficiency with adenosine deaminase deficiency
Immunodeficiency with nucleotide phosphorylase deficiency
Biotin-dependent multiple carboxylase deficiency
Phagocytic Dysfunction: Primary Immunodeficiency Diseases
Chronic granulomatous disease associated with recurrent infections with bacteria that are sensitive to killing by oxygen dependent mechanisms due to lack of oxidative burst for bacterial killing, i.e. absence of production of reactive oxygen species (e.g. hydrogen peroxide)
Chédiak-Higashi syndrome (autosomal recessive disorder, defective intracellular transport protein encoded by LYST, inability to kill bacteria)
Myeloperoxidase deficiency
Severe congenital neutropenia associated with recurrent life-threatening infections
Hyper-IgE syndrome (Job syndrome characterized by defective STAT3, block in CD4+ T cell (Th17) differentiation, elevated IgE, susceptible to extracellular bacteria and fungi)
Elevated IgE defective chemotaxis, and recurrent infections
Tuftsin deficiency disease in children associated with infections

Contd...

Table 4.39 Classification of immunodeficiency diseases (*Contd...*)

Complement System Defects: Primary Immunodeficiency Diseases
Defective production in C2, C3, and C5 associated with recurrent life-threatening bacterial infections
Defective production of C2 and C4 associated with development of systemic lupus erythematosus due to improper clearance of immune complex
Defective production of C5b–C9 membrane attack complex (MAC) associated with recurrent disseminated infections with Neisseria infections
Hereditary deficiency of C1 inhibitor (C1 INH) associated with laryngeal edema
PIG-A gene mutation encoding phosphatidylinositol-linked membrane proteins (paroxysmal nocturnal hemoglobinuria due to uncontrolled activation of complement associated with recurrent bouts of intravascular complement mediated hemolysis)

Table 4.40 Primary immunodeficiency diseases

Defects in Lymphocytes and other Molecules	Disorder	Comments
Defects in maturation of lymphocytes		
B cell	X-linked agammaglobulinemia of Bruton	▪ Lack of B cells and plasma cells ▪ Loss of BTK tyrosine kinase ▪ Recurrent life-threatening bacterial and viral infections
T cell	DiGeorge syndrome	▪ Thymic hypoplasia ▪ Autosomal dominant
T cell	Bare lymphocyte syndrome	Defect in MHC class II promoter
T cell, B cell and NK	Severe combined immuno-deficiency syndrome (SCID)	▪ RAG 1/RAG 2 (lack of TCR or Ig rearrangement) ▪ Adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) deficiency which affects development of T cells and B cells due to toxic metabolites ▪ JAK3 and IL-2R-γ deficiency (defective signals from IL-2, IL-4, IL-7, IL-9, IL-15) ▪ ZAP-70 deficiency results in defective signal from TCR
Defects in activation and function of lymphocytes		
B cell	Hyper-IgM syndrome	▪ Deficiencies of CD40, CD40 ligand and NEMO ▪ Absence of isotope switching and/or somatic hypermutation plus T cell defects ▪ Recurrent life-threatening bacterial infections (*Pneumocystis jirovecii*, *Cryptosporidium parvum*)
B cell	Common variable immuno-deficiency	▪ ICOS deficiency ▪ Defective IgA and IgG production ▪ Recurrent life-threatening bacterial and viral infections
B cell	Selective IgA deficiency	▪ MHC class molecules-linked disorder ▪ Absence of IgA synthesis ▪ Recurrent life-threatening respiratory tract infections
T cells and NK cells	X-linked lymphoproliferative disorders	▪ SAP (SH2D1A) mutant ▪ Disruption in regulation of B cell growth ▪ Epstein-Barr B cell tumors, fatal infectious mononucleosis
CD4+ helper T cells (Th17)	Hyper-IgE syndrome (Job syndrome)	▪ Defective STAT3 ▪ Blockage in CD4+ helper T cell (Th17) differentiation ▪ Elevated IgE ▪ Recurrent extracellular bacterial and fungal infections

Contd...

Table 4.40 Primary immunodeficiency diseases (*Contd...*)

Defects in Lymphocytes and other Molecules	Disorder	Comments
CD4+ helper T cells	MHC class II deficiency	▪ Lack of CD4+ helper T cells ▪ Lack of MHC class II expression ▪ Recurrent respiratory and skin infections
CD8+ cytotoxic T cells	MHC class II deficiency	▪ Lack of CD8+ cytotoxic T cells ▪ TAP mutation ▪ Lack of MHC class I expression ▪ Recurrent pathogen infections
Immunodeficiencies associated with systemic manifestations		
T cells and platelets	Wiskott-Aldrich syndrome	▪ X-linked disorder ▪ Defective WASP gene encoding cytoskeleton protein (CD43) ▪ Impaired T cell activation and CD8+ regulatory T cells dysregulation ▪ Recurrent encapsulated extracellular bacterial and viral infections (e.g. HSV, EBV)
B cells and T cells	Ataxia-telangiectasia	▪ Autosomal recessive disorder ▪ Mutation of cell-cycle kinase domain of ATM, low IgG and IgE ▪ Recurrent life-threatening respiratory tract infections
Phagocytic immunodeficiencies		
Neutrophils	Chédiak-Higashi syndrome	▪ Autosomal recessive disorder ▪ Defect in killing of bacteria due to mutation in LYST gene coding for defective intracellular transport protein
	Severe congenital neutropenia	▪ Autosomal dominant disorder or sporadic/ELA2 mutation, HAX1 mutation, Gfi1 mutation ▪ Severe neutropenia, developmental arrest of myeloid precursors usually at promyelocytic stage and increased apoptosis
	Myeloperoxidase (MPO) deficiency	▪ Neutropenia associated with frequent fungal infections especially *Candida albicans*
	Neutrophil specific granule deficiency	▪ Autosomal recessive/C/EBP-epsilon mutation ▪ Absence of defensin and gelatinase enzyme in specific granules results in recurrent infections
	Cyclic neutropenia	▪ Autosomal dominant disorder or sporadic/ELA2 mutation ▪ Fluctuations between normal granulocyte counts and severe neutropenia with 21-day periodicity and increased apoptosis in myeloid precursors
	Glucose-6-phosphatase deficiency	▪ Autosomal recessive/G6PC3 mutation ▪ Increased susceptibility to apoptosis
	P14 deficiency	▪ Autosomal recessive/p14 (MAPBPIP) mutation ▪ Defective lysosome function, neutropenia, hypogammaglobulinemia, short stature and hypopigmentation
	Leukocyte adhesion deficiency I (LAD-I)	▪ Autosomal recessive/TBG2 mutation ▪ Defective integrin β_2 (CD18) ▪ Defective adhesion and migration
	Leukocyte adhesion deficiency II (LAD-II)	▪ Autosomal recessive/GDP-fucose transporter mutation leads to congenital disorder of fucosylation ▪ Granulocytes unable to bind to selectins on endothelium ▪ Defective adhesion and migration
	Leukocyte adhesion deficiency III (LAD-III)	▪ Autosomal recessive/RASGRP2 mutation ▪ Defective integrin activation ▪ Defective adhesion and migration ▪ High peripheral blood neutrophil count with severe bleeding

Contd...

Table 4.40 Primary immunodeficiency diseases (*Contd...*)

Defects in Lymphocytes and other Molecules	Disorder	Comments
Macrophages	WHIM deficiency	▪ Autosomal recessive/autosomal dominant/CXCR4 mutation ▪ Warts, hypogammaglobulinemia, infections and myelokathexis
	Wiskott-Aldrich deficiency (WAS deficiency)	▪ X-linked/WAS mutation ▪ Dysfunctional actin polymerization in hematopoietic cells, defects of cell activation, adhesion, migration and phagocytosis
	Rac2 deficiency	▪ Autosomal dominant disorder/Rac2 mutation ▪ Abnormal granulocyte chemotaxis, respiratory burst and degranulation ▪ High neutrophil counts
	Chronic granulomatous disease	▪ Autosomal recessive disorder ▪ Phagocytes unable to produce superoxide linked to defective oxidative burst for bacterial killing
	Many disorders affecting macrophage functions	Defect in release of macrophage-activating factors, migration, phagocytosis and killing of pathogens
Complement protein deficiencies		
Complement cascade protein deficiencies	▪ C2, C3 and C5 deficiencies	▪ Recurrent infections with select groups of bacteria with polysaccharide capsules
	▪ C2, and C4 deficiencies	▪ Systemic lupus erythematosus due to improper clearance of immune complex
	▪ C5b–C9 membrane attack complex (MAC) deficiency	▪ Recurrent disseminated infections with Neisseria infections
	▪ Hereditary C1 inhibitor (C1-INH) deficiency	▪ Laryngeal edema
	▪ PIG-A gene mutation encoding phosphatidylinositol-linked membrane proteins	▪ Uncontrolled activation of complement leads to paroxysmal nocturnal hemoglobinuria (PNH) characterized by recurrent bouts of intravascular complement-mediated hemolysis

until after the six months of age due to persistence of maternal antibodies. Patients develop recurrent bacterial especially sinopulmonary infections such as pneumococci, staphylococci, streptococci and *Haemophilus influenzae*. These patients are usually resistant to viral infections except Coxsackie virus and ECHO virus.

Laboratory Diagnosis

There is an absence of both mature B cells in peripheral blood and plasma cells in lymphoid tissues. Lymph nodes lack well defined germinal centers. T cells are unaffected.

X-Linked Hyper-IgM Syndrome

X-linked hyper-IgM syndrome is more common than autosomal variant. In normal health, cytokine is necessary for immunoglobulin class switching to IgG, IgA or IgE X-linked. Hyper-IgM syndrome occurs due to mutated CD40L on CD4+ helper T cells or CD40 receptor on B cells. Second signal cannot be delivered to CD4+ helper T cells during B cells activation. Consequently, cytokine necessary for immunoglobulin class switching is not produced, and the patients have normal or increased concentration of IgM, and low levels IgG, IgA or IgE, hence prone to pyogenic infections.

Selective IgA Deficiency

Selective IgA deficiency is inherited disorder characterized by inability of IgA secreting B cells to mature into IgA producing plasma cells. Other immunoglobulin levels remain within normal limits.

- Patients are usually asymptomatic, but may also be associated with frequent episodes of diarrhea and recurrent infections especially those involving mucosal surfaces.
- The patients lacking in IgA may develop anaphylactic reactions to transfused blood due to increased IgE concentration. This sensitization can result in susceptibility to anaphylaxis on subsequent blood transfusion.

Selective IgM Deficiency

Selective IgM deficiency is a rare immunodeficiency disorder that has been reported in association with

serious recurrent pyogenic infections, such as bacteremia. The disorder can affect infants, children and adults, who have normal IgG subclasses and IgA, normal vaccination responses, absence of T cell defects and absence of causative external factors. Patient presents with recurrent otitis media, chronic sinusitis, bronchitis, bronchiectasis, pneumonia and urinary tract infections. Adults with selective IgM deficiency are at risk of developing allergic and autoimmune diseases.

Selective IgG Subclass Deficiency

IgG is the main immunoglobulin in human blood that provides protection against many infectious agents. IgG is a combination of four subclasses: IgG1 (60–70%), IgG2 (20–30%), IgG3 (5–8%) and IgG4 (1–3%).

- Normally, subclasses IgG1 and IgG3 are effective against toxins produced by *Corynebacterium diphtheriae* and *Clostridium tetani*. In contrast, IgG2 subclass is effective against polysaccharide capsule of certain disease-producing *Haemophilus influenzae* and *Streptococcus pneumoniae*.
- The term 'selective IgG subclass deficiency' refers to significant decrease in the serum concentrations of one or more subclasses of IgG in a patient whose total IgG concentration is within normal range. Occasionally, patients may suffer from infections.

Transient Hypogammaglobulinemia of Infancy

Transient hypogammaglobulinemia of infancy (THI) is a primary immunodeficiency disorder caused by a transient drop of levels of IgG in an infant between 5 months to 2 years of age. IgG levels typically return to reference range between 2 and 6 years of age.

- Clinically, THI is characterized by recurrent infections, although some infants may be asymptomatic.
- Treatment with antibiotics and replacement immunoglobulin therapy is of foremost importance in the management of symptomatic infants.

Common Variable Immunodeficiency

Common variable immunodeficiency (CVID) is a primary immunodeficiency disease characterized by low levels of protective immunoglobulins and an increased risk of infections in children and adults.

- The hallmark of CVID is recurrent or severe bacterial and viral infections of upper airway, sinuses and lungs. Respiratory tract infection can result in pneumonia, chronic bronchitis and bronchiectasis.
- Patient presents with chronic cough, runny nose, fatigue, fever, diarrhea, painful swollen joints in the knee, ankle, elbow or wrist, lymphadenopathy and splenomegaly. In addition, persons with CVID, there is higher risk of developing malignant tumors.

X-Linked Lymphoproliferative Syndrome

X-linked lymphoproliferative (XLP) syndrome is primary immunodeficiency disorder characterized by a defective immune system.

- Persons with X-linked lymphoproliferative syndrome exposed to Epstein-Barr virus can result in severe, life-threatening fulminating hepatitis. There is increased risk of developing various infections, lymphoid malignancies and other abnormalities. In most cases, patient presents with fatigue, epistaxis and infections anytime between 6 months to 10 years of age.
- Diagnosis of XLP syndrome is based on clinical manifestations, laboratory findings and identification of genetic mutations.

T CELL DEFECTS: PRIMARY IMMUNODEFICIENCY DISEASES

Primary immunodeficiency diseases due to T cell defects include DiGeorge syndrome, also known as congenital thymic aplasia and chronic mucocutaneous candidiasis with or without endocrinopathy, T cell deficiency associated with purine nucleoside phosphorylase deficiency; T cell deficiency associated with absent membrane glycoprotein, and T cell deficiency associated with absent MHC class I or class II antigens or both (base lymphocyte syndrome). Deficiency in cell-mediated immunity is associated with recurrent viral, fungal and protozoal diseases.

DiGeorge Syndrome (Congenital Thymic Hypoplasia)

In both DiGeorge syndrome (congenital thymic hypoplasia) and Nezelof syndrome, there is failure of maturation of T cells, but B cells remain unaffected. Majority of persons with DiGeorge syndrome have deletion of a small piece of chromosome 22 known as 22q11.2, which is also known as 22q11.2 deletion syndrome.

Pathogenesis

In DiGeorge syndrome, aberrant embryonic development of third and fourth branchial arches, results in congenital thymic hypoplasia and parathyroid glands as well as anomalies of aortic arch, mandible and ear. It can be summed up by **CATH22**, which denotes cardiac defects, abnormal facies, thymic hypoplasia, cleft palate, hypocalcemia and microdeletion of chromosome 22q11. In about 30% of cases, DiGeorge syndrome is also associated with behavior disorders and psychosis (bipolar disorder and schizophrenia) that develop during adolescence.

Clinical Features

DiGeorge syndrome affected children develop recurrent infections (bacterial, fungal and viral), pneumonias and tetany from hypoparathyroidism with hypocalcemia,

breathing problems, developmental disabilities, congenital cardiac defects, cleft lip and palate.

Chronic Mucocutaneous Candidiasis

Chronic mucocutaneous candidiasis is characterized by persistent or recurrent symptomatic Candida infection occurs due to inherited T cell defects and low levels of immunoglobulins in childhood, that affects mucosal sites, skin, and nails. It can be either autosomal dominant or autosomal recessive inheritance pattern.

- Autosomal dominant chronic mucocutaneous candidiasis is associated with gain-of-function mutations of **STAT1** gene resulting in IL-17 deficiency, which is associated with malfunctioning of endocrine glands such as hypothyroidism, hypoparathyroidism, hypoadrenalism and type 1 diabetes mellitus. Patients can develop autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) syndrome, in which there is mutation of AIRE gene.
- Chronic mucocutaneous candidiasis usually presents before the age of three years with one or more of the symptoms: chronic oral thrush, napkin dermatitis, paronychia, widespread crusted plaques on the scalp, trunk, hands and feet, stenosis of esophagus, larynx or vagina and increased susceptibility to infection with dermatophyte fungi (tenia) and human papillomavirus.
- Chronic mucocutaneous candidiasis may lead to premature death due to disseminated Candida infection, sepsis, pneumonia or mycotic aneurysms. Patients are more prone to develop carcinomas of skin, nose, ear and esophagus.

COMBINED T CELL AND B CELL DEFECTS: PRIMARY IMMUNODEFICIENCY DISEASES

Primary immunodeficiency diseases due to combined T cell and B cell defects include severe combined immunodeficiency disease, Wiskott-Aldrich syndrome, immunodeficiency with ataxia-telangiectasia, immunodeficiency with thymoma, immunodeficiency with short-limbed dwarfism, immunodeficiency with adenosine deaminase deficiency, immunodeficiency with nucleotide phosphorylase deficiency and biotin-dependent multiple carboxylase deficiency.

Severe Combined Immunodeficiency Syndrome

Severe combined immunodeficiency syndrome (SCID) is X-linked disorder or an autosomal recessive disorder caused by mutations in different genes involved in the development and function of B cells and T cells. Infants with SCID appear healthy at birth but are highly susceptible to severe infections due to defective combined humoral and cell-mediated immunity. X-linked SCID occurs due to defects in IL-2 receptor. Swiss type SCID occurs due to adenosine deaminase (ADA) deficiency. Death occurs during the first year of life unless given immunoglobulin and bone marrow transplantation. SCID is often called 'bubble boy disease' since 1970s and 1980s, when world knew David Vetter, a boy with X-linked SCID, who survived for 12 years in a plastic, germ-free bubble.

- **X-linked SCID:** In normal health, MHC class II molecules are essential for activation of CD4+ T helper cells and cytokine production. Cytokine signaling is essential for proliferation and maturation of T cells and B cells.
  - In normal health, enzyme ADA prevents formation of toxic products to lymphocytes.
  - Defect in cytokine IL-2 receptor plays important role in pathogenesis of X-linked SCID.
  - Patients are treated by enzyme ADA replacement therapy (polyethylene glycol-modified bovine ADA (PEG-ADA) and bone marrow transplantation.
- **Swiss type SCID:** Approximately 50% of Swiss type SCID patients have autosomal recessive inheritance due to ADA deficiency.
  - In normal health, ADA protects T cells from toxic metabolic products.
  - ADA deficiency results in accumulation of deoxyadenosine, which in turn leads to buildup of deoxyadenosine triphosphate (dATP) in all cells, which inhibits ribonucleotide reductase enzyme and prevents DNA synthesis, hence cells are unable to divide. Accumulation of toxic metabolic products makes the T cells nonfunctional.
- **Radiosensitive SCID:** Mutations in genes encoding proteins involved in nonhomologous end joining DNA repair pathway prevent resolution of rearrangement intermediates by ionization radiation, which is called radiosensitive-sensitive SCID. Patients have very few T cells and B cells due to failure of DNA rearrangement. Patients develop carcinoma.
- **Omenn syndrome:** Omenn syndrome (leaky SCID) was first described by **Gilbert Omenn** in 1965, partial or impaired V(D)J recombinational activity occurs due to mutations in either in RAG1 or RAG2 genes encoding small quantity of RAG protein. Protein loss through the skin and gastrointestinal tract leads to generalized metabolic derangements.
  - Patient presents with chronic diarrhea, failure to thrive, recurrent infections (*Pneumocystis jirovecii*, cytomegalovirus) lymphadenopathy, hepatomegaly, erythroderma, desquamation, alopecia, and loss of eyebrows, including eyelashes. Tissue damage occurs due to abnormal expansion of Th1 and Th2 cells.
  - Patient outcome is fatal unless corrected by bone marrow transplantation. Laboratory investigations

include normal T cell count, depressed T cell proliferation to antigens, depressed humoral immunity, normal natural killer cells, hypereosinophilia, elevated serum IgE.

Pathology Pearls: Mechanisms of Severe Combined Immunodeficiency (SCID)

Lymphocyte Precursor Cell Death due to Purine Metabolism Defects

Adenosine deaminase (ADA) or purine nucleoside phosphorylase (PNP) deficiency results in accumulation of toxic products, which adversely affect developing B cells and T cells.

Defective Signaling through the Common γ-Chain Dependent Cytokine Receptors

- Several cytokine receptors share the common γ-chain subunit: IL-2 (peripheral T cell homeostasis), IL-4 (class-switch recombination), IL-7 (T cell development), IL-9 (hematopoiesis), IL-15 (natural killer cell development, NK cell requires TAM receptor and IL-15 R) and IL-21 (B cell maturation).
- Mutations in the common γ-chain result in absence of both mature T cells and NK cells. This is the most frequent form of SCID called SCIDX1.
- Because Janus kinase 3 (JAK3) mediates signaling from the γ-chain receptors, mutations in JAK3 result in a SCID phenotype that is undistinguishable from SCIDX1. KAK3 mutation is autosomal recessive SCID. γ-Chain mutation is X-linked SCID. IL-7Rα-chain deficient SCID lacks T cells but has B cells and natural killer cells.

Defective V(D)J Rearrangement

- Mutations in either RAG1 or RAG2 genes impair the development of T cells and B cells and not natural killer cells. SCID patients lack T cells and B cells but natural killer cells are present.
- Omenn syndrome (leaky SCID) was first described by Gilbert Omenn in 1965, partial or impaired V(D)J recombinational activity occurs due to mutations in either in RAG1 or RAG2 genes encoding small quantity of RAG protein. Protein loss through the skin and gastrointestinal tract leads to generalized metabolic derangements.

Defective pre-TCR/TCR Signaling

- T cells are key mediators in mounting an effective adaptive cell-mediated immune response. T cell signaling is essential in T cell development.
- Thymocytes bearing TCR with a high affinity for self-peptide MHC complex undergoes apoptosis (negative selection), whereas those bearing low-affinity TCR survive and differentiate into mature T cells (positive cells), which ensures only those T cells that are self-tolerant survive while eliminating the self-reactive T cells.
- Abnormal TCR rearrangement results in low-affinity limited repertoire TCRs. Dysregulation of this pathway can cause defective T cell survival and activation.

Clinical Features

Severe combined immunodeficiency disorder patient presents with failure to thrive and increased susceptibility to bacterial, fungal, and viral infections. There is increased risk for development of carcinomas. Graft-versus-host disease occurs as a result of blood transfusions. Patient lacks no adaptive immune response with fatal outcome by two years of age without bone marrow transplantation or gene therapy based on maturation of donor lymphoid progenitor cells. Thymic hypoplasia with absent or greatly reduced thymic lymphoid component, hypoplasia of lymph nodes, tonsils, and other lymphoid tissues.

Treatment

Treatment is sterile isolation of baby and stem cell transplantation. Gene therapy is a technique by which faulty gene is replaced by a normal healthy gene. Normal ADA gene is isolated, cloned and inserted into retrovirus vector, which has a packing sequence but no viral genes. Bone marrow cells of the patient are aspirated, and treated with retrovirus containing ADA gene. These bone marrow cells treated with retrovirus containing ADA gene are reinjected into the patient. T cells in bone marrow now become fully functional normal ADA gene and hence the immune system. Patient is observed for expression of normal gene. Gene therapy in severe combined immunodeficiency (SCID) disease is shown in Fig. 4.44.

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome is X-linked disorder associated with defects in both B cell and T cell functions (i.e. humeral and cellular immunity) due to mutation in WASP gene, which is characterized by low levels of IgM, recurrent infections, thrombocytopenia and eczema.

- Both T cells and platelets show absence of certain surface glycoprotein (CD43), which is ligand for intercellular adhesion molecule 1 (CAM-1). It has been suggested that a defect of glycosylation especially sialylation of these cell surfaces.
- Serum IgM level is low keeping in view of poor response to polysaccharide antigen. IgA and IgE concentrations are raised. However, IgG level remains within normal range.

Clinical Features

Patient presents with eczema, thrombocytopenia, bloody diarrhea, recurrent infections and poor antibody response to polysaccharide antigens. Patient has fatal outcome before six years of age due to bleeding, infection or malignancy (most often lymphoma).

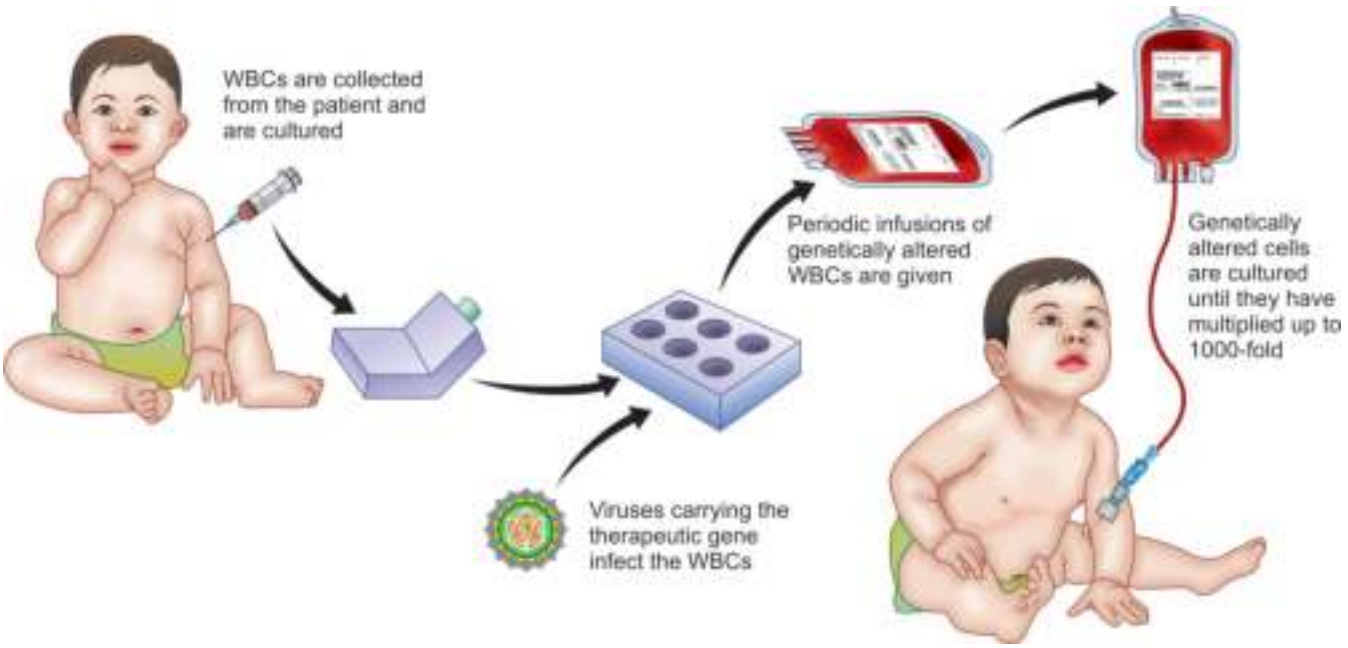


Fig. 4.44: Gene therapy in severe combined immunodeficiency (SCID). Gene therapy for SCID involves isolation and molecular correction of gene mutations in the patients' own pluripotent hematopoietic stem cells (HSCs), followed by transplantation of the functional cells back into the SCID patients.

Ataxia-Telangiectasia

Ataxia-telangiectasia is autosomal recessive trait associated with chromosomal breakages. There is increased risk of development of malignant neoplasm especially lymphomas.

- Patient presents with triad of features: (a) cerebellar degeneration and spinocerebellar atrophy, (b) dilated blood vessels on the flexor surface of forearms and conjunctiva, and (c) diminished resistance to infection.
- Cerebellar degeneration and spinocerebellar atrophy lead to appearance of choreoathetoid movements in early life. Dilated blood vessels are present on the flexor surface of forearms and conjunctiva.
- Diminished resistance to infection occurs due to decreased plasma levels of IgA and IgE and impaired cell-mediated immunity. Affected patients are prone to recurrent infections of sinuses and respiratory tract resulting to bronchiectasis.

PHAGOCYTIC DYSFUNCTION: PRIMARY IMMUNODEFICIENCY DISEASES

Phagocytes (neutrophils, macrophages) are cells that protect the body by phagocytosis and destroying harmful foreign particles, microbes and dead cells. Primary immunodeficiency diseases due to phagocyte defects include chronic granulomatous disease and leukocyte adhesion deficiency syndrome.

Defects in Adhesion of Leukocytes

Leukocytic adhesion is impaired in diabetes mellitus and patient on steroid therapy and hemodialysis. Under physiologic state, **β_2-integrins** (LFA-1 and Mac-1) expressed on leukocytes participate in adhesion of leukocytes to venular endothelium. Gene mutation of LAD-I causes deficiency of β_2-integrins resulting in recurrent bacterial infections, delayed wound healing and delayed separation of umbilical cord in newborns. Gene mutation of LAD-II causes mild disorder. LAD-III deficiency is characterized by both severe bacterial infections and a severe bleeding disorder.

Defects in Chemotaxis of Leukocytes

Chemotaxis is the directed migration of a cell in response to a chemical stimulus, which uses cytokines and chemokines to attract neutrophils and macrophages to the site of tissue injury, ensuring that pathogens in the area will be eliminated. Chemotaxis is impaired in diabetes mellitus, cancers, sepsis, immunodeficiency disorders and thermal injuries.

Defects in Opsonization of Injurious Agents

Opsonization is a process by which injurious stimulus coated by opsonins, i.e. IgG, C3b and complement mannose-binding lectin (MBL).

- Opsonization enhances binding of injurious agent to membrane receptors (e.g. FcR1, CR1 and MBL) expressed on activated neutrophils.

- Defects in opsonization results in 'Bruton's agammaglobulinemia', an X-linked inherited agammaglobulinemia is characterized by the absence of mature B cells which in turn leads to severe immunoglobulin deficiency and recurrent infections.

Defects in Phagocytosis

Phagocytosis is impaired in leukemias, diabetes mellitus, malnutrition, neonates and Chédiak-Higashi syndrome. Mutation in LYST gene is associated with Chédiak-Higashi syndrome. In normal health, phagocytic vesicle fuses with neutrophilic membrane of lysosome to form phagolysosome. LYST gene located on chromosome 1q42 encodes a protein essential for assembly of microtubules in the cytoplasm. Mutation in LYST gene results in defective fusion of phagosome with lysosome (phagolysosome).

- Chédiak-Higashi syndrome also causes defective degranulation of neutrophils, dysfunctional platelet (bleeding), impaired membrane fusion of lysosomes with melanosomes (albinism), Schwann cells (neuropathy), natural killer cells, and CD8+ cytotoxic T cells (aggressive lymphoproliferative disorder).
- In Chédiak-Higashi syndrome, neutrophils contain giant granules due to aberrant organelles. Defective degranulation of neutrophils leads to impaired microbial killing, and recurrent bacterial infections (*Staphylococcus aureus*) forming soft tissue abscess.

COMPLEMENT SYSTEM DEFECTS: PRIMARY IMMUNODEFICIENCY DISEASES

Primary immunodeficiencies due to complement proteins deficiency include hereditary angioedema, deficiency of C3, deficiency of membrane attack complex (MAC), and C2 deficiency or C4 deficiency secondary to autoimmunity.

- Complement system consists of wide variety of proteins, which perform many functions: (a) defense against pyogenic bacterial infection by opsonization, chemotaxis, activation of leukocytes and degradation of microbes, (b) bridging innate and adaptive immunity and (c) disposal of immune and inflammatory products by clearance of immune complexes from the tissues and removal of apoptotic cells. The proteins involved in activating the complement system are themselves activated by three convergent pathways termed classical, alternative and mannose-binding lectin (MBL).
- In complement system cascade, C3b participates in opsonization and phagocytosis of microbes. C3a and C5a play key role in adhesion, transmigration and chemotaxis of leukocytes. C3a and C5a increase capillary permeability. C5a acts on lipoxygenase pathway to synthesize arachidonic acid metabolites. C5b–C9 membrane attack complex (MAC) degrades of microbes via enhancing arachidonic acid metabolism and producing reactive oxygen metabolites.
- Patients develop increased susceptibility to infections due to defects in complement proteins, pathologic activation and deficiency of regulatory proteins. Defective formation of membrane attack complex (MAC) leads to increased susceptibility to Neisseria organisms. C3a, C5a and C4 deficiency leads to anaphylactic shock due to excessive release of histamine. Deficiency of complement system cascade linked to diseases in human beings is given in Table 4.41.

Recurrent Infections

Patient presents with increased susceptibility to infection due to deficiency of complement proteins such as C2, C3, and C5. Defective formation of membrane attack complex (MAC) leads to increased susceptibility to Neisseria organisms.

Hereditary Angioneurotic Edema

In normal health, classical pathway involves fixation antibodies (IgG and IgM) and complement (C1). C1

Table 4.41 Deficiency of complement system cascade linked to diseases in human beings

Disorder	Complement System Cascade Defects
Recurrent infections	C2, C3, and C5 deficiency
Neisseria infections	C3b-inactivator, C6, C7 and C8 deficiency, and C5b–C9 defective formation of membrane attack complex (MAC)
Laryngeal edema	Hereditary deficiency of C1 inhibitor (C1 INH)
Systemic lupus erythematosus (SLE)	C1, C2, C3, C4, C5 and C6, deficiency leads to improper clearance of immune complex
Paroxysmal nocturnal hemoglobinuria	▪ PIG-A gene mutation encoding phosphatidylinositol linked membrane proteins leading to uncontrolled activation of complement resulting to recurrent bouts of intravascular complement mediated hemolysis ▪ Flow cytometry demonstrating diminished CD55 and CD59 expression on red blood cells, leukocytes and platelets

is inhibited by plasma protein C1 inhibitor (C1 INH). Hereditary deficiency of C1 inhibitor (C1 INH) causes improper activation of C1 by immune complex resulting in excessive breakdown of C4 and C2. Complement C2 molecule generates vasoactive peptide (bradykinin), which produces painless non-pitting edema of soft tissues especially laryngeal edema, which may be life-threatening.

Systemic Lupus Erythematosus

Patient with C1, C2, C3, C4, C5 and C6 deficiency may develop autoimmune disease systemic lupus erythematosus (SLE) resulting from failure to clear immune complexes.

Paroxysmal Nocturnal Hemoglobinuria

In normal health, glycosylphosphatidylinositol (GPI) anchor regulatory proteins such as CD55, CD59, and C8, which are required for the protection of red blood cells, granulocytes, and platelets from complement-mediated lysis. CD55 known as DAF (decay accelerating factor) cleaves C3b. **CD59** known as membrane inhibitor reactive lysis (MIRL) participates in cleaving C5–9.

Gene Mutation

PIG-A gene mutation encoding phosphatidylinositol linked membrane proteins leads to uncontrolled activation of complement system resulting to recurrent bouts of intravascular complement-mediated hemolysis.

Clinical Features

Paroxysmal nocturnal hemoglobinuria (PNH) is often marked by the passage of hemoglobin-containing urine on awakening. During hemolytic episodes, patients develop normocytic or macrocytic anemia, accompanied by an appropriate reticulocyte's response. Paroxysmal nocturnal hemoglobinuria may develop as a primary disorder or evolve from preexisting cases of aplastic anemia.

Laboratory Diagnosis

Flow cytometry demonstrates diminished CD55 and CD59 expression on red blood cells, leukocytes and platelets in cases of paroxysmal nocturnal hemoglobinuria. Traditional diagnostic test has based on hemolysis in sucrose (**sucrose hemolysis test**) or acidified serum (**Ham test**) *in vitro*.

SECONDARY IMMUNODEFICIENCY DISEASES

Secondary immunodeficiency diseases are more frequent than primary immunodeficiency diseases. Secondary immunodeficiency diseases occur in the settings of malnutrition, cancer, aging, immunosuppressive agents and acquired immunodeficiency syndrome (AIDS). Causes of secondary immunodeficiency diseases are given in Table 4.42.

Table 4.42 Causes of secondary immunodeficiency diseases

Cancers
Chemotherapeutic drugs
Exposure to radiation
Malnutrition
Splenectomy
Immunosuppressive therapies
Infections
Aging process
Acquired immunodeficiency syndrome (AIDS)

ACQUIRED IMMUNODEFICIENCY SYNDROME

Acquired immunodeficiency syndrome (AIDS) is a chronic potentially life-threatening condition caused by the human immunodeficiency virus (HIV). HIV is transmitted by sexual contact, infected blood, sharing needles in drug addicts and vertical transmission from mother to fetus/child during pregnancy, childbirth or breastfeeding.

- HIV enters the body through mucosal tissues and blood and first infects CD4+ helper T cells, dendritic cells. Langerhans' cells, and macrophages. The infection becomes established in lymphoid tissues, where it remains latent for long periods until activated by cytomegalovirus or Epstein-Barr virus (EBV).
- CD4+ helper T cells play a central role in immune response. HIV targets CD4+ helper T cells resulting in marked depletion (<200 cells/mm^3). Cellular and humoral immunity are impaired. These patients are susceptible to opportunistic infections and carcinomas. The vast majority of AIDS cases in the United States and Europe are caused by infection with the retrovirus HIV-1.
- Without medication, it may take years before HIV weakens immune system to the point of developing AIDS, that causes profound immunosuppression leading to opportunistic infections, secondary neoplasms, and neurologic manifestations.
- There is no cure for HIV/AIDS, but medications can control infection and prevent progression of the disease. Antiviral treatments for HIV have reduced AIDS mortality around the world.
- HIV infection is diagnosed by enzyme-linked immunosorbent assay (ELISA) test; Western blot and direct assessment of viral RNA.

Pathology Pearls: HIV Evasion Mechanisms

- The rapid rate of mutations enables the HIV antigens to escape immune recognition. HIV makes 10 billion copies/day leading to depletion of CD4+ helper T cells.
- HIV integrates into host genome resulting in formation of abnormal proteins. It can remain hidden in resting cells.
- There is downregulation of MHC class I process, impairment of Th1 response of CD4+ helper T cells. HIV infects central nervous system, where antibodies have poor penetration.
- Disrupted immune system fails to control intracellular HIV infection. These reasons also contribute to the difficulty of HIV vaccine research.

Structure of HIV

Human immunodeficiency viruses are about 100–140 nm in diameter. HIV-1 and HIV-2 are members of the family of retroviruses. Retroviruses use RNA to encode their genetic information rather than DNA, as human cells do.

- HIV is composed of a capsid core, which contains the genetic material that has been surrounded by a lipid envelope, in which are embedded the trimeric transmembrane glycoprotein (gp41) to which the surface glycoprotein (gp120) is attached. These viral proteins (gp41 and gp120) are responsible for attachment to the host cell and are encoded by env gene of viral RNA genome.
- Beneath the envelope, is the matrix protein p17, the core proteins p6 and p24 and the nucleocapsid protein p7 (bound to the RNA). All these proteins are encoded by viral gag gene. Within the viral core, lies two copies of positive-sense, viral RNA genome (diploid RNA genome), together with protease, integrase and reverse transcriptase enzymes encoded by the viral pol gene.
- There are several other proteins with various immunomodulatory or regulatory functions in both HIV-1 and HIV-2 including **vif** (viral infectivity factor), **vpr** (viral protein R), **tat** (transactivator transcription), **rev** (regulator of expression of viral protein) and **nef** (negative regulatory factor). An additional **vpu** (viral protein U) is present in HIV-1 and not in HIV-2. Similarly, **vpx** (viral protein X) is present only in HIV-2 and not in HIV-1.
- HIV infects CD4+ helper T cells via high-affinity interaction between the virion envelope glycoprotein (gp120) and the CD4 molecule.
  - HIV integrates into host DNA (CD4+ helper T cells, macrophages, microglial cells) and undergoes high rate of mutation. The infection of CD4+ helper T cells is assisted by the T cell coreceptor called CXCR4; while HIV infects monocytes by interacting with CCR5 coreceptor.
  - HIV can remain dormant within cell for many years, especially memory CD4+ helper T cells. HIV induces a cytokine environment that helps in replication of virus. Either way, this subset of CD4+ helper T cells could become a target for eradicating HIV.
- HIV-1 can cause AIDS in people worldwide. HIV-2 has been isolated in West Africa, India. HIV-2 causes AIDS much more slowly than HIV-1 but otherwise clinically similar. By damaging the immune system, HIV interferes with body's ability to fight infection and disease. Structure of human immunodeficiency virus (HIV) is shown in Fig. 4.45. Summary of HIV-1 gene products and their functions is given in Table 4.43.

Routes of Transmission

Routes of transmission of human immunodeficiency virus include homosexual or bisexual men (75%), intravenous drug abusers (15%), multiple blood transfusion recipients (2%), hemophilic patients (1%) and transplacental route. Risk of human immunodeficiency virus has been greatly diminished by screening donor blood for anti-HIV antibodies, HIV p24 antigen, and HIV-1 RNA. HIV screening and heat inactivation of HIV in factor VIII concentrates have become universal in hemophilic patients.

Sexual Transmission

Sexual transmission is most common mode of transmission of HIV infection. In more than 75% cases, HIV is carried in the semen, and enters the recipient's body through abrasion in rectal or oral mucosa or by direct contact with mucosal lining cells.

Parenteral Transmission

Parenteral transmission occurs in individual's intravenous drug abusers, random recipients of blood transfusion. Mechanism of viral transmission includes sharing of needles, syringes and blood transfusion products contaminated with HIV.

Vertical Transmission

Mother-to-infant transmission is the major cause of pediatric AIDS. Infected mothers can transmit the infection to their offspring by three routes: (a) *in utero* by transplacental spread, (b) during delivery through an infected birth canal, and (c) after birth by ingestion of breast milk. These routes of transmission during birth (intrapartum) and in the immediate period thereafter (peripartum) is considered to be the most common mode of transmission. Higher the risk of transmission is associated with high maternal viral load and low CD4+ helper T cell counts as well as chorioamnionitis.

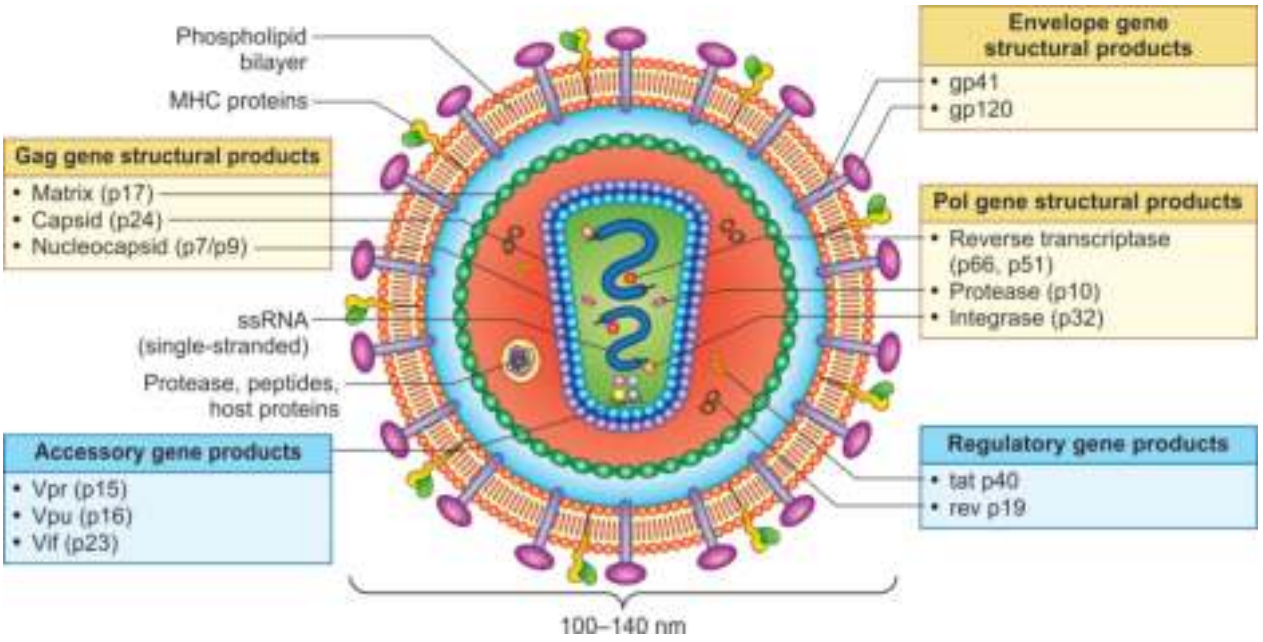


Fig. 4.45: Structure of human immunodeficiency virus (HIV). Each HIV virion is composed of numerous glycoproteins such as gp120 and gp41. The gp41 is a transmembrane molecule that crosses the lipid bilayer of the envelope. The gp120 is nonvalently associated with gp41 and serves as the viral receptor for CD4+ helper T cells of host. The viral envelope also contains some host-cell membrane proteins such as MHC class I and class II molecules. Within the envelope is viral core, or nucleocapsid, which includes a layer of protein called p17 and inner layer protein called p24. The HIV genome consists of two copies of double-stranded RNA (ssRNA), which are associated with two molecules of reverse transcriptase p64 and nucleoid proteins p10, a protease and p32 integrase. These glycoproteins play a vital role when HIV binds to and enters certain target cells.

Table 4.43 Summary of HIV-1 gene products and their functions

Viral Gene	Viral Protein	Designation	Function
Structural proteins			
gag gene	Capsid (CA)	p24	Viral capsid 24 plays a key role in pathogenesis of HIV that mediates gag oligomerization and assembly
	Nucleocapsid (NC)	p7	Viral nucleocapsid p7 is an RNA binding protein that packages the genome in the core; mediates gag oligomerization and assembly
	HIV budding p6	p6	HIV budding p6 is essential for budding of viral particles from plasma membrane that promotes membrane fusion of nascent virions via PTAP-Tsg101 recruitment of the ESCRT complex binds Vpr protein and packages it in the core
env gene	Surface envelope glycoprotein (SU)	SU, gp120	Surface envelope protein gp120; binds to CD4 receptor and chemokine receptors on target cell
	Transmembrane envelope glycoprotein (TM)	gp41	Transmembrane envelope gp41 protein; mediates fusion via fusion peptide
pol gene	Reverse transcriptase (RT)	p66, p51	RNA-dependent-DNA-polymerase converts viral ssRNA to linear DNA
	Integrase (IN)	p32	Integrase p32 protein facilitates integration of HIV DNA into host genome; involved in dynein-mediated transport of the RTC
	Protease (PR)	p10	Protease p10 protein play key role in cleavage of polyprotein components of gag-pol
Regulatory proteins			
tat gene	Trans-activator of transcription (Tat)	p40	Activator of transcription of HIV ssRNA is essential for virus replication
rev gene	Regulator of virions protein expression (Rev)	p19	Regulator of virion p19 protein is a post-transcriptional regulation of viral genes; including export of viral RNA from the nucleus

Contd...

Table 4.43 Summary of HIV-1 gene products and their functions (*Contd...*)

Viral Gene	Viral Protein	Designation	Function
Accessory proteins			
vpr gene	Viral protein R (vpr)	p15	Viral p15 protein assists in nuclear transport of HIV post-entry; induces G cell cycle arrest in infected cells
vpu gene	Viral protein U (vpu)	p16	Viral p16 protein enhances the release of virus particles by counter-acting the interferon-induced restriction factor tether in; promotes CD4 degradation
vif gene	Virion infectivity factor (vif)	p23	Viral p23 protein counteracts the anti-viral activity of APOBEC3 protein group. The latter protein groups are HIV restriction factors that hypermutate HIV genomes and render them nonfunctional

There are several other proteins with various immunomodulatory or regulatory functions in both HIV-1 and HIV-2 including vif (viral infectivity factor), vpr (viral protein R), tat (transactivator transcription), rev (regulator of expression of viral protein) and nef (negative regulatory factor). An additional vpu (viral protein U) is present in HIV-1 and not in HIV-2. Similarly, vpx (viral protein X) is present only in HIV-2 and not in HIV-1.

HIV Life Cycle

Life cycle of HIV comprises binding of HIV to CD4+ helper T cells, fusion of HIV gp120 to host cell membrane, internalization of HIV into host cell, synthesis of proviral DNA, entry of viral DNA into CD4+ helper T cells and creating new virus particles virions. Active viral replication is associated with more infection of cells and progression to AIDS. HIV does not infect the naïve T cells, which contain an enzyme, which inactivates the viral genome. With the activation of T cells, there is upregulation of NF-κB causing cell lysis. Life cycle of HIV is shown in Fig. 4.46.

Binding, Fusion and Internalization of HIV Glycoprotein (gp120) to Host Cell Membrane

The main attachment receptor for HIV is the CD4 molecule belonging to immunoglobulin superfamily, that is present on the CD4+ helper T cells, macrophages and microglial cells.

- The HIV-1 cell's envelope surface glycoprotein (gp120) initially binds to CD4+ molecule, which triggers a conformational change in the host cell membrane and virus envelope that allows binding of the co-receptor (i.e. CCR5 on macrophages and CXCR4 on CD4+ helper T cells), which is required for fusion between host cell membrane and virus envelope.
- Macrophages possess the CCR5 co-receptor, hence HIV strains requiring the CCR5 co-receptor for entry into the macrophages, also referred to as '**macrophage-tropic**' although macrophages also infect CD4+ helper T cells. Certain mutations in the CCR5 receptor are associated with totally resistant to some strains of HIV in homozygotes in the absence of the CCR5 molecule on their macrophages. But heterozygotes with mutation of CCR5 receptor develop a more slowly progressive disease.
- The 'lymphotropic' HIV strains use CXCR4 co-receptor on lymphocytes, for binding and entry into the host cells. So far, CXCR4 co-receptor deficient persons have not been found.

Proviral DNA (Double-stranded DNA) Synthesis

All retroviruses encode reverse transcriptase enzyme that reads the viral RNA sequence and transcribes into double-stranded deoxyribonucleic acid (ssDNA), which is then integrated, via the action of the integrase enzyme into the host cell genome.

- The provirus DNA or viral integrated dsDNA then acts as a template for viral genomic and messenger RNA (mRNA) transcription by the host cell's nucleic acid replicating machinery.
  - Recombination between these two RNA strands during viral replication, coupled with error-prone action of the reverse transcriptase enzyme, gives rise to the extreme genetic diversity of human immunodeficiency virus (HIV).
  - Integration of the provirus double-stranded DNA (dsDNA) into human genome of host cell establishes infection.
- Viral replication is enhanced by coinfection with other microorganisms, inflammatory cytokines and cellular activation. During cellular replication, provirus is transcribed by the host cell RNA polymerase II enzyme, and translated into proteins by viral messenger RNA, genomic RNA and cellular RNAs.

Creation of New Virus Particles

The gag-pol precursor polypeptide encoded by viral messenger RNA, is cleaved by viral-encoded protease enzyme to produce the gag and pol viral proteins.

- In addition, viral messenger RNA is also spliced to produce other viral proteins **tat, rev, vif, vpr** for

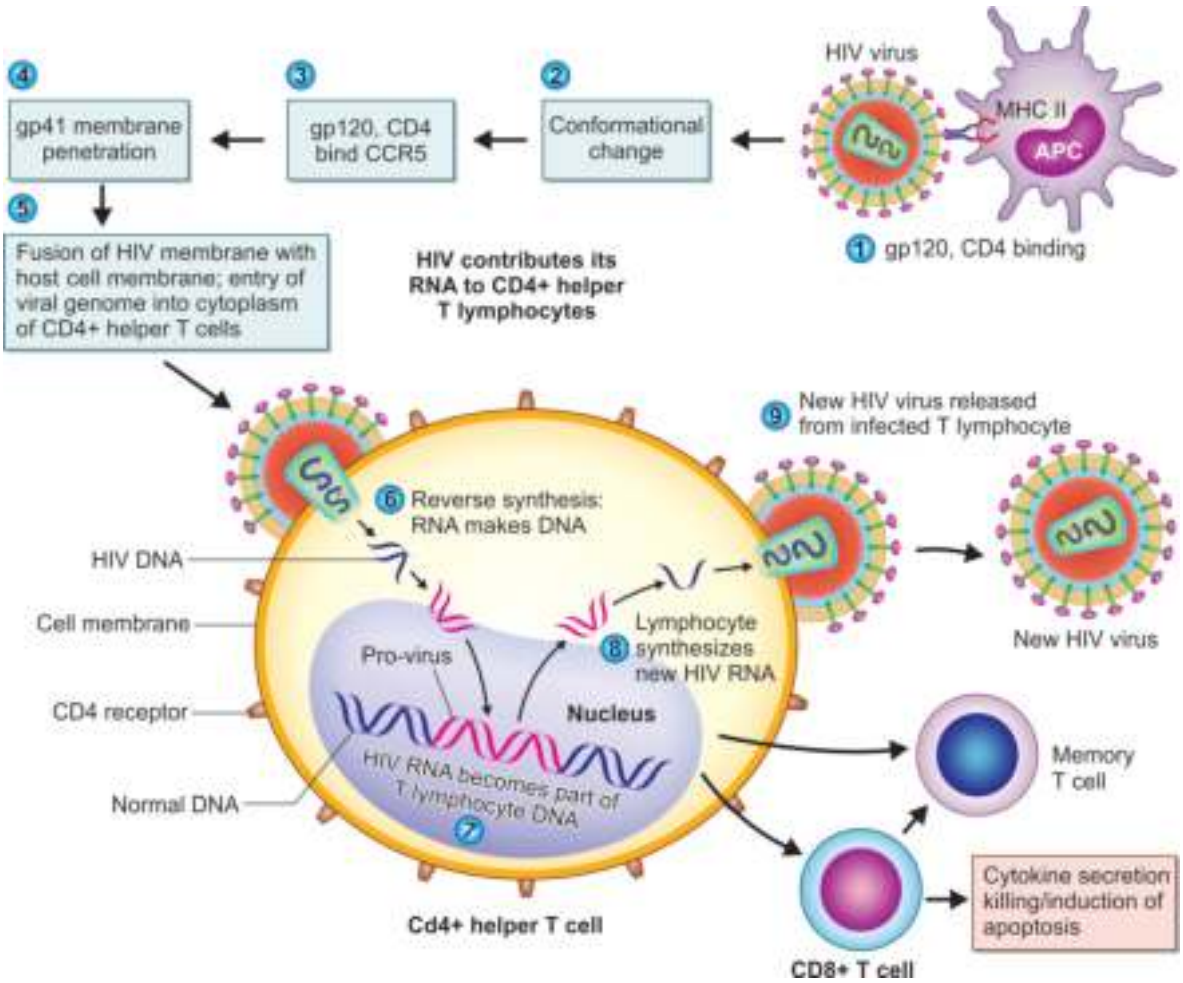


Fig. 4.46: Life cycle of human immunodeficiency virus (HIV) in the host cell. Various stages of the HIV life cycle in the host cell are: binding, fusion, reverse transcription, integration, replication, assembly and budding. HIV uses CD4+ helper T cells to replicate and each infected CD4+ helper T cells produces hundred of new copies of new HIV particles. The process is called the HIV life cycle. Each replication cycle only lasts for one to two days.

HIV-1, as well as the env precursor polypeptide. Ultimately, the env precursor polypeptide is cleaved by host cellular proteases, producing the envelope glycoproteins (gp120 and gp41).

- These viral proteins, together with the replicated diploid viral genomic RNA, are assembled and enveloped by budding through the host cell membrane, producing complete human immunodeficiency virus (HIV) virions.
- The new HIV virions (virus particles) are released from the CD4+ helper T cell. HIV is found in blood, semen, vaginal secretions, breast milk, and saliva.

HIV Natural History and Immunopathogenesis

In normal health, CD4+ helper T cells play important role in cell-mediated response, recognize antigens and utilize major histocompatibility complex (MHC) class II molecules. CD4+ helper T cells are differentiated according to the type of 'help'. Th1 cells activate CD8+ cytotoxic T cells, which utilize major histocompatibility complex (MHC) class II molecules to destroy infected cells. Th2 cells activate B cells, promoting antibody-mediated immunity.

- HIV integrates into host DNA (CD4+ helper T cells, dendritic cells, Langerhans' cells, and microglial cells of the central nervous system). Host mounts cell-mediated and humoral immune responses against HIV. Cell-mediated immune response is most important against HIV. Antibodies have many roles to control HIV infection. But these antibodies are less effective in controlling HIV infection as compared to cell-mediated immune response by two mechanisms: (a) antibodies formed against proteins on surface of virus block attachment of the virus to the host cell receptor, and (b) antibody-dependent cell-mediated cytotoxicity (ADCC) occurs by binding of Fc portion of antibody to natural killer cells (NK cells) leading to destruction of infected cells by indirect mechanism.
- The pathology of HIV infection is usually characterized by declining CD4+ helper T cell counts in the

peripheral blood, wasting disease and neurological disease. The neurological disease is related to infection of macrophages and microglia.

- Three stages of HIV infection include: (a) primary infection (acute HIV infection), (b) clinical latent infection (chronic HIV), and (c) acquired immunodeficiency syndrome (AIDS). There is no permanent cure for HIV. Symptoms depend on the stage of HIV infection.
- Antiretroviral therapy (ART) can slow or prevent HIV from advancing from one stage to the next. In primary HIV infection, about 50% of infected persons remain asymptomatic. Rest symptomatic infected persons present with fever and lymphadenopathy.
- HIV disease may be asymptomatic for many years. Before fully developed AIDS occurs, there is acute illness resembling infectious mononucleosis; a long latent phase followed by generalized lymphadenopathy; and a chronic HIV stage marked by fever, weight loss and diarrhea, illness and then AIDS as a result of disruption of immune system by HIV.
- HIV seropositivity begins soon after initial HIV infection. Antibodies to the proteins encoded by the genes of retroviral gag, env and pol regions can be demonstrated, especially antibodies to the gp120 and p24 proteins. Initial HIV infection occurs with mild illness in 70% of cases. HIV infection can also be demonstrated by amplification of viral genetic sequences by polymerase chain reaction (PCR) or by viral culture.
- The late stage, defined as AIDS, is marked by HIV infection complicated by specified secondary opportunistic infections or malignant neoplasms.

Seroconversion and Acute HIV Infection Stage

After seroconversion, when anti-HIV antibodies in response to acute HIV infection are detectable, patients remain asymptomatic for 2–15 years. During this stage, viral replication continues at a high rate up to 1010 infectious virions/day, leading to infection of 108–109 CD4+ helper T cells/day. Simultaneously, there is rapid turnover of CD4+ helper T cells.

- The rapid turnover of CD4+ helper T cells and its enormous diversity underlie the difficulty in producing antiretroviral drugs with long-term efficacy and development of an effective vaccine against HIV.
- Depletion of HIV-infected CD4+ helper T cells occurs through several mechanisms: (a) HIV has direct cytopathic effect, (b) CD8+ cytotoxic T cells destroy HIV-infected cells, and (c) lymphocytic activation and cytokines cause apoptosis of CD4+ helper T cells. Over this period, there is steady decline in the CD4+ helper T cell count.
- Some infected persons present with flu-like illness such as fever, headache, muscle aches and joint pain, cough, night sweats, skin rash, sore throat, lymphadenopathy, diarrhea and weight loss within two to four weeks after HIV infection. Symptoms may persist for few weeks. High number of infectious virions and rapid depletion of CD4+ helper T cells lead to progression of the disease.

Chronic HIV Infection Stage

Chronic HIV infection stage is also called asymptomatic HIV infection or clinical latency. HIV is still active but viral load is low. Persons remain asymptomatic during this stage. Without taking antiretroviral therapy (ART), some persons may progress to the next stage faster. Persons can transmit HIV to others during this stage. At the end of this stage, viral load goes up and the CD4+ helper T cell goes down. Persons who take antiretroviral therapy may never progress to next stage (i.e. AIDS).

Acquired Immunodeficiency Syndrome (AIDS)

Acquired immunodeficiency syndrome is the most severe stage of HIV infection, that disrupts immune system leading to opportunistic infections and tumors. CD4+ helper T cell count drops below 200 cells/mm. Persons can have a high viral load, that is very infectious. Without antiretroviral therapy, persons with AIDS can survive for three years.

Opportunistic Infections in AIDS

Decline in immune status parallels the decline in CD4+ helper T cells number and function. Loss of CD4+ helper T cells results in failure of normal Th1 response and cell-mediated immunity that is necessary for controlling intracellular infections.

- Children have a high incidence of opportunistic bacterial infections, such as otitis media, sepsis, chronic salivary gland enlargement, *Mycobacterium avium-intracellulare* complex function, and pneumonias, including lymphoid interstitial pneumonia and *Pneumocystis jirovecii* pneumonia.
- HIV causes direct injury to nervous system (encephalopathy and peripheral neuropathy), kidney (nephropathy), heart (cardiomyopathy), and gonads in both sexes (hypogonadism) and gastrointestinal tract (dysmotility and malabsorption). HIV causes indirect injury to various organs by opportunistic infections and malignant tumors as a consequence of immunosuppression. Opportunistic infections and malignant tumors associated with AIDS are shown in Fig. 4.47. Opportunistic infections in AIDS are given in Table 4.44.

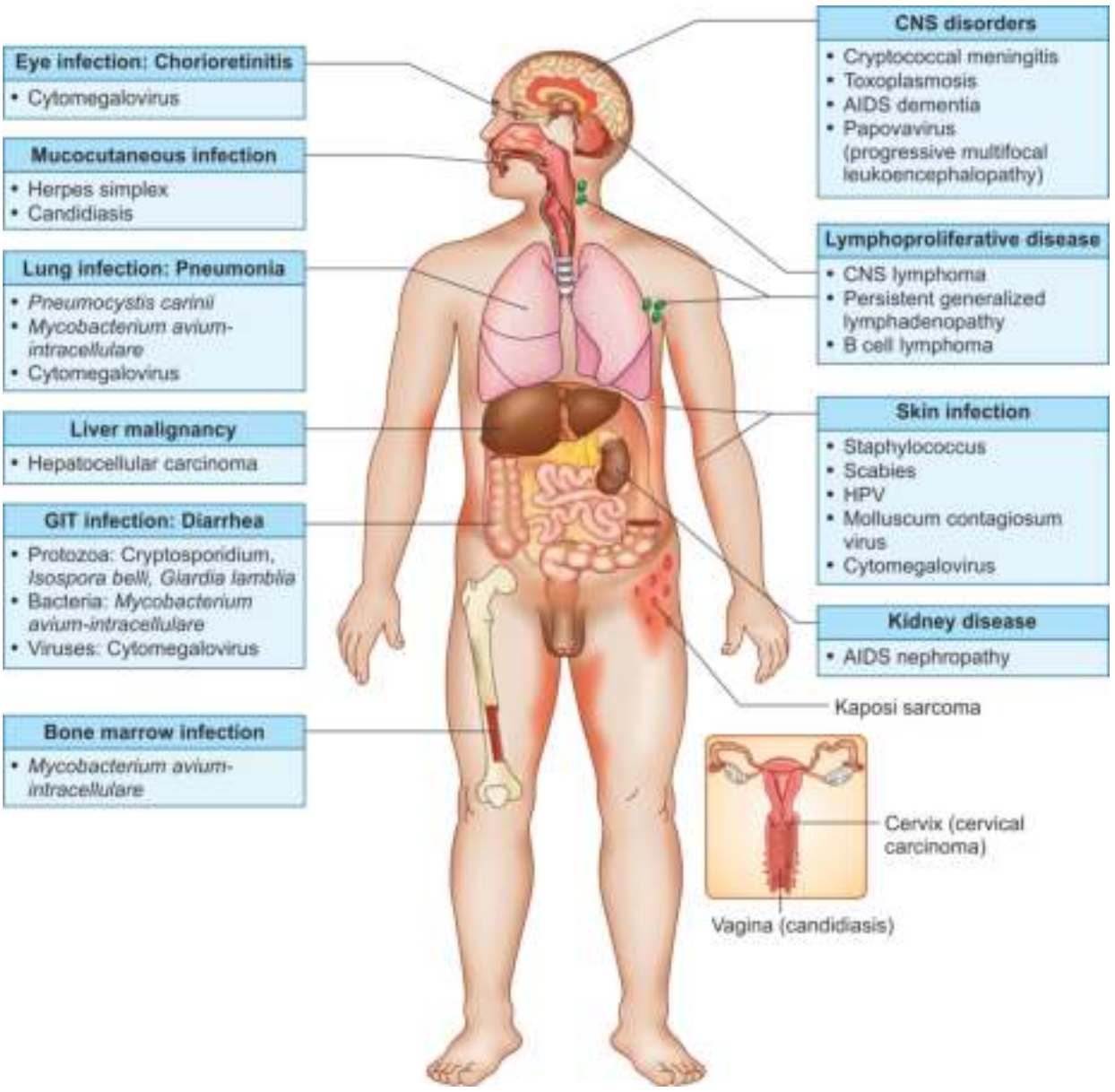


Fig. 4.47: Opportunistic infections and carcinomas associated with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS). HIV damages the immune system that fails to fight of opportunistic infections. HIV-related opportunistic infections include *Streptococcus pneumoniae*, Salmonella infection, candidiasis, tuberculosis and toxoplasmosis. HIV/AIDS patients are at risk of development of Kaposi sarcoma, non-Hodgkin lymphoma, Hodgkin disease, cervical carcinoma and carcinomas of the lung, liver, mouth, throat and anus.

Table 4.44 Opportunistic infections in AIDS

Organs Involved	Opportunistic Infections
Central nervous system	▪ Cryptococcus (meningitis) ▪ *Toxoplasma gondii* (AIDS dementia) ▪ Papovavirus (progressive multifocal leukoencephalopathy)
Eye (chorioretinitis)	Cytomegalovirus
Mucocutaneous (recurrent aphthous ulcers, angular cheilitis, necrotizing ulcerative gingivitis and periodontitis)	▪ Viral infections (herpes simplex virus, herpes zoster virus, cytomegalovirus) ▪ Fungal infections (*Candida albicans*, *Histoplasma capsulatum*, *Cryptococcus neoformans* and aspergillosis) ▪ Bacterial infections (*Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*)

Contd...

Table 4.44 Opportunistic infections in AIDS (*Contd...*)

Organs Involved	Opportunistic Infections
Lungs consolidation (pneumonia)	▪ *Pneumocystis jirovecii* ▪ *Mycobacterium avium-intracellulare* ▪ Cytomegalovirus
Skin (dermatitis, folliculitis and impetigo)	▪ Staphylococcus ▪ Scabies ▪ Human papillomavirus ▪ Molluscum contagiosum ▪ Cytomegalovirus
Small intestine (malabsorption syndrome)	▪ Protozoa (Cryptosporidium, *Isospora belli*, *Giardia lamblia*) ▪ *Mycobacterium avium-intracellulare* ▪ Cytomegalovirus
Large colon	Colitis by opportunistic infections
Kidneys	HIV nephropathy
Vagina	*Candida albicans*
Bone marrow	*Mycobacterium avium-intracellulare*

Pathology Pearls: Opportunistic Infections in AIDS

Fungal Infections

- Fungal infections are a major cause for opportunistic pneumonitis in immunocompromised persons.
- Gross examinations of lungs show irregular yellowish-gray infiltrate with a granular dry and firm on cut surface.
- Opportunistic infections in AIDS include *Pneumocystis jirovecii, Mucor* species, *Candida albicans*, Cryptosporidium, Coccidioides, *Cryptococcus neoformans*, and *Histoplasma capsulatum.*

Bacterial Infections

Bacterial infections include Mycobacterium tubercle bacilli both typical and atypical such as *Mycobacterium avium-intracellulare*.

Viral Infections

- Cytomegalovirus is member of the herpes family, which may cause retinitis, blindness, colitis, pneumonia and infection of adrenal glands.
- Patients are prone to herpes simplex virus, varicella zoster virus, JC virus, Epstein-Barr virus and poxviruses.

Protozoal Infections

Patient with HIV infection may develop giardiasis, leishmaniasis and toxoplasmosis.

Pneumocystis jirovecii

- *Pneumocystis jirovecii* causes pneumonia in immunocompromised persons.
- Patient presents with fever, non-productive cough (viscous sputum), dyspnea, weight loss and night sweats.
- *Pneumocystic jirovecii* may invade liver, spleen and kidney in minority of cases.

Cancers in AIDS

Decline in immune status parallels the decline in CD4+ helper T cells number and function results in failure of normal Th1 response and cell-mediated immunity that is necessary for prevention of development of tumorigenesis. HIV dysregulates immune system, activates cellular genes or proto-oncogenes or inhibit tumor suppressor genes resulting in genomic instability. HIV may also induce abnormalities of endothelial and epithelial cells resulting in development of malignant tumors. HIV / AIDS-related malignant tumors are given in Table 4.45.

Kaposi Sarcoma

Kaposi sarcoma is most often associated with acquired immunodeficiency syndrome (AIDS) especially in male homosexual persons. Kaposi sarcoma is vascular neoplasm involving mucocutaneous, lymphatic, gastrointestinal tract and lungs.

- Kaposi sarcoma is caused by human herpesvirus 8 (HHV) also known as Kaposi sarcoma herpesvirus (KSHV). HHV-8 encodes proteins that interfere with the p53 and pRb tumor suppressor pathways.
- Human herpesvirus 8 (HHVS) and HIV induce angiogenic and inflammatory cytokines responsible for tumor progression.
- Patient presents with reddish purple macules in distal lower extremities. AIDS-associated Kaposi sarcoma often involves lymph nodes and disseminates widely to viscera early in its course.

Lymphomas Associated with HIV

Lymphomas associated with HIV include Burkitt's lymphoma, diffuse large B cell lymphomas (HHV-8), immunoblastic lymphoma (primary CNS), primary

Table 4.45 HIV/AIDS-related malignant tumors

Organs	Cancers
AIDS defined carcinomas	
Skin	Kaposi sarcoma (HHV8), vascular tumor that may involve mucocutaneous, lymphatic, gastrointestinal tract, and pulmonary sites
Central nervous system	Immunoblastic lymphoma HHV8
Lymph nodes	▪ Burkitt's lymphoma (associated with translocations of MYC gene on chromosome 8, surface IgM, CD19, CD20, CD10, and BCL-6, phenotype consistent with a germinal center B cell origin involving CNS, jaw and facial bones of orbit) ▪ Diffuse large B cell lymphoma (CD20 positive, occurs due to dysregulation of BCL-6 at chromosome 3q27) ▪ Primary effusion lymphomas (pleural and peritoneal effusion having clonal IgH gene rearrangements, absence of lymphadenopathy) ▪ Plasmablastic lymphoma ▪ Persistent generalized lymphadenopathy
Cervix	Cervical carcinoma (HPV)
Less common carcinomas	
Anal region	Anal carcinoma (HPV)
Liver	Hepatocellular carcinoma
Lymph node	▪ Hodgkin's disease mixed cellularity EB virus in 70% cases) CD15+, CD30+ ▪ Hodgkin's disease depletion type (EB virus) CD15+, CD30+
Conjunctiva	Squamous cell carcinoma of conjunctiva
Uterus	Leiomyosarcoma (pediatric age group)

Extranodal involvement occurs in central nervous system, liver, bone marrow and gastrointestinal tract.

effusion lymphomas (pleural and peritoneal effusion having clonal IgH gene rearrangements), plasmablastic lymphoma (advanced stage) and Hodgkin's disease. Extranodal involvement occurs in central nervous system, liver, bone marrow and gastrointestinal tract.

- **Burkitt's lymphoma:** Burkitt lymphoma is associated with reciprocal chromosome translocation t(8;14) involving the heavy chain c-Myc oncogene on chromosome 8 and heavy immunoglobulin chain locus on chromosome 14. Expression of surface IgM, CD19, CD20, CD10, and BCL-6 phenotyping is consistent with a terminal center B cell origin neoplasm involving CNS, jaw and orbit.
- **Primary effusion lymphoma:** Primary effusion lymphoma is malignant large B cell lymphoma associated with pleural or peritoneal effusion in the absence of lymphadenopathy in advanced HIV infection or older adults. The tumor cells are often anaplastic in appearance and typically fail to express surface B or T cell markers, but have clonal IgH gene rearrangements. In all cases, the tumor cells are infected with KSHV/HHV-8, which appears to have a causal role.
- **Plasmablastic lymphoma (PBL):** Plasmablastic lymphoma is diffuse proliferation of neoplastic cells most of which are resembling B immunoblasts affecting oral cavity in AIDS persons of 50 years age group. Immunophenotyping reveals positivity with CD138, CD38, and IRF4/MUM1. PBL also shows positivity with CD79a in 50–85% cases.
- **Hodgkin's disease (mixed cellularity type):** More than 50% of patients present as stage III or IV disease. Males are more affected than females with peak in young adults and again in adults older than 55 years of age. EB virus plays role in the pathogenesis of Hodgkin's disease. On histopathologic examination of lymph node, tumor is composed of Reed-Sternberg cells admixed with T lymphocytes, macrophages, plasma cells and eosinophils. Immunophenotyping of Reed-Sternberg cells reveals positivity with CD15 and CD30 in 70% of cases.
- **Hodgkin's disease (lymphocytic depletion type):** Hodgkin's disease (lymphocytic depletion type is more common in older males. EB virus plays role in the pathogenesis of Hodgkin's disease. On histopathologic examination of lymph node, it is composed of Reed-Sternberg cells and paucity of background reactive cells. Immunophenotyping of Reed-Sternberg cells reveals positivity with CD15 and CD30 more common in older males. HIV-infected individuals in developing countries often present with advanced disease.

Other Cancers

Patient may develop hepatocellular carcinoma, HPV-related carcinomas of cervix and anus including lymphoma of central nervous system.

Lymphomatoid Granulomatous

Lymphomatoid granulomatous is an angiocentric and angiodestrictive lymphoproliferative disease involving extranodal sites and composed of EBV positive B cells along with reactive T cells. It has a spectrum of histologic grade and clinical aggressiveness, which is related to the proportion of large B cells.

HIV Testing for Persons

Recommended groups for HIV testing include homosexual men, commercial sex workers, unprotected sex, illness or fever without cause, high-risk needle sharing, perinatal exposure and patient request. Purpose of HIV testing includes blood safety, blood donation safety, identification of asymptomatic individuals and diagnosing clinically suspected cases. HIV testing is useful for prophylaxis, management and treatment of HIV-related illnesses.

WHO Definition of AIDS Patient

An adult or adolescent (>12 years) is considered to have AIDS if a test for HIV antibody is positive and one or more of the following conditions is/are present, which include >10% weight loss or cachexia with diarrhea/fever or both, cryptococcal meningitis, pulmonary or extrapulmonary tuberculosis, Kaposi sarcoma, neurological impairment, esophageal candidiasis, life-threatening or recurrent pneumonia with/without known etiology and invasive cervical carcinoma.

Laboratory Testing

Samples are collected with universal precautions for HIV testing include blood, serum, plasma, saliva or urine. Approximately 3–5 ml of blood sample may be taken as whole or anticoagulated blood. Specimen should be transported and processed within 24–48 hours. If serum/plasma has been separated then it should be refrigerated at –20 degree for a week.

Window Period

The window period in untreated HIV infection begins at the time of infection, which can last 4–8 weeks. During window period, a person is infected, infectious and viremic, with a high viral load and a negative HIV antibody test. The point when the HIV antibody test becomes positive is called the **point of seroconversion**. Window period in untreated HIV infection is shown in Fig. 4.48.

Reporting Procedure

Results of HIV testing is kept confidential. At least three screening HIV testing should be performed, which can be negative or positive or intermediate. Negative HIV report is mentioned if the initial screening report is

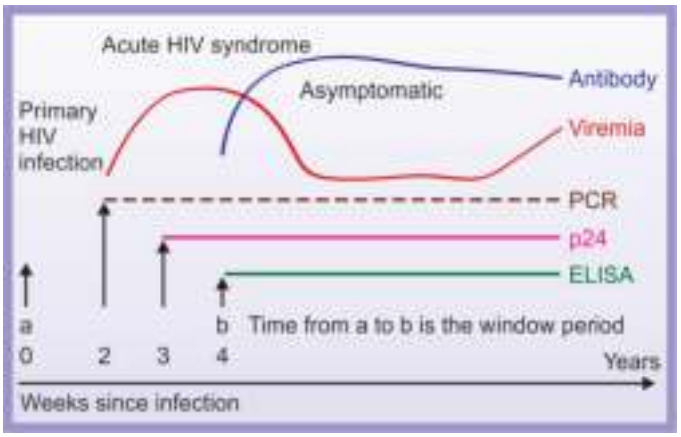


Fig. 4.48: Window period in untreated HIV infection. Normal HIV blood tests detect the presence of antibodies in human body as a part of their immune response to fight HIV. In most cases, HIV persons develop antibodies to HIV within 28 days of infection. During this period, HIV persons experience the so-called 'window period', the antibodies have not been produced in high quantity to be detected by standard tests and when the HIV persons may not show clinical signs of HIV infection but can transmit the disease to others.

nonreactive. At least three screening tests show reactive positive results concordantly, subsequent sample is retested before reporting. Discordant results by three screening tests, follow-up samples to retest at 2 weeks, 3 months, 6 months, 12 months before report dispatched. If the HIV result remains indeterminate even after a year, the person is considered to be HIV negative.

Screening of HIV Infection

Screening of HIV infection is done by analysis of enzyme-linked immunosorbent assay (ELISA) and particle agglutination (PA), antigen capture and immune complex disassociation methods. Screening of HIV infection is given in Table 4.46.

Confirmatory Tests for HIV Infection

HIV infection is confirmed by Western blot, immunofluorescence, nucleic acid testing, HIV RNA detection and HIV DNA detection. Confirmatory tests for HIV infection are given in Table 4.47. Testing of body fluids for HIV is given in Table 4.48.

- **Enzyme-linked immunosorbent assay (ELISA) test:** ELISA test is done for HIV infection. False positive ELISA test is seen in persons immunized against influenza vaccine up to 3 months, presence of rheumatoid factor, chronic alcoholism, HLA-DR antibodies in multigravida, autoimmune disorders and hemodialysis. False negative ELISA test is seen in advanced AIDS and during window period in 6–20% of cases. Unusual serotypes may give rise to negative results in screening. ELISA test for HIV infection

Table 4.46 Screening of HIV infection

Testing Categories	Analyte Detection	Use
ELISA	IgG/IgM	Standard screening using spectrophotometry
Simple ELISA	IgG/IgM	No special equipment required
Rapid ELISA	IgG/IgM	Results obtained in 10–30 minutes
Particle agglutination (PA)	IgG/IgM	ELISA with visual read out of particle clumping
Antigen capture	p24	HIV p24 detection
Immune dissociation	p24	Disrupts p24 antigen–antibody complex permitting capture

Table 4.47 Confirmatory tests for HIV infection

Testing Categories	Analyte Detection	Use
Western blot technique	IgG	Confirmation of HIV infection
Immunofluorescence (IF) microscopy	IgG	Confirmation of HIV infection
Nucleic acid technique (NAT)	HIV RNA	PCR used to detect HIV nucleic acid in donor screening
HIV RNA detection technique	HIV RNA viral load	Useful in resolving indeterminate cases
HIV DNA detection technique	HIV DNA	RT-PCR used to detect HIV DNA in neonatal diagnosis

Table 4.48 Body fluids for HIV testing

Source	ELISA	Rapid ELISA	Western Blot	Immunofluorescence (IF)	Nucleic Acid Testing (NAT)
Whole blood	+	+	–	–	–
Dried blood	+	–	–	–	–
Plasma	+	+	+	+	+
Serum	+	+	+	+	+
Oral fluid	+	–	+	–	–
Urine	+	–	+	–	–

using HIV antibody against immobilized antigens is given in Table 4.49.

- **Particle agglutination assays:** Particle agglutination assays are based on the ability of sera containing HIV antibodies to cross-link small particles containing HIV antigen on the surface. It has high sensitivity and specificity and easy to perform. Its disadvantages include subject to reader interpretation and no permanent records available.
- **Nucleic acid test (NAT):** NAT is adjunct but not a replacement of serological tests. Various formats include PCR and RT-PCR, NASBA (nucleic acid sequence-based amplification) and bDNA (branched-chain DNA). NAT is best in detecting HIV during seronegative period, which has a small window period. Its disadvantage is its high cost.
- **Rapid visual test (tri-dot test)**: Rapid visual test (tri-dot test) is a visual rapid and sensitive immunoassay

Table 4.49 Enzyme-linked immunosorbent assay (ELISA) test for HIV infection using HIV antibody against immobilized antigens

Generation of ELISA	Component	Approximate Window Period	Advantages
First generation	Infected cell lysates	63 days	Laboratory diagnosis of HIV infection
Second generation	Recombinant proteins and peptides	42 days	Decreases false positive
Third generation	Sandwich with laboratory antigen	22 days	Short window period
Fourth generation	p24 antigen sandwich	16 days	Short window period

for differential detection of HIV-1 and HIV-2 antibodies (IgG) in human serum against antigens (gp41 C terminal of gp120 and gp36). Principle of rapid visual test is based on reaction of immobilized antigens with antibodies present in the serum. Conjugate binds to the Fc portion of the HIV antibody to give distinct pinkish purple dot against a white background. Results are confirmed by assay be performed on fresh samples.

- **Immunofluorescence (IF) microscopy:** Immunofluorescence identifies the presence of antibodies by their specific ability to react with antigens on infected cells. Characteristic staining pattern of reactivity provides additional specificity to the interpretation. Conditions interfering with immunofluorescence microscopy include lipemia, hyperfibrinogenemia, paraproteinemia and autoimmune disease.
- **Radioimmunoprecipitation:** Radioimmunoprecipitation (RIPA) identifies antibodies by their ability to react with radiolabeled antigens, which helps to resolve indeterminate Western blot in early infection.
- **CD4+ helper T cells count:** CD4+ helper T cell count is the best indicator for immediate state of the immune system. Normal CD4+ helper T cell count (500–1600 IU/L) and CD8+ cytotoxic T cell count (375–1100 IU/L) (CD4+/CD8+) ratio ranges from 0.9–1.9. It correlates very well with level of immunologic tolerance. CD4+ helper T cell count is performed at every 3–6 months interval. CD4+ helper T cell count is most reliable short-term indicator of disease progression. Changes in CD4+ helper T cells and CD8+ cytotoxic T cells occur over the time span of an HIV infection and its relationship to AIDS diagnosis. CDC classification of HIV categories is given in Table 4.50.
- **CDC classification of HIV categories:** CDC classification of HIV categories includes CD4+ helper T cell count, asymptomatic acute HIV infection, symptomatic HIV infection and AIDS associated with opportunistic infection and carcinomas. Gradual loss of CD4+ helper T cells over the time span of an HIV infection and its relationship to AIDS diagnosis is shown in Fig. 4.49.

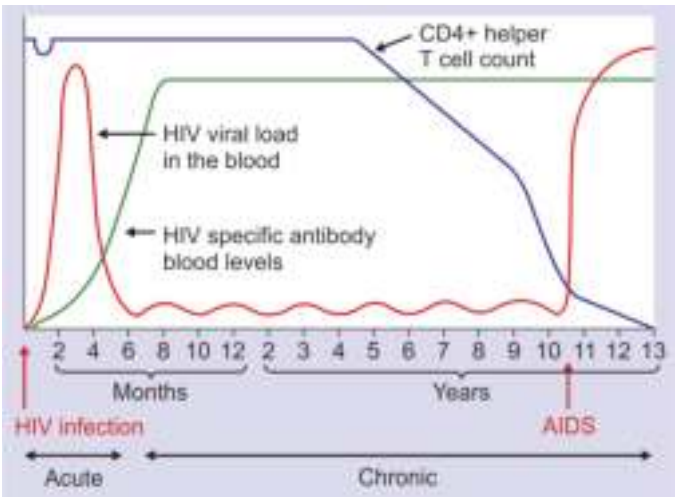


Fig. 4.49: Gradual loss of CD4+ helper T cells over the time span of an HIV infection and its relationship to AIDS diagnosis. The hallmark of human immunodeficiency virus (HIV) infection is gradual loss of CD4+ helper T cells and imbalance of CD4+ helper T cell homeostasis, high HIV viral load and HIV specific antibody with progressive impairment of immunity that results in death. HIV infection in human is caused by two related yet distinct HIV-1 and HIV-2 viruses. HIV-1 is more virulent than HIV-1 and permits the host to mount less effective adaptive immune response.

HIV RNA Determination

HIV RNA determination is highly sensitive and precise, which becomes essential component in monitoring HIV infection. It predicts the outcome of CD4+ T cell count in future. It provides very important prognostic information. During therapy levels of HIV RNA must be measured every 3–4 months.

- **HIV RNA viral loads after infection:** It can be used to assess the viral set point and predict the likelihood of progression to AIDS in the next 5 years. The higher the viral set point, there is rapid fall of CD4+ helper T cell count and rapid progression of HIV infection to AIDS.
- **Rate of disease progression:** It is determined by the patient's viral load. With levels between 1,000 and 10,000 viral copies, the likelihood of AIDS in 5 years is 8%. At 10,000 to approximately 50,000, the likelihood is 26%. At 50,000 to <100,000 it is 49%. Between 100,000 to 1,000,000 the likelihood is 62% at 5 years.

Table 4.50 CDC classification of HIV categories

CD4+ T Cell Count Categories	A. Asymptomatic Acute HIV Infection	B. Symptomatic HIV Infection	C. AIDS Indicator State (Opportunistic Infections and Cancers)
>500/µl	A1	B1	C1
200–499/µl	A2	B2	C2
<200/µl	A3	B3	C3

HIV Vaccines

There are a number of factors that cause development of an HIV vaccine to differ from the development of other classic vaccines. Classic vaccines mimic natural immunity against reinfection generally seen in individuals recovered from infection; there are almost no recovered AIDS patients.

- Most vaccines provide protection against disease, not against infection. HIV infection may remain latent for long periods before causing AIDS. Most effective vaccines are whole-killed or live-attenuated organisms; killed HIV-1 does not retain antigenicity and the use of a live retrovirus vaccine raises safety issues.
- Most vaccines provide protection against infections through mucosal surfaces of the respiratory or gastrointestinal tract; the great majority of HIV infection is through the genital tract.

HYPERSENSITIVITY REACTIONS

Immune system protects our body from outside harmful invaders such as bacteria, viruses and fungi. Hypersensitivity reactions occur when the normally protective immune system responds abnormally in response to an exogenous antigen or allergen, potentially harming the body, whereas autoimmune diseases arise from an abnormal immune response to endogenous substances (**autoantigens**).

- A symptomatic hypersensitivity reaction only occurs in sensitized persons, who must have had at least one prior asymptomatic exposure with the offending exogenous antigen.
- Hypersensitivity reactions are commonly classified into four types by Gell and Coombs: IgE-mediated type 1, IgG-mediated type 2, immune complex-mediated type 3 and cell-mediated type 4. Types 1, 2, and 3 are known as immediate hypersensitivity reactions, which require the active production of IgG, IgM and IgE antibodies by plasma cells, the terminally differentiated B cells. Type 4 hypersensitivity is delayed type of cell-mediated that occurs due to interaction of T cells and macrophages.
  - Type 1 hypersensitivity reaction is an immediate immune reaction mediated by immunoglobulin E (IgE) attached to mast cells, which degranulate in response to subsequent exposure to soluble antigen or allergen and release histamine and other chemical mediators leading to tissue injury. Examples of type 1 hypersensitivity reaction are hay fever (allergic rhinoconjunctivitis due to inhaled allergens), urticaria (hives), eczema, food allergy (nuts, shellfish, eggs, wheat), drug allergy (penicillin), insect venom allergy (bee, wasp), angioneurotic nonpitting subcutaneous edema over face, bronchial asthma, anaphylaxis (cutaneous and anaphylactic shock) and anaphylactic transfusion reactions (in patients with IgA deficiency).
  - Type 2 hypersensitivity reaction refers to an immediate antibody-mediated hypersensitivity reaction in which antibodies (IgG or IgM) are directed against cellular or extracellular matrix antigens with the resultant cellular functional loss or damage to tissue antibody-dependent cellular cytotoxicity (ADCC) mechanisms (e.g. autoimmune hemolytic transfusion reaction, hemolytic disease of the fetus and newborn, autoimmune hemolytic anemia, immune thrombocytopenia, drug-induced neutropenia and agranulocytosis, Hashimoto's thyroiditis, pernicious anemia, hyperacute transplant rejection, rheumatic fever, Goodpasture syndrome, Graves' disease, Lambert-Eaton myasthenic syndrome, pemphigus vulgaris, bullous pemphigoid).
  - Type 3 hypersensitivity reaction is an immediate immune complex-mediated hypersensitivity reaction, which causes tissue damage (skin, joints, blood vessels and glomeruli) due to antigen–antibody complex deposition. Examples of type 3 hypersensitivity reactions are polyarteritis nodosa, drug-induced hypersensitivity vasculitis, post-streptococcal glomerulonephritis, IgA nephropathy, membranous glomerulonephritis, systemic lupus erythematosus, serum sickness, Arthus reaction, hypersensitivity pneumonitis.
  - Type 4 hypersensitivity reaction is delayed type of cell-mediated hypersensitivity reaction that occurs at least 48 hours after exposure to an antigen, which involves sensitized CD4+ helper T cells, which release (cytokines, chemokines), macrophages and CD8+ cytotoxic T cells. Delayed hypersensitivity and epithelioid cell granulomas play a pivotal role in tissue damage observed during infections with slow-growing intracellular pathogens such as *Mycobacterium tuberculosis* (tuberculosis), *Mycobacterium leprae* (leprosy) and *Histoplasma capsulatum*. Examples of type 4 hypersensitivity reaction are contact dermatitis, tuberculin skin test reaction and granulomatous inflammation.
  - Type 5 is stimulatory hypersensitivity reaction, in which antibodies act as agonists for cell surface

receptors present on a cell, which a hormone would normally activate. Antibodies formed against thyroid-stimulating hormone receptors of thyroid follicular cells lead to **Graves' disease**. Comparison of different hypersensitivity reactions is given in Table 4.51.

Table 4.51 Comparison of different hypersensitivity reactions

Type 1 Hypersensitivity Reaction	Type 2 Hypersensitivity Reaction	Type 3 Hypersensitivity Reaction	Type 4 Hypersensitivity Reaction
Response time			
Less than 30 minutes (15–30 minutes)	Less than 8 hours (minutes to <8 hours)	Less than 8 hours (3–8 hours)	24 to 72 hours (acute cases). More than one week in chronic cases
Antigens			
Non-self (heterologous)	Self (autologous cells)	Self or non-self	Self or non-self
Antibody's role			
IgE	IgG, IgM	IgG, IgM	T cells (none Ig)
Complement system's role			
No	Yes or no	Yes	No
Cells involved in initiation			
Mast cells and basophils	None	None	APC, T helper cell 1
Inflammatory cells			
Eosinophils	Neutrophils	Neutrophils	Macrophages, T cells, keratinocytes
Mechanism			
First allergen exposure: IgE binds to mast cells	IgG, IgM antibodies cause cell injury by three mechanisms	Insoluble antigen–antibody (IgG, IgM) complexes are deposited in vessel wall and serosal surfaces or other tissues	Direct destruction of target antigenic cells by binding of cytotoxic T cells, FAS-related induction of apoptosis, and/or release of perforins and granzymes
Subsequent exposure to same allergen causes mast cell degranulation and release histamine, PAF and other chemical mediators that cause itching, bronchospasm, wheezing, shortness of breath and anaphylactic shock	▪ Direct cellular cytotoxicity ▪ Complement-mediated induced phagocytosis ▪ Autoantibodies block/inactivate cell surface receptors	▪ Immune complexes activate complement system resulting in chemotaxis of neutrophils ▪ PMN cells attempt to phagocytose immune complex resulting in liberation of hydrolytic enzymes and chemical mediators causing tissue damage	▪ T cell cytokine response activates macrophages and results in epithelioid granulomas formation (IFN-γ and TNF-α) ▪ T cell cytokine response activates mast cells (IL-3 and IL-5), synthesis of vasoproliferative factors (IL-3. and IL-8)
Chemical mediators			
▪ Early phase: histamine, ECF, PAF, heparin ▪ Late phase: LTC4, LTD4, LTDE4, TXA2, and prostaglandins	C3a, C5a: chemotaxis of neutrophil	C3a, C5a: chemotaxis of neutrophils	Monocyte chemotactic factors (MCF), monocyte inhibitory factors (MIF), IFN-γ, TNF-α, IL-3, GM-CSF
Target tissue			
Vascular endothelium, bronchial smooth muscle	Blood or tissue cells	Vascular endothelium and epithelial cells	Modified self, infected cells, allografts

Contd...

Table 4.51 Comparison of different hypersensitivity reactions (*Contd...*)

Type 1 Hypersensitivity Reaction	Type 2 Hypersensitivity Reaction	Type 3 Hypersensitivity Reaction	Type 4 Hypersensitivity Reaction
Primary results			
Vascular permeability, transudation, erythema, edema, wheal, bronchospasm, wheal and flare	Blood or tissue cell lysis, phagocytosis, inflammation, decreased serum complement	Necrotizing vasculitis (fibrinoid necrosis) vasoconstriction, thrombi, necrosis, decreased serum complement	Erythema, induration, granuloma formation
Histology			
Eosinophils Basophils	Antibody and complement	Complement and neutrophils/ macrophages	Monocytes/macrophages, lymphocytes
Beneficial effects			
Antiparasitic response and toxin neutralization	Direct lysis and phagocytosis of extracellular bacteria (gram-positive) and other susceptible bacteria and virus neutralization	Acute inflammatory neutrophils recruitment at the site of extracellular microbes and their clear microbes	Protection against fungi, intracellular bacteria (*M. tuberculosis*) and viruses, other intracellular pathogens
Pathologic disorders			
▪ Hay fever ▪ Urticaria ▪ Food allergy ▪ Drug allergy ▪ Insect venom allergy ▪ Angioneurotic edema ▪ Bronchial asthma ▪ Anaphylactic shock	▪ Autoimmune hemolytic anemia ▪ Immune thrombocytopenia ▪ Acute hemolytic transfusion reaction ▪ Hemolytic disease of the fetus and newborn ▪ Hashimoto's thyroiditis ▪ Pernicious anemia ▪ Systemic lupus erythematosus ▪ Hyperacute graft rejection ▪ Acute rheumatic fever ▪ Goodpasture syndrome ▪ Myasthenia gravis ▪ Pemphigus vulgaris ▪ Bullous pemphigoid	▪ Systemic lupus erythematosus ▪ Rheumatoid arthritis ▪ Immune complex-mediated glomerular diseases ▪ Serum sickness ▪ Arthus reaction ▪ Polyarteritis nodosa ▪ Farmer's lung disease	▪ Contact dermatitis ▪ Tuberculin skin test ▪ Chronic granulomatous diseases (tuberculosis, leprosy, *Histoplasma capsulatum*, sarcoidosis, Crohn's disease)

TYPE 1 HYPERSENSITIVITY REACTION

Type 1 hypersensitivity reaction is also known as an immediate reaction mediated by immunoglobulin E (IgE) attached to mast cells, which degranulate in response to subsequent exposure to soluble antigen or allergen and release histamine and other chemical mediators leading to tissue injury.

- Examples of IgE-mediated type 1 hypersensitivity reaction are hay fever (allergic rhinoconjunctivitis due to inhaled allergens), urticaria (hives), eczema, food allergy (nuts, shellfish, eggs, wheat), drug allergy (penicillin), insect venom allergy (bee, wasp), angioneurotic nonpitting subcutaneous edema over face, bronchial asthma, anaphylaxis (cutaneous and anaphylactic shock) and anaphylactic transfusion reactions in patients with IgA deficiency.
- **Anaphylaxis** is a rapid generalized immunologic reaction after exposure to antigens in a sensitized person, with at least two clinical manifestations: (a) airway compromise from swelling or wheezing, (b) hypotension or cardiovascular collapse, and (c) diffuse cutaneous findings (urticaria, angioedema, +/− erythroderma).
- Anaphylactoid reaction presents similar to anaphylaxis, expressed by similar chemical mediators, but not triggered by IgE and not necessarily due to prior exposure to the inciting antigen. Urticaria refers to diffuse patchy erythematous pruritic skin rashes with raised borders. Angioedema is nonpitting subcutaneous edema over face, mouth or peri-airway tissue.
- Type 1 hypersensitivity reaction is also referred to as atopy, which is triggered within minutes of subsequent exposure to a variety of environmental antigens such as pollen and house dust mite. Immediate hypersensitivity reaction has strong genetic link and caused by an overproduction of IgE. Common allergens classified by portal of entry associated with type 1 hypersensitivity reactions are given in Table 4.52.

Table 4.52 Common allergens classified by portal of entry associated with type 1 hypersensitivity reactions

Portal of Entry	Allergens
Inhalation	Plant pollens (rye grass, ragweed, timothy grass, birch trees), dust mold spores, dander, animal hair, formalin, drugs
Ingestion	Food (milk, peanuts, wheat, shellfish, soya beans, nuts, eggs), food additives, drugs (aspirin, penicillin, sulphonamide)
Contact	Cosmetics, heavy metals, detergents, formalin, rubber, solvents, dyes
Injections	Hymenopterans venom (bee, wasp, ant, dust mites, cockroach calyx), drugs, vaccines, foreign serum, enzymes, hormones

CATEGORIES OF IgE-MEDIATED TYPE 1 HYPERSENSITIVITY REACTION

Categories of IgE-mediated type 1 hypersensitivity reactions include allergic (atopic) and anaphylaxis. Allergic (atopic) disorders most commonly affect the nose, eyes, skin and lungs, which include conjunctivitis, atopic dermatitis, urticaria, angioedema and allergic bronchial asthma. Anaphylaxis is a severe, potentially life-threatening allergic reaction, which can occur within seconds or minutes of exposure to allergens. Categories of IgE-mediated type 1 hypersensitivity reactions are given in Table 4.53.

Allergic (Atopic) Reactions

Allergic (atopic) reactions include hay fever (allergic rhinitis), bronchial asthma, urticaria (hives), eczema, food allergy, drug allergy. A positive family history of allergy is found in 50% of atopic individuals. Tissue damage and necrosis are present in acute inflammation, while absent in atopic reactions.

Allergic Rhinitis (Hay Fever)

Allergic rhinitis (hay fever) is most common disorder in adults, which is a seasonal reaction to airborne inhaled plant pollen or molds or house dust. Antigens inhaled react with the IgE attached to mast cells/basophils in the nasal mucosa, thereby triggering the release of histamine stored in cytoplasmic granules. Histamine increases the permeability of mucosal blood vessels, causing edema and sneezing. The targets are typically respiratory membranes. Patient presents with nasal congestion, sneezing, coughing, profuse mucus secretion, itchy red and teary eyes and mild bronchoconstriction.

Bronchial Asthma

Bronchial asthma is characterized by episodes of impaired breathing due to severe bronchoconstriction. The airways are responsive to minute amounts of inhaled allergens. Thick mucus plugs are present in airways. Patient presents with shortness of breath, wheezing, cough and ventilatory rales, which may have

Table 4.53 Categories of IgE-mediated type 1 hypersensitivity reactions

Diseases	Clinical Manifestations
Allergic (atopic) reactions	
Hay fever (allergic rhinitis) seasonal reaction to airborne inhaled plant pollen or molds or house dust	Nasal congestion, sneezing, coughing, profuse mucus secretion, itchy red and teary eyes and mild bronchoconstriction
Bronchial asthma	Bronchoconstriction and increased mucus secretion
Urticaria (hives)	Skin hives
Eczema (atopic dermatitis)	Itchy inflammation of skin characterized by dry scaly lesions on the face, scalp, neck, and inner surfaces of the limbs and trunk
Food allergy	Cramping, vomiting, diarrhea and abdominal pain
Drug allergy (penicillin, sulphonamide, aspirin, opiates, contrast media)	Affecting 5–10% of hospitalized patients
Anaphylaxis	
Cutaneous anaphylaxis (injection of allergen)	Wheal and flare reaction
Anaphylactic shock (injection of antibiotics or serum administration)	Acute potentially life-threatening characterized by rapidly progressive urticaria, bronchospasm, laryngeal edema, vasodilatation, increased vascular permeability, intravascular volume depletion and vascular shock with fatal outcome within a few minutes

fatal outcome. Bronchial asthma has been discussed in details under type 1 hypersensitivity reaction.

Atopic Dermatitis

Atopic dermatitis is an itchy inflammatory disease of skin, known as eczema. Sensitization occurs through ingestion, inhalation and occasionally skin contact with allergens. It usually begins in infancy with reddened, vesicular, encrusted skin lesions followed by dry scaly lesions on the face, scalp, neck, and inner surfaces of the limbs and trunk during in children and adults.

Food Allergy

Food allergens enter via ingestion, but can affect respiratory tract and skin. Gastrointestinal tract symptoms include vomiting, diarrhea and abdominal pain. In severe cases, young children present with failure to thrive and growth retardation.

Drug Allergy

Approximately 5–10% of hospitalized patients may develop drug allergy. Compounds implicated most often are antibiotics (penicillin), sulphonamide, aspirin, opiates, contrast media used in imaging techniques. Some forms of penicillin sensitivity occur due to the presence of small amounts of drug in meat, milk and other foods. Exposure to penicillium mold in the environment also causes drug allergy.

Anaphylaxis

Clinical types of anaphylaxis seen in human beings include cutaneous and systemic anaphylaxis. Cutaneous anaphylaxis is the wheal and flare reaction to the injection of allergen. Systemic anaphylaxis is an acute potentially life-threatening type 1 hypersensitivity reaction, which typically occurs within minutes but can occur up to an hour after re-exposure to the offending antigen. Clinical manifestations of anaphylactic shock are shown in Fig. 4.50.

- The allergens and route of entry are variable, i.e. injection of antibiotics or serum administration.
- Anaphylaxis is characterized by rapidly progressive urticaria (vascular swelling in the skin accompanied by itching), bronchospasm, laryngeal

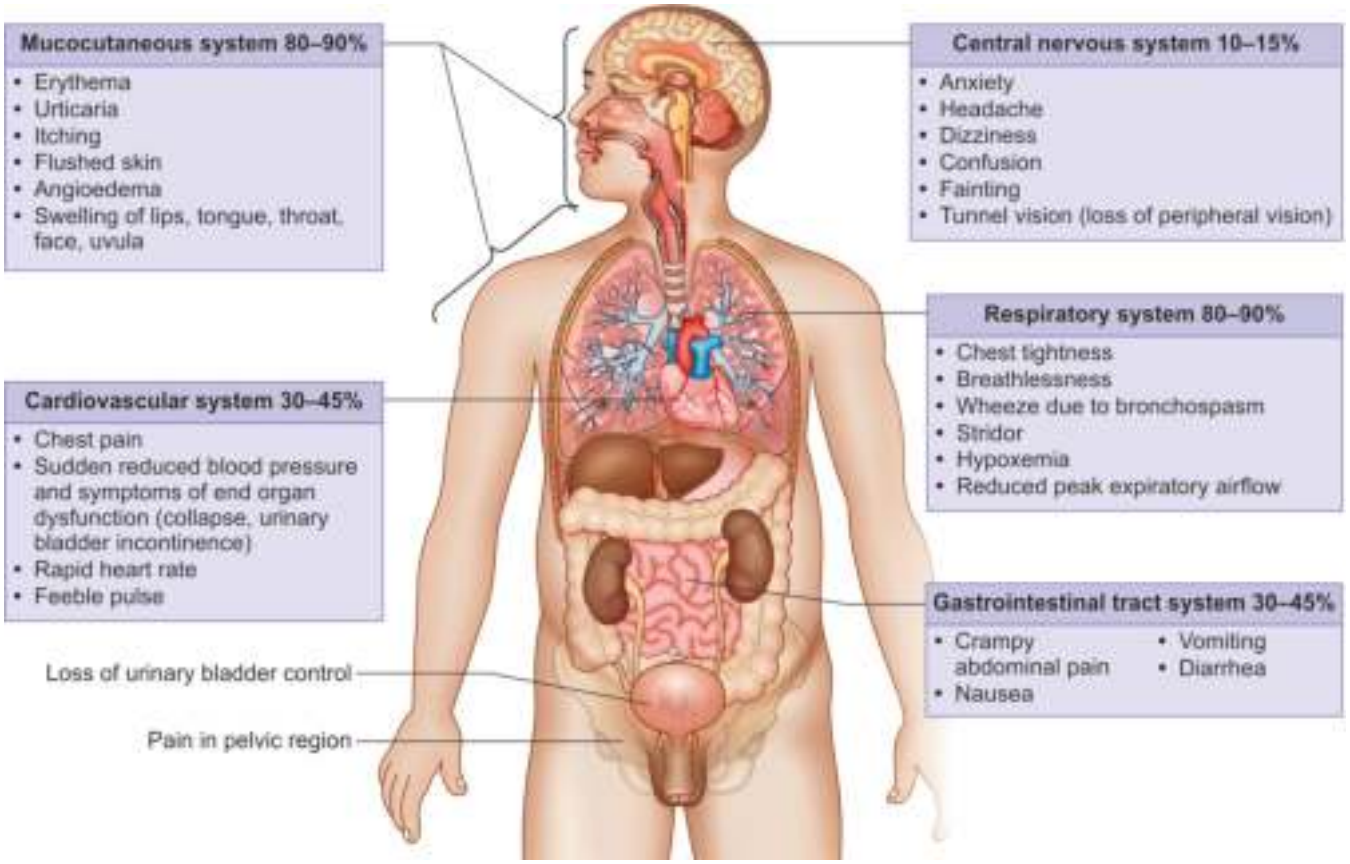


Fig. 4.50: Clinical manifestations of anaphylactic shock. Anaphylaxis is a serious, potentially life-threatening multi-systems allergic reaction on exposure to allergens such as foods, insect stings, medications. Anaphylactic shock is a complication of anaphylaxis that occurs when the blood pressure drops very low leading to poor tissue perfusion of the body. Patient presents with faintness, fat, shallow breathing, wheezing, fast heartbeat, clammy skin, confusion and low of consciousness.

edema, and vascular shock with fatal outcome within a few minutes.
- Children are more likely to experience food-related anaphylaxis, who develop severe stomach cramps, nausea, and diarrhea.
- Adults are more likely to experience anaphylaxis related to antibiotics, radiocontrast media, anesthetic agents and insect stings. Bee venom containing several allergens and enzymes may create sensitivity after exposure leading to anaphylaxis.

BRONCHIAL ASTHMA

Bronchial asthma is an example of type 1 hypersensitivity reaction, which occurs due to increased responsiveness of the airways to a variety of stimuli. Mast cells degranulation release chemical mediators, which stimulate bronchial mucus production and bronchoconstriction.
- Mast cells play central role in the development of type 1 hypersensitivity reaction. Mast cells are derived from circulating basophils and present near blood vessels and nerves in subepithelial tissue.
- Degranulation of mast cells and basophils is triggered by various stimuli such as anaphylatoxins (C4a, C3a, C5a), IL-8, physical stimuli (heat, cold and sunlight), drugs (morphine, codeine), bee venom (mallettin, adenine) and calcium ionophores.
- Mast cells release preformed and newly synthesized chemical mediators such as histamine, serotonin, leukotrienes and eosinophilic chemoattractant factor A (ECF-A) responsible for clinical manifestations of bronchial asthma.
- Differences between extrinsic and intrinsic bronchial asthma are given in Table 4.54. Pathogenesis of type 1 hypersensitivity reaction is shown in Fig. 4.51.

First Time Exposure to Antigen (Sensitization)

First time exposure to exogenous antigen (via skin, inhalation or ingestion), antigen processing cells (macrophages, dendritic cells) process the allergen (antigen) and present to CD4+ helper T cells that stimulate B cells to form immunoglobulin secreting plasma cells, which synthesize IgE that binds to high affinity Fc receptors on the surface of tissue mast cells and basophils.
- CD4+ helper T cells synthesize interleukins (IL-4, IL-5, and IL-6).
  - IL-5 that primes basophils to release histamine and leukotriene. IL-5 participates in maturation, chemotaxis, activation, and survival of eosinophils.
  - IL-6 promotes mucus production.
  - IL-4 switches B cells to IgE antibody synthesis.
- **Tumor necrosis factor-α** synthesized by macrophages activates neutrophils, attracts monocytes/macrophages and enhances production of other cytokines by T cells.

Subsequent Encounter to Same Exogenous Antigen

Subsequent encounter with same provocative dose of allergen (antigen) that binds to the IgE–mast cell complex results in influx of calcium, which is crucial process. Free antigen binds to two adjacent IgE antibodies (cross-linking) and triggers the mast cell degranulation leading to synthesis of preformed and newly synthesized chemical mediators.
- The reaction is amplified by platelet activating factor (PAF) that causes platelet aggregation and release of histamine, heparin and vasoactive and chemotactic factors attract eosinophils and neutrophils which release various hydrolytic enzymes that cause necrosis.
- Cell-bound IgE on the surface of mast cells and basophils of sensitized persons binds a substance

Table 4.54 Differences between extrinsic and intrinsic bronchial asthma

Characteristics	Extrinsic Asthma	Intrinsic Asthma
Definition	Caused by type 1 hypersensitivity reaction induced by exposure to an extrinsic antigen	Caused by diverse nonimmune mechanisms as a result of intrinsic stimuli
Age of clinical presentation	Childhood	Adult
Family history	Present	Absent
Preceding allergic reactions	Present in the form of rhinitis, urticaria, and eczema	Absent
Drug hypersensitivity	Absent	Present
Serum IgE level	Increased serum IgE level	Normal serum IgE level
Skin test	Positive skin test	Negative skin test
Emphysema	Unusual	Common
Associated chronic bronchitis	Absent	Present
Examples	▪ Atopic/allergic asthma ▪ Occupational asthma ▪ Allergic bronchopulmonary aspergillosis	Aspirin ingestion, pulmonary infection especially viral, cold, inhaled irritants, stress, exercise

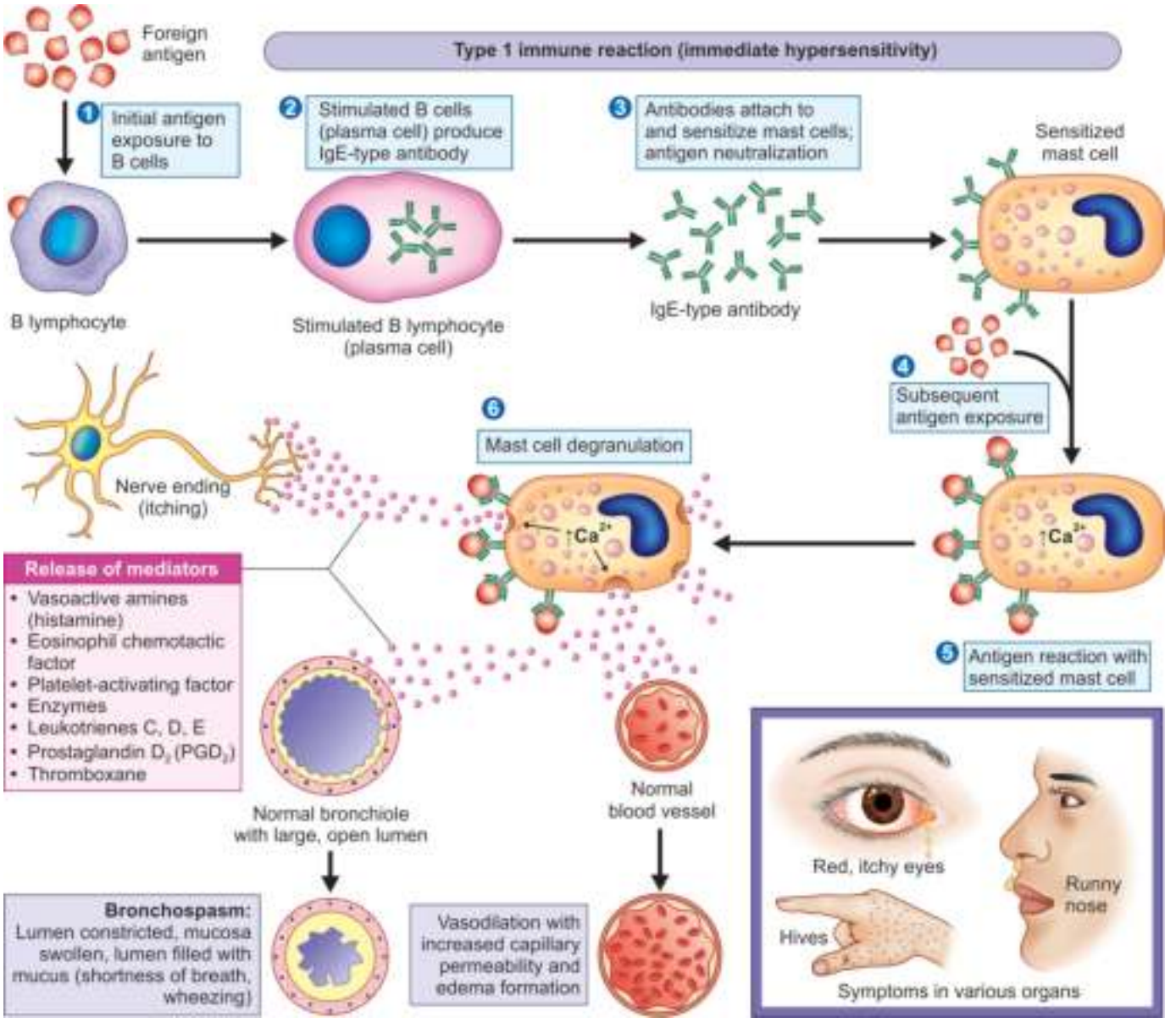


Fig. 4.51: Type 1 hypersensitivity reactions. Initial exposure to antigen (sensitizing dose) stimulates synthesis of IgE antibody. This IgE binds to surface of mast cells. On subsequent exposure to same antigen (provocative dose), it binds with IgE antibody attached to the mast cells and stimulate mast cells to release cytoplasmic granules containing chemical mediators responsible for itching, bronchospasm, wheezing, shortness of breath and anaphylactic shock.

called histamine releasing factor possibly produced by macrophages and B cells causing further release of histamine.

- Mast cells degranulation and their actions are shown in Fig. 4.52. Mast cells preformed and newly synthesized chemical mediators and their actions are given in Table 4.55.

Preformed Chemical Mediators of Mast Cells

Mast cells release preformed chemical mediators such as histamine, serotonin, leukotrienes, prostaglandin, platelet activating factor (PAF), heparin, tryptase and eosinophilic chemotactic factor (ECF), which induce smooth muscle contraction of bronchi, abdominal cramps, peripheral vasodilation, increased vascular permeability, hypovolemia and hypotension.

- Mast cell degranulation is preceded by increased Ca^{++} influx, which is crucial process. The reaction is amplified by platelet activating factor (PAF) that causes platelet aggregation and release of histamine, heparin and vasoactive and chemotactic factors attract eosinophils and neutrophils which release various hydrolytic enzymes that cause tissue necrosis.

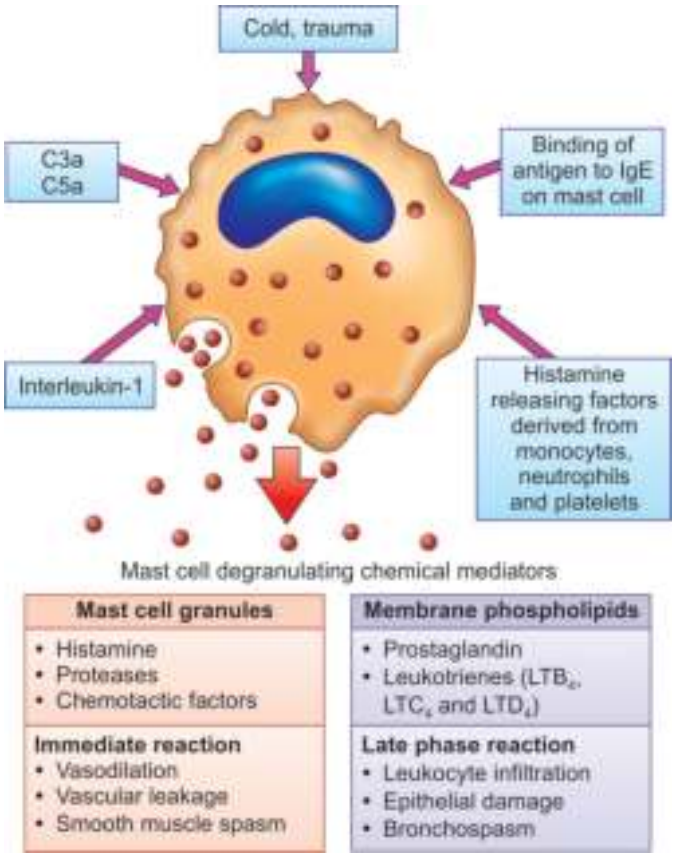


Fig. 4.52: Mast cells degranulation and their actions. Mast cell degranulation is a central event in the development of lesions in urticaria, and histamine levels are elevated in biopsies skin. Upon stimulation by an allergen, mast cells degranulate and release contents into surrounding tissues.

- The chemical mediators cause the **early phase** of allergic reactions that appears within minutes after exposure to the antigen. Late phase of allergic reactions may begin several hours after exposure to antigen.
- Cell-bound IgE on the surface of mast cells and basophils of sensitized persons binds a substance called histamine releasing factor possibly produced by macrophages and B cells causing further histamine release.
  - **Histamine:** Histamine is synthesized by mast cells, basophils and platelets,which is the most profuse and fastest-acting allergic chemical mediator. Histamine causes constriction of the smooth muscle layer of the bronchiole and intestine, thereby causing labored breathing. Histamine increases mucus secretion and synthesis of 'eotaxin', which attract **eosinophils**.
  - **Mast cell enzymes:** Mast cells release neutral protease, chymase, tryptase and hydrolytic enzymes, which cause tissue damage and proteolysis. These enzymes synthesize kinins and C3a. Tryptase is a good marker of mast cell activation.
  - **Eosinophilic chemotactic factor A (ECF-A):** Eosinophilic chemotactic factor A participates in chemotaxis of eosinophils and neutrophils. Major basic protein synthesized by eosinophils and hydrolytic enzymes by neutrophils cause significant tissue damage/necrosis in the late phases of allergic reactions.
  - **Kininogenase:** Kininogen causes vasodilation, vascular permeability and edema.

Newly Synthesized Chemical Mediators of Mast Cells

Mast cells also synthesize new chemical mediators such as leukotrienes, prostaglandins, platelet activating factor, adenosine, bradykinin and cyclic nucleotides.

- **Leukotrienes:** Leukotrienes are 1000 times more potent than histamine, but their actions are similar to histamine. LTC_4 and LTD_4 cause bronchoconstriction, increase vascular permeability and mucus secretion of respiratory airways. LTB_4 is chemotactic for eosinophils, PMN cells, basophils and monocytes.
- **Prostaglandins:** PGD_2 and $PGF_{2\alpha}$ cause bronchoconstriction. PGD_2 enhances histamine release from basophils, and causes edema and pain. PGD_2 inhibits platelet aggregation, whereas $PGF_{2\alpha}$ promotes platelet aggregation.
- **Platelet activating factor:** Platelet activating factor (PAF) causes bronchoconstriction, increased vascular permeability, and chemotaxis as well degranulation of eosinophils and neutrophils.
- **Adenosine:** Adenosine causes bronchoconstriction, which potentiates IgE-induced mast cell mediator release.
- **Bradykinin:** Bradykinin causes bronchoconstriction and increased vascular permeability.
- **Cyclic nucleotides:** Cyclic nucleotides modulate type 1 hypersensitivity reaction.

Phases of Allergic Reactions

Phases of allergic reactions are of two types: immediate and late. Immediate and late response in allergic reactions is given in Table 4.56.

Immediate Response in Allergic Reaction

Immediate allergic response occurs due to release of histamine, chemotactic factors for eosinophils, and proteases resulting into intense immediate reactions within 5–30 minutes. These chemical mediators produce tissue swelling by increasing vascular permeability, hypersecretion of bronchial mucosal glands and bronchoconstriction. Eosinophils are recruited in the tissues, which may be demonstrated by histopathologic examination of biopsy taken from allergic reaction site. Peripheral blood shows eosinophilia.

Table 4.55 Mast cells preformed and newly synthesized chemical mediators and their actions

Mediators	Actions	
Preformed chemical mediators of mast cells		
Histamine	▪ Bronchoconstriction ▪ Increased mucus secretion	▪ Synthesis of 'eotaxin', which attracts eosinophils
Enzymes (neutral protease, chymase and tryptase)	Tissue damage	
Eosinophil chemotactic factor A (ECF-A)	Chemotaxis of eosinophils	
Kininogenase	▪ Vasodilatation	▪ Increased vascular permeability and edema
Newly synthesized chemical mediators of mast cells		
Leukotrienes	▪ LTC_4, LTD_4 and LTE_4 (bronchoconstriction, increase vascular permeability and mucus secretion of respiratory airways)	▪ LTB_4 (chemotactic for eosinophils, PMN cells, basophils and monocytes)
Prostaglandins	▪ PGD_2 and $PGF_{2\alpha}$ cause bronchoconstriction	▪ PGD_2 stimulates basophils to release histamine that results in edema and pain
Platelet activating factor (PAF)	▪ Bronchoconstriction ▪ Increased vascular permeability	▪ Chemotaxis of eosinophils and neutrophils and degranulation
Adenosine	▪ Bronchoconstriction	▪ Potentiates IgE-induced mast cell mediator release
Bradykinin	▪ Bronchoconstriction	▪ Increased vascular permeability
Cyclic nucleotides	Modulation of immediate hypersensitivity reaction	

Table 4.56 Immediate and late response of allergic reactions

Parameters	Immediate Response	Late Phase Response
Onset of response	Within 5–30 minutes	Within 2–24 hours and lasts for several days
Chemical mediators	Histamine, chemotactic factors for eosinophils, and proteases	Prostaglandins and leukotrienes
Clinical manifestations	Tissue swelling by increasing vascular permeability, hypersecretion of bronchial mucosal glands and bronchoconstriction	Tissue destruction (mucosal and epithelial tissue)

Late Response in Allergic Reaction

Late allergic response occurs due to the synthesis and release of prostaglandins and leukotrienes, which enhance and prolong acute inflammatory reaction. Time interval between previous and subsequent exposure to same allergen can be many years. Allergen enters the body and produces changes in bronchopulmonary tree within 2–24 hours and lasts for several days. The tissues are infiltrated by eosinophils, neutrophils, monocytes and CD4+ helper T cells. Tissue destruction (mucosal and epithelial tissue) occurs in bronchopulmonary tree.

Actions of Chemical Mediators on Bronchopulmonary Tree

Chemical mediators synthesized by mast cells participate in bronchoconstriction, increased vascular permeability and chemotaxis of eosinophils. It is worth mentioning that histamine binds to histamine H1 receptors and induces bronchoconstriction and increased vascular permeability. Actions of chemical mediators in type 1 hypersensitivity reaction are given in Table 4.57.

- **Smooth muscle contraction:** Histamine, leukotrienes (LTC_4, LTD_4, and LTE_4), platelet activating factor (PAF), and prostaglandins intensify response by causing bronchoconstriction. Leukotrienes also act on alveoli.
- **Vascular changes:** Histamine, leukotrienes (LTC_4, LTD_4, and LTE_4), platelet activating factor (PAF), neutral protease and PGD_2 act on blood vessels. Histamine, prostaglandins and bradykinin increase vascular permeability resulting in fluid leakage into alveoli.
- **Cellular infiltration:** IL-4, IL-5, IL-6, TNF-α, LTB_4 and ECF-A participate in chemotaxis of eosinophilic and neutrophils.

Table 4.57 Actions of chemical mediators in type 1 hypersensitivity reaction

Chemical Mediators	Actions
Histamine, leukotrienes (LTC_4, LTD_4, and LTE_4), PAF, and prostaglandins	Bronchoconstriction
Histamine, prostaglandins and bradykinin	Increased vascular permeability
IL-4, IL-5, IL-6, TNF-α, LTB_4 and ECF-A	Chemotaxis of eosinophils and neutrophils

Pathology Pearls: Histologic Changes in Bronchial Asthma

Bronchi/Bronchioles Changes

Bronchi/bronchioles changes include bronchial smooth muscle hypertrophy, hyperplasia of goblet cells, thickening and hyalinization of basement membranes.

Bronchial Wall

Bronchial wall is infiltrated by eosinophils, mast cells, macrophages and lymphocytes.

Bronchial Lumen

- Bronchial mucosa shows edema, focal ulceration. Bronchial lumen is occluded with mucus plugs containing whorl-like accumulations of epithelial cells.
- 'Curschmann's spirals' and crystalloids of eosinophils-derived 'major basic protein and cationic proteins' coalesce to form 'Charcot-Leyden crystals' demonstrated in the sputum of bronchial asthma patients.

Clinical Pearls: Bronchial Asthma

Clinical Features

- Bronchial asthma attack may last for hours or even days.
- Due to narrowing of airways, patient presents with sudden dyspnea, episodic expiratory wheezing (inspiratory in severe cases), and tightness in the chest, nocturnal coughing, and tachypnea with use of accessory muscles for breathing.
- After the bronchial asthmatic attack is over, there is prolonged coughing up of tenacious secretions.
- Anteroposterior diameter of chest is increased due to air trapping and increase in residual volume. Initially, patient develops respiratory alkalosis. If bronchospasm is not relieved, patient may develop respiratory acidosis. Such patients need tracheal intubation and mechanical ventilation.

Complications

- Patient may develop pneumothorax, pneumomediastinum, status asthmaticus and fatal outcome.
- When severe acute asthma is unresponsive to therapy. Patient is referred to as status asthmaticus.
- Light microscopy of lung in status asthmaticus often shows a bronchus containing a luminal mucous plug, submucosal gland hyperplasia, smooth muscle hyperplasia, basement membrane thickening, and increased numbers of eosinophils.

Therapeutic Agents

- Therapeutic agents such as epinephrine, isoproterenol and phenoxybenzamine increasing intracellular cAMP provide relief in bronchial asthma.
- Therapeutic agents such as norepinephrine, phenylephrine, propranolol, acetylcholine and carbachol decreasing intracellular cAMP or stimulate cGMP aggravate these allergic conditions. Relationship between allergic symptoms and cyclic nucleotides are given in Table 4.58.

Diagnostic Tests

Diagnosis of allergy can be made by skin scratch testing. IgE estimation and histamine release test on basophils.

- **Skin scratch testing:** The forearm or back is mapped and then injected with a selection of allergen extracts. The allergist must be fully aware of potential anaphylaxis attacks triggered by these injections. Approximately 20 minutes after antigenic challenge, histamine-mediated wheal-and-flare response is studied depending on size on a scale of 0 (no reaction) to 4+ (>1.5 cm).
- **Radioallergosorbent test (RAST):** Radioallergosorbent test is used to detect specific IgE antibodies in serum formed against suspected allergen.

Table 4.58 Relationship between allergic symptoms and cyclic nucleotides

Allergic Symptoms	Molecular Level	Therapeutic Agent	Mechanism of Action
Improvement of allergic symptoms	Elevation of cyclic-AMP	▪ Epinephrine or isoproterenol ▪ Phenoxybenzamine	▪ Stimulation of β-adrenergic receptors ▪ Blockage of α-adrenergic receptors
Worsening of allergic symptoms	Lowering of cAMP or elevation of cGMP	▪ Norepinephrine, or phenyl-epinephrine ▪ Propranolol ▪ Acetylcholine or carbachol	▪ Stimulation of α-adrenergic receptors ▪ Blockage of β-adrenergic receptors ▪ Stimulation of γ-cholinergic receptors

- **Enzyme-linked immunosorbent assay (ELISA) test:** Enzyme-linked immunosorbent assay test is used to estimate IgE antibodies in an atopic condition. IgE elevation is also seen in multiple myeloma and helminths infestations.

Therapeutic Correlation

Various drugs are administered to interrupt allergic response at certain points, i.e. interfering action of histamine, release of chemical mediators from mast cells; and inflammation. Therapeutic agents used to relieve bronchopulmonary symptoms in bronchial asthma are given in Table 4.59.

- **Antihistaminic drugs:** Antihistaminic drugs such as azelastine, cetirizine, desloratadine, and fexofenadine are beneficial in patients of bronchial asthma. These drugs act by blocking receptors expressed on mast cells thus inhibit mast cell degranulation. These drugs have some side effects.
- **Glucocorticoids and disodium cromoglycate:** These agents prevent mast cells degranulation by inhibiting transmembrane influx of calcium ions needed to trigger mast cells degranulation.
- **Singulair or accolate agents:** These agents block the leukotriene receptors expressed on target cells. It is worth mentioning that leukotrienes cause bronchoconstriction.
- **Zileuton agent:** Zileuton drug prevents synthesis of prostaglandins, TXA_2 and prostacyclin by inhibiting cyclooxygenase pathway.
- **Terbutalin or albuterol agents:** These are short acting bronchodilators derived from isoproterenol.
- **Theophylline agent:** Theophylline inhibits cAMP-phosphodiesterase and intracellular Ca^{++} release resulting in elevation of cyclic adenosine monophosphate (cyclic AMP) in the cells. Through the action of protein kinase A enzyme, cyclic AMP activates target enzymes in the cells and opens ion channels in the cell membrane. The final result is smooth muscle relaxation and bronchodilation.
- **Desensitization therapy:** Administration of purified allergens repeatedly block the primary sensitization process. Desensitization by injecting purified allergens and drug therapy to prevent allergy is shown in Fig. 4.53. The blocking antibody mechanism for allergic desensitization is shown in Fig. 4.54.

Table 4.59 Therapeutic agents used to relieve bronchopulmonary symptoms in bronchial asthma

Therapeutic Agents	Mechanism of Action
Antihistaminic drugs	Blocking receptors expressed on mast cells
Glucocorticoids and disodium cromoglycate	Preventing mast cells degranulation by inhibiting transmembrane influx of calcium ions needed to trigger degranulation
Singulair or accolate agents	Blocking the leukotriene receptors expressed on target cells (leukotrienes cause bronchoconstriction)
Zileuton agent	Preventing synthesis of prostaglandins, TXA_2 and prostacyclin by inhibiting cyclooxygenase pathway
Terbutaline or albuterol agents	Short acting bronchodilators derived from isoproterenol
Theophylline agent	▪ Elevation of cAMP by inhibiting cAMP-phosphodiesterase ▪ Inhibiting intracellular Ca^{++} release
Desensitization therapy	Administration of purified allergens repeatedly to block the primary sensitization process

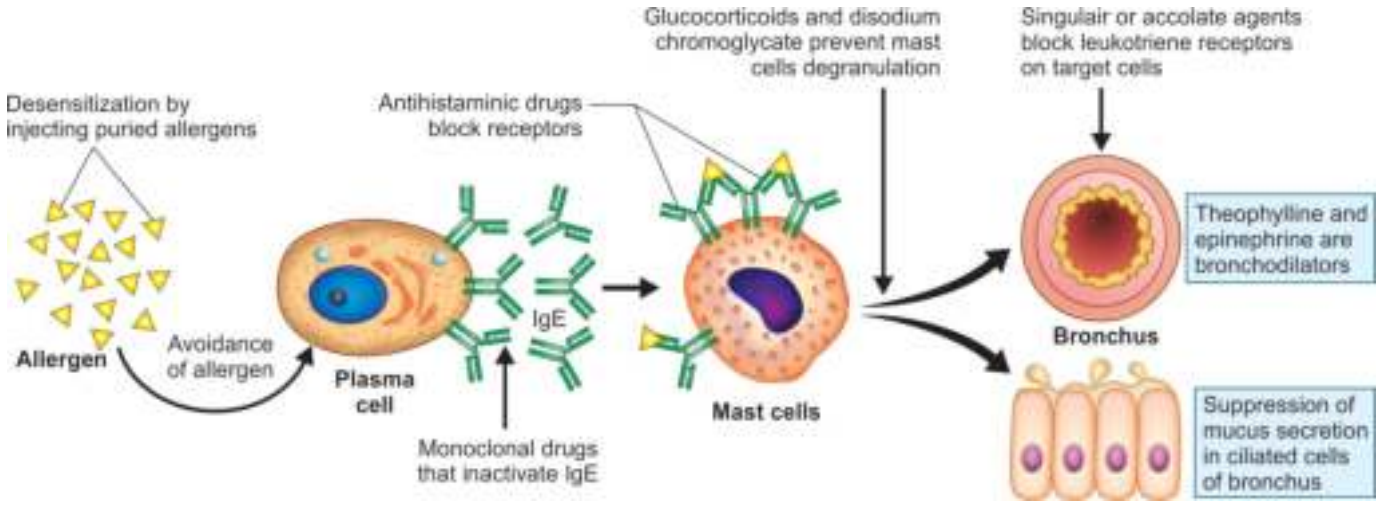


Fig. 4.53: Desensitization by injecting purified allergens and drug therapy to prevent allergy. Type 1 hypersensitivity is interrupted by injecting purified allergens and various drugs to prevent mast cells degranulation that interrupt release of inflammation inducing chemical mediators.

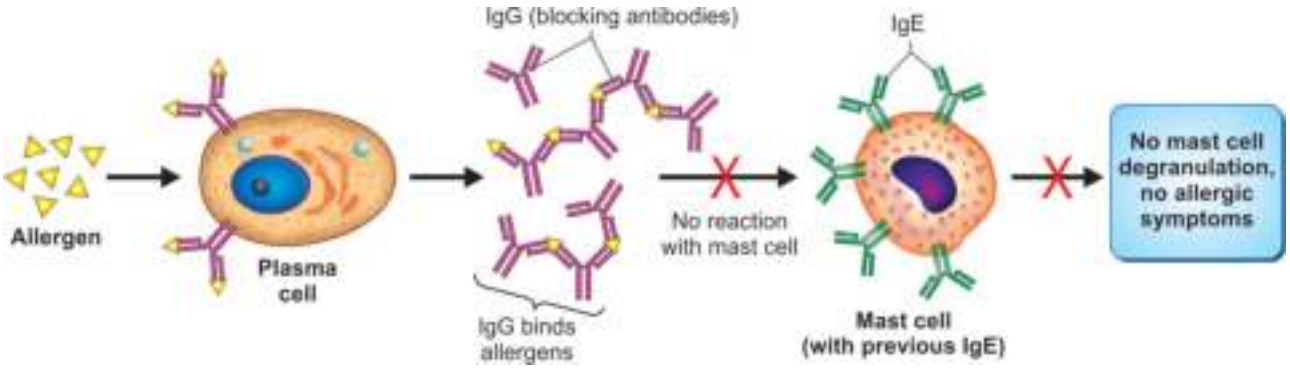


Fig. 4.54: The blocking antibody mechanism for allergic desensitization. An injection of allergen causes IgG antibodies to be formed instead of IgE; these blocking antibodies cross-link and effectively remove allergen before it can react with IgE in the mast cells.

TYPE 2 HYPERSENSITIVITY REACTION

Type 2 hypersensitivity reaction is also known as cytotoxicity hypersensitivity reaction. Antigen-presenting cells (macrophages and dendritic cells) process cell-bound antigen either self antigen (intrinsic) or planted (extrinsic usually a drug such as benzylpenicillin or methyldopa) antigen bound to cell membrane or basement membrane; and present to CD4+ helper T cells resulting in production antibody secreting plasma cells.

- IgG and IgM mistakenly bind to surface antigens on the cells in the body, which are responsible for a number of autoimmune disorders limited to a particular organ/tissue damage in one of three mechanisms: (a) antibody-dependent cellular cytotoxicity, (b) complement-mediated increased susceptibility to phagocytosis of target cells, and (c) Fc-receptor-mediated antibody-dependent cytotoxicity.
- Examples of type 2 hypersensitivity reaction include autoimmune hemolytic transfusion reaction, hemolytic disease of the fetus and newborn, autoimmune hemolytic anemia, immune thrombocytopenia, drug-induced neutropenia and agranulocytosis, Hashimoto's thyroiditis, pernicious anemia, hyperacute transplant rejection, rheumatic fever, Goodpasture syndrome, Graves' disease, Lambert-Eaton myasthenic syndrome, pemphigus vulgaris, bullous pemphigoid.
- Clinical features, diagnostics and treatment of type 2 hypersensitivity reaction depend on the underlying etiology. Diagnosis may involve autoantibody testing and Coombs' test. Pathogenesis of type 2 hypersensitivity reaction is shown in Fig. 4.55A to C.

ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

Preformed antibodies IgG or IgM in low concentration bind to cell bound intrinsic antigen and coat it. Fc portion of IgG or IgM binds to Fc receptors on cytotoxic leukocytes such as natural killer cells (most important), macrophages, neutrophils and eosinophils. These cytotoxic leukocytes release hydrolytic enzymes and oxygen-derived free radicals resulting to tissue damage. Antibody-dependent cellular cytotoxicity mechanism in type 2 hypersenstivity reaction does not involve fixation of complement. Diseases caused by antibody-dependent cellular cytotoxicity (ADCC) mechanism in type 2 hypersensitivity reaction are given in Table 4.60.

Antibody-dependent Cellular Cytotoxicity Disorders

Antibody-dependent cellular cytotoxicity disorders include systemic lupus erythematosus, Hashimoto's thyroiditis, and acute rheumatic fever. Treatment involves anti-inflammatory and immunosuppressive agents.

Systemic Lupus Erythematosus

Autoantibodies develop to range of nuclear antigens in systemic lupus erythematosus (SLE), and causes non-organ specific autoimmune disease. Defective clearance of apoptotic cells and immune complexes are important contributors to the development of SLE. The loss of immune tolerance, increased antigenic load, excess CD4+ helper T cell, defective B cell suppression and shifting of CD4+ T helper 1 cell (Th1 cell) and CD4+ T helper 2 cell (Th2 cell) immune responses leads to B cell hyperreactivity and production of pathogenetic autoantibodies. Finally, certain environmental factors may be required to trigger the disease.

Hashimoto's Thyroiditis

Hashimoto's thyroiditis often affects HLA-DR5 and HLA-B5 persons, which is an autoantibody dependent lymphocytic cytotoxicity disorder, in which anti-TSH receptor autoantibody of IgG class to cell surface antigen stimulate natural killer cells, lymphocytes, which destroy sensitized cells. Circulating autoantibodies (anti-TSH receptor, anti-thyroglobulin, anti-peroxidase, anti-microsomal and anti-iodine receptor autoantibodies) are demonstrated in Hashimoto's thyroiditis.

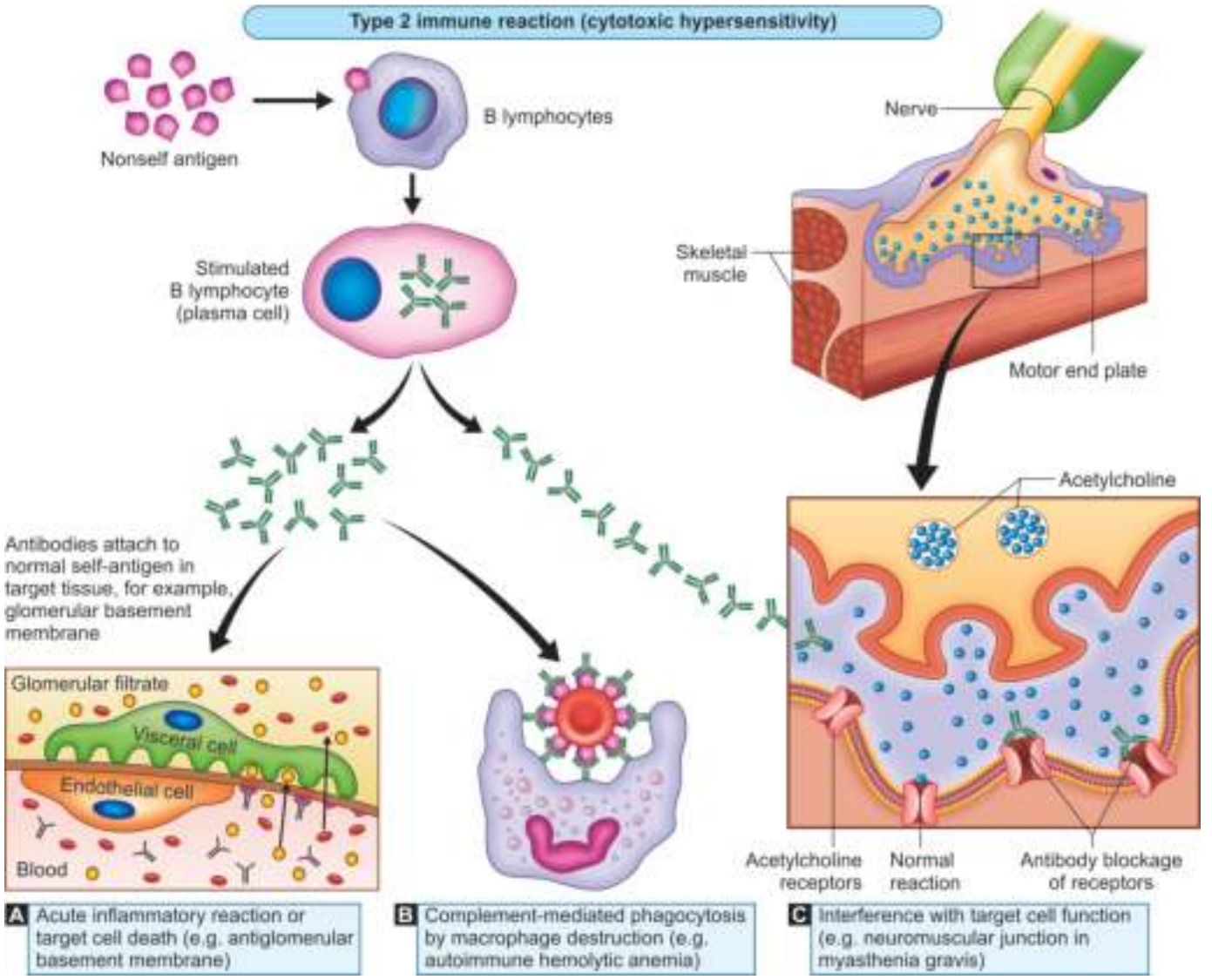


Fig. 4.55: Pathogenesis of type 2 hypersensitivity reaction. (A) Target tissue inflammation or cell death. Antibodies attach to target antigen, which is destroyed by inflammation (glomerular basement membrane) or phagocytosis (RBCs), (B) complement-mediated phagocytosis by macrophage resulting in tissue damage, (C) interference with target cell function. Antibodies attach to target cell receptors and interfere with function (blockage of signal transmission from nerves to muscle in myasthenia gravis.

Table 4.60 Diseases caused by antibody-dependent cellular cytotoxicity (ADCC) mechanism in type 2 hypersensitivity reaction

Disease	Target Antigens	Characteristics
Systemic lupus erythematosus	▪ Systemic ▪ Nuclear antigens (DNA to anti-DNA)	Inflammation of many organs, antibodies against red and white blood cells, platelets, clotting factors, nucleus DNA
Hashimoto's thyroiditis	Thyroid gland	Destruction of the thyroid follicles
Acute rheumatic fever	Antibodies against streptococcal cell wall cross react with myocardial and joint antigens	Pancarditis and migratory polyarthritis

Type 2 and 3 hypersensitivity reactions participate in the pathogenesis of systemic lupus erythematosus.

Acute Rheumatic Fever

Autoantibodies develop against streptococcal cell wall (Group A Streptococcus-β hemolyticus) cross react with myocardial antigens and joints. This multisystem autoimmune disorder affecting periarteriolar connective tissue.

- Patient develops pancarditis, transient mild migratory polyarthritis, chorea, erythema marginatum and subcutaneous nodules.
- Diagnostic tests for acute rheumatic fever include detection of circulating antibody against the tissues involved and the presence of antibody in the lesion (biopsy) by immunofluorescence microscopy.

COMPLEMENT-MEDIATED WITH INCREASED SUSCEPTIBILITY TO PHAGOCYTOSIS OF TARGET CELLS

Complement-mediated with increased susceptibility to phagocytosis of target cells is also known as complement-fixing antibody-mediated cytotoxicity. Antibodies (IgG, IgM) bind to fixed antigens localized to tissue, platelets and red blood cell membranes resulting in complement activation by classical pathway. The cells opsonized by antibodies and complement proteins are destroyed by splenic macrophages. Serum complement is characteristically decreased in these patients. Diseases caused by complement-mediated with increased susceptibility to phagocytosis of target cells in type 2 hypersensitivity reactions are given in Table 4.61.

Goodpasture Syndrome

Goodpasture syndrome is characterized by nephritic syndrome and pulmonary hemorrhage (hemoptysis). Goodpasture antigen is normally present in glomerular basement membrane and lung alveoli. Autoantibodies cross react with antigens located in glomerular basement membrane and pulmonary alveoli. Immunofluorescence study reveals smooth and linear staining pattern due to antibody and complement in renal and lung basement membrane of Goodpasture syndrome.

Drug-induced Autoimmune Hemolytic Anemia or Thrombocytopenia

Sometimes a drug or its metabolite such as benzylpenicillin may bind firmly to the cell surface to give a highly immunogenic epitope. Individuals produce IgG cytotoxic autoantibodies to their own drug coated red blood cells or platelets resulting in their destruction, especially in the spleen. Patients present with anemia and thrombocytopenia-related manifestations.

Warm Antibody-mediated Autoimmune Hemolytic Anemia

IgG autoantibody is reactive at 37°C '**warm autoantibody**' and causes autoimmune disorders. IgM autoantibody is active at 4°C, which becomes less active at higher temperature; but is still able to bind complement and agglutinate red blood cells at the temperature 30°C of the peripheral tissues (e.g. hands, feet, nose and ears).

- RBCs coated by IgG or IgM autoantibodies become bound to receptors on macrophages because they have receptors for the Fc fragments of immunoglobulins. Once activated, complement system cascade leads to the destruction of the red blood cells through formation of a membrane attack complex (MAC).
- IgM antibodies, which are powerful agglutinins, may agglutinate red blood cells in the red pulp of spleen, resulting in cellular destruction. IgM may also activate complement, resulting in cellular lysis.
- Such autoantibodies are detected by Coombs' test (agglutination test).

Table 4.61 Diseases caused by complement-mediated with increased susceptibility to phagocytosis of target cells in type 2 hypersensitivity reactions

Disease	Target Antigens	Characteristics
Goodpasture syndrome	Goodpasture antigen in noncollagenous proteins in basement membrane of kidney glomeruli and lung alveoli	Antibodies to basement membrane of glomeruli and alveoli
Drug-induced (benzylpenicillin) autoimmune hemolytic anemia or thrombocytopenic purpura	RBCs or platelets membrane proteins	Antibodies to surface RBCs or platelets. Opsonization and phagocytosis of RBCs and platelets
Warm antibody-mediated hemolytic anemia IgG autoantibody is reactive at 37°C	RBCs coated by IgG or IgM autoantibodies become bound to receptors of macrophages because they have receptors for the Fc fragments of immunoglobulins	Antibodies to surface RBCs
Blood transfusion reactions	IgG or IgM antibodies attach to the foreign cells of donor, resulting in complement fixation	The resultant formation of membrane attack complexes lyses the donor cells
Hemolytic disease of newborn or erythroblastosis fetalis	RBCs membrane proteins (Rh blood group antigen)	Opsonization and phagocytosis of RBCs results in hemolytic disease of newborn

Blood Transfusion Reactions

In blood transfusion reactions, recipient humans may become sensitized to special antigens on the surface of red blood cells of donor humans. Most common type 2 hypersensitivity reaction occurs when mismatched blood is transfused to the recipient's ABO typing or IgM antibodies attach to the foreign cells, resulting in complement fixation, formation of membrane attack complexes and lysis of donor cells. Crossmatching of donor and recipient blood is necessary to determine which transfusions are safe to perform.

Hemolytic Disease of Fetus (Erythroblastosis Fetalis) or Newborn

Hemolytic disease of fetus or newborn most commonly occurs with Rh blood group incompatibility between mother and fetus, which occurs when the mother is Rh– and baby is Rh+, having inherited an allele for one of the Rh antigens from the father. An Rh –ve person, if exposed to Rh +ve blood, will form specific IgG antibodies against the Rh antigens. Therefore, Rh group should also be matched before transfusion. Hemolytic disease of the fetus or newborn is shown in Fig. 4.56.

- **During first pregnancy, at birth of newborn:** A small quantity of fetal red blood cells usually leaks across the placenta into the maternal bloodstream. Upon exposure to Rh antigen, the mother's immune system responds by making anti-Rh antibodies. Because the baby is already born, it suffers no damage. Prevention of hemolytic disease of fetus or newborn involves therapy with Rh immune globulin after first delivery.
- **During subsequent pregnancy:** The maternal antibodies cross the placenta into the fetal red blood cells. If the second foetus is Rh+, the ensuring antigen–antibody reaction causes hemolysis of fetal RBCs.
  - The result is hemolytic disease of fetus or newborn (HDN). Newborn baby develops severe anemia and jaundice.

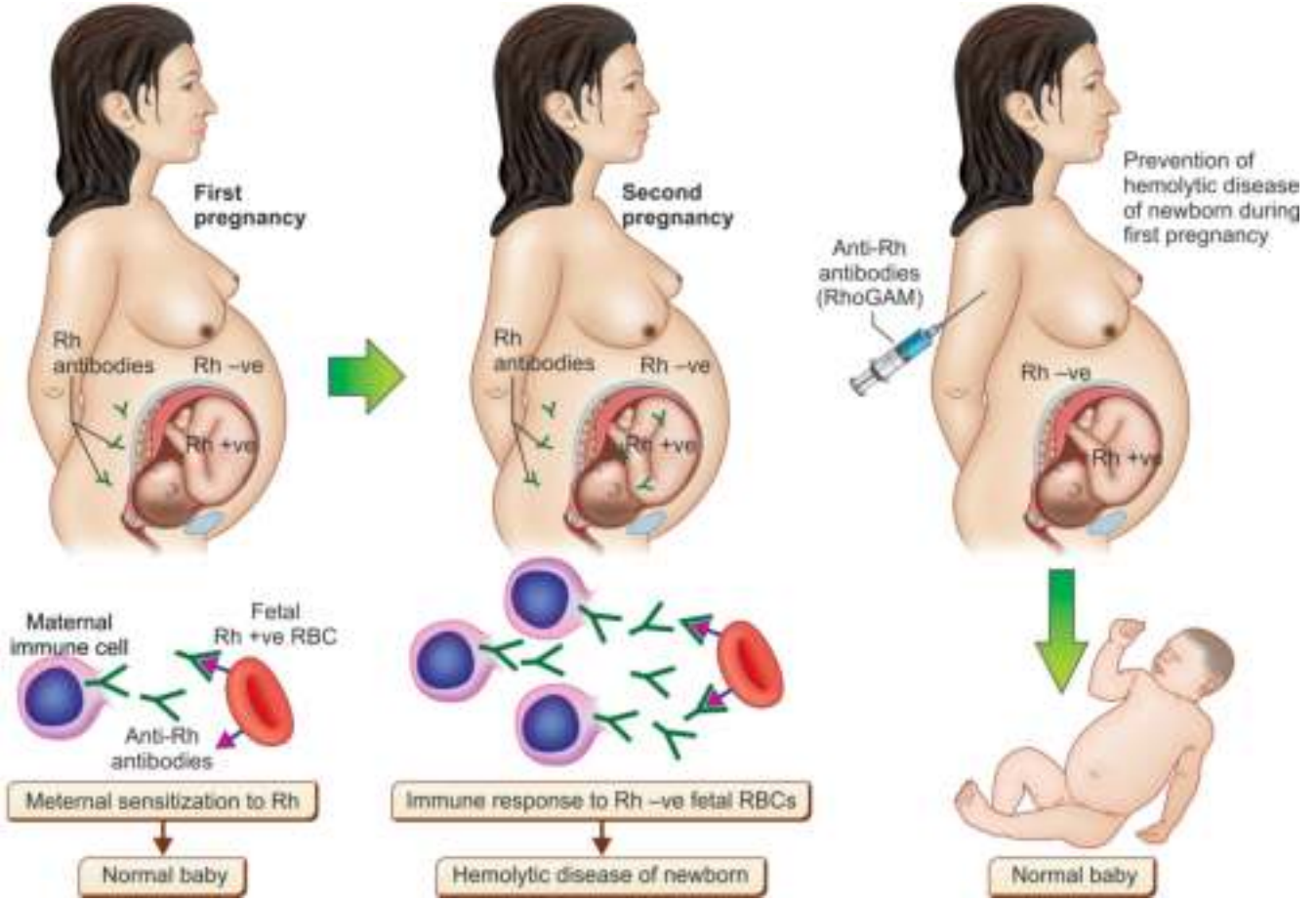


Fig. 4.56: Hemolytic disease of the fetus or newborn. Rh factor incompatibility can cause lysis of red blood cells. A naturally occurring red blood cell incompatibility results when a fetus Rh +ve develops within Rh –ve mother, initial sensitization of the maternal immune system occurs when fetal blood passes the placental barrier. In most cases, the fetus develops normally. However, a subsequent pregnancy with Rh +ve fetus results in a severe fetal red blood cells hemolysis. Control of incompatibility is done by administration of anti-Rh antibody (RhoGAM) to Rh –ve mothers during pregnancy to inactivate and remove any Rh factor that may be transferred from the fetus in maternal circulation. In some cases, anti-Rh antibody (RhoGAM) is administered before sensitization occurs.

- Development of cardiac failure in newborn is called erythroblastosis fetalis.
- Direct Coombs' test detects IgG and/or C3b attached to RBCs. Indirect Coombs' test detects antibodies in serum (e.g. anti-D).

ANTI-RECEPTOR ANTIBODY-MEDIATED TYPE 2 HYPERSENSITIVITY REACTION

In some persons, autoantibodies are formed against cell surface receptors, which impair normal cellular functions of receptors without causing cell injury. Examples of anti-receptor antibody-mediated type 2 hypersensitivity reaction include Graves' disease, myasthenia gravis, insulin-resistant diabetes mellitus, pernicious anemia, pemphigus vulgaris, Lambert-Eaton myasthenic syndrome and rheumatoid arthritis. Diseases caused by anti-receptor antibody-mediated type 2 hypersensitivity reaction are given in Table 4.62.

Graves' Disease

Antibodies to the TSH receptor are seen in patients with Graves' disease an autoimmune disorder that mimic the action of TSH, but are not regulated by natural negative feedback controls, which leads to thyroid follicular hyperplasia and increased synthesis of thyroid hormones resulting to features of hyperthyroidism.

- Patient also develops **exophthalmos** due to infiltration by lymphocytes in retrobulbar ocular muscles in 60–90%.
- Circulating autoantibodies demonstrated in Graves' disease are thyroid stimulating immunoglobulin (**TSI**) mimicking TSH), thyroid growth immunoglobulin (**TGI**), antimicrosomal and antithyroglobulin.

CTL4 or PTPN22 gene mutation increases risk of Graves' disease.

Myasthenia Gravis

Myasthenia gravis is a type 2 hypersensitivity disorder caused by autoantibodies that bind to the acetylcholine receptors in the motor end plates of skeletal muscles. The autoantibodies bind to postsynaptic receptors and block neurotransmission resulting to progressive skeletal muscle weakness involving particularly the external ocular, eyelids and proximal limb muscles. Myasthenia gravis may cause death due to respiratory muscles paralysis. Pathogenesis of myasthenia gravis is shown in Fig. 4.57A and B.

Lambert-Eaton Myasthenic Syndrome

Lambert-Eaton myasthenic syndrome (LEMS) is a rare autoimmune disease of presynaptic neuromuscular transmission due to impaired acetylcholine and autoantibodies directed against calcium channel, which results in the gradual onset of muscle weakness in patients associated with small cell lung carcinoma. Paraneoplastic syndrome manifestations of small cell lung carcinoma are often mistaken for myasthenia gravis.

Insulin-resistant Type 2 Diabetes Mellitus

Antibody inhibits binding of insulin to the insulin receptors on adipose cells and skeletal muscles leading to insulin-dependent diabetes mellitus.

- Obesity, lack of physical activity, cigarette smoking, and genetic predisposition contribute to insulin-dependent diabetes mellitus. Patient develops hyperglycemia prone to ketoacidosis.

Table 4.62 Diseases caused by anti-receptor antibody-mediated type 2 hypersensitivity reaction

Disease	Target Antigens	Characteristics
Graves' disease	Thyroid gland	Antibodies against thyroid-stimulating hormone receptors
Myasthenia gravis	Acetylcholine receptors at neuromuscular junction	Antibody inhibits acetylcholine binding, down-modulation receptors
Insulin-dependent diabetes mellitus	Pancreas	Antibodies cause destruction of insulin secreting cells
Pernicious anemia	Stomach lining	Antibodies against intrinsic factor synthesized by parietal cells prevent transport of vitamin B_{12}
Pemphigus vulgaris	Desmoglein-3 proteins in intercellular junctions of epidermis	Antibody-mediated activation of proteases, disruption of intercellular adhesions results in skin vesicles (bullae)
Lambert-Eaton myasthenic syndrome	Calcium channels	Antibodies to calcium channels formed in patients with Lambert-Eaton myasthenic syndrome associated with small cell carcinoma of the lung resulting to muscle weakness
Rheumatoid arthritis	Fc portion of IgG	Rheumatoid factor represents multiple antibodies directed against the Fc portion of IgG and is seen in patients with rheumatoid arthritis and many other collagen vascular diseases

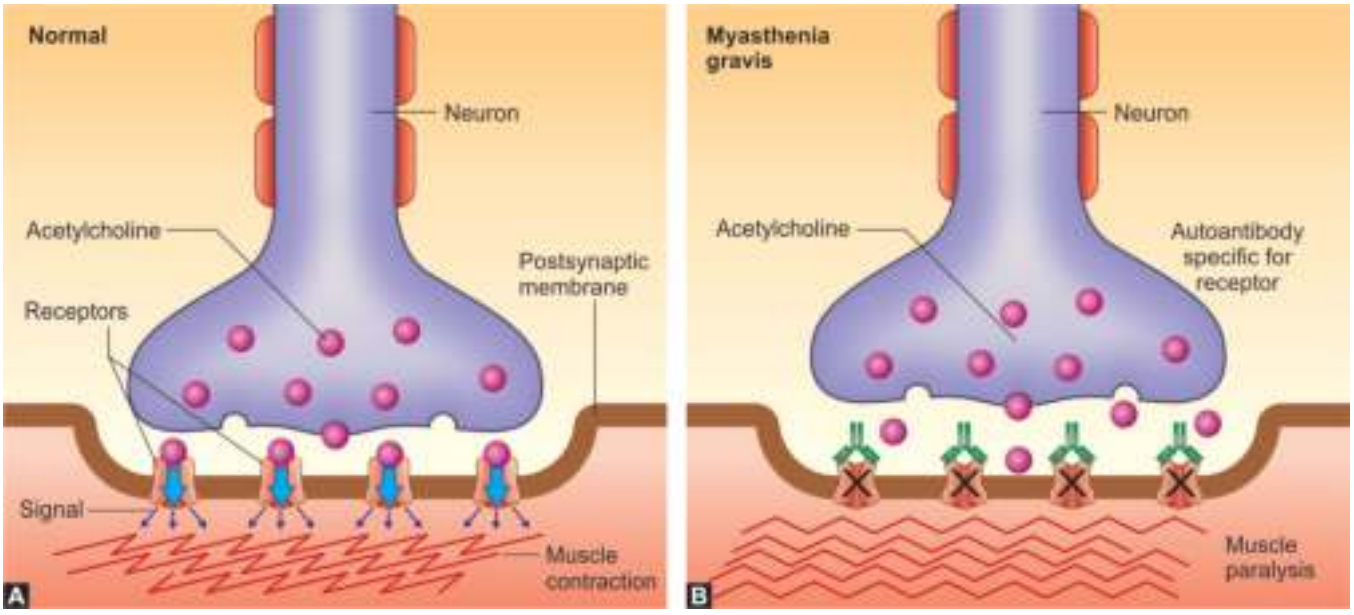


Fig. 4.57: Mechanism for involvement of autoantibodies in myasthenia gravis. (A) Normal neuromuscular junction, (B) in myasthenia gravis, antibodies formed against receptors on the postsynaptic membrane block them so that acetylcholine cannot bind resulting in inhibition of muscle contraction.

- Diagnostic tests such as standard fasting blood glucose and glycosylated hemoglobin (HbA1c) measure the average amount of glucose in the bloodstream over a period of three months in diabetic patients. HbA1c is analyzed in percentages, under 5.7% is considered healthy, a result 5.7–6.4% suggests prediabetes and higher than 6.5% is diabetes mellitus.

Pernicious Anemia

Pernicious anemia is important cause of megaloblastic anemia resulting from vitamin B_{12} deficiency due to lack of intrinsic factor (IF) normally synthesized by gastric parietal cells. Impaired intrinsic factor production can occur in adults due to autoimmune destruction of gastric parietal cells, which synthesize intrinsic factor. Gastrectomy can significantly reduce the production of intrinsic factor.

Rheumatoid Arthritis

Rheumatoid arthritis is an autoimmune disease that causes painful swelling of bilateral joints of the fingers, hands, wrists, knees, ankles, feet and toes.

- A number of genetic and environmental factors play role in the pathogenesis of rheumatoid arthritis.
- Rheumatoid factor (RF) represents multiple antibodies directed against the Fc portion of IgG and seen in patients with rheumatoid arthritis and many other collagen vascular diseases.
- Patients present with pain, swelling, stiffness and tenderness in many joints, stiffness especially in the morning hours, fatigue, weakness and fever.
- About 80% of rheumatoid arthritis cases are positive for rheumatoid factor (RF). Antibodies to cyclic citrullinated peptides are demonstrated in 60–70% of patients. Elevated erythrocyte sedimentation rate confirms inflammation of joints.

Pemphigus Vulgaris

Autoantibodies to desmoglein-3 disrupt intercellular junctions in epidermis are found in patients with pemphigus vulgaris, an autoimmune skin disorder associated with formation of blistering (vesicles). Immunofluorescence study reveals smooth and linear staining pattern in pemphigus vulgaris (skin intercellular protein, desmosomes).

TYPE 3 HYPERSENSITIVITY REACTION

Type 3 hypersensitivity reaction, also referred to as immune complex-mediated reaction, is characterized by immune complex deposition, complement fixation, and localized inflammation of tissues/organs. Examples of type 3 hypersensitivity reaction include Arthus reaction, serum sickness, systemic lupus erythematosus, immune complex-mediated glomerular diseases (poststreptococcal glomerulonephritis, membranous glomerulonephritis and lupus nephropathy), and polyarteritis nodosa. Clinical features, diagnostics and treatment of disorder related to type 3 hypersensitivity reaction dependent on the underlying etiology.

PATHOGENESIS OF IMMUNE COMPLEX-MEDIATED TISSUE INJURY AND ORGANS DAMAGE

Normally, antigen–antibody immune complexes formed in small quantity are cleared by reticuloendothelial

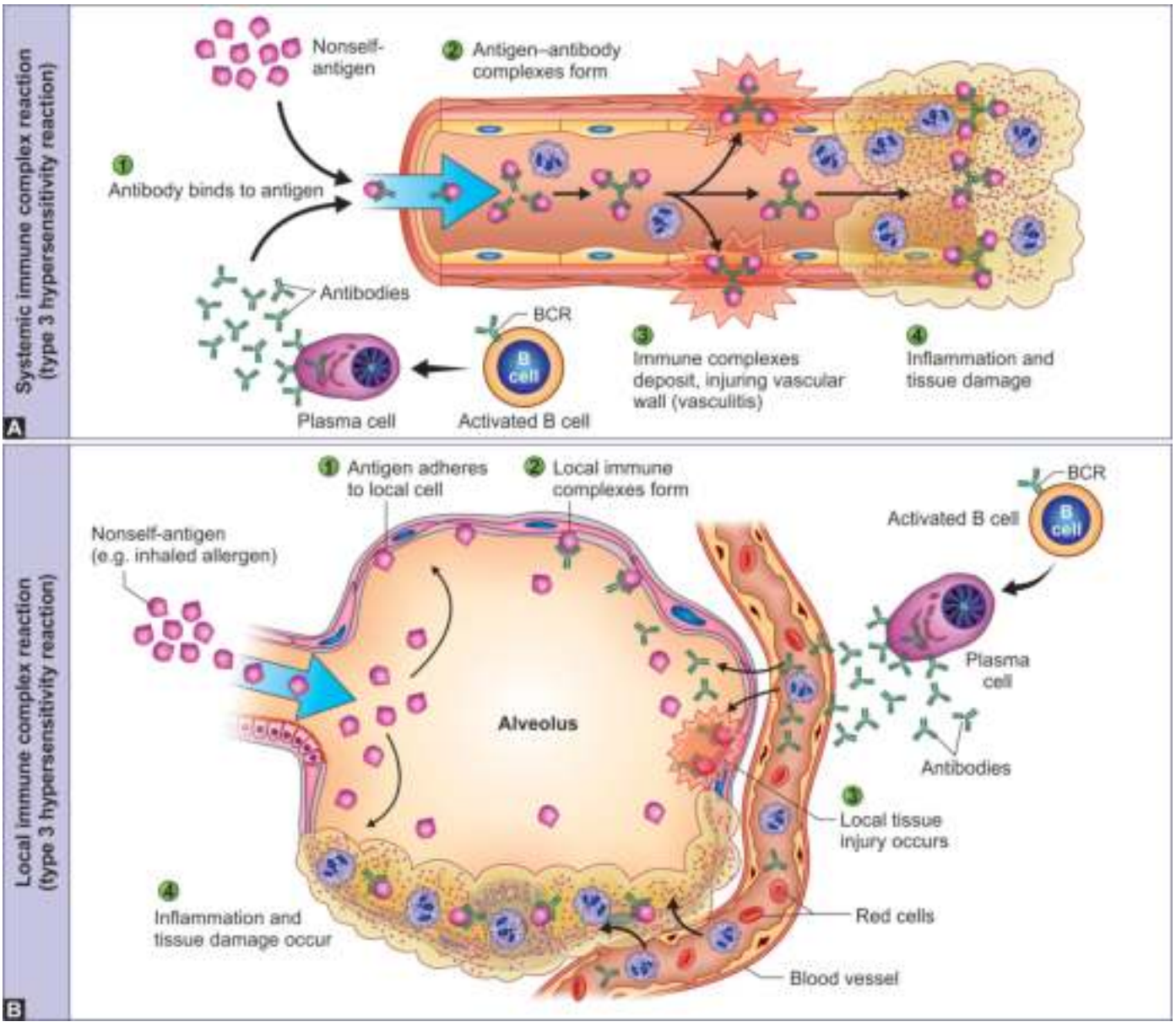


Fig. 4.58: Pathogenesis of type 3 hypersensitivity reaction. Antigen and antibody bind together to create an immune complex. (A) Systemic immune complex reaction. Antibody binds to nonself-antigen and enter circulation. Antigen–antibody complexes are formed. (B) Local immune complex occurs following injection of antigen or organ transplantation.

system. Pathogenesis of type 3 hypersensitivity reaction is shown in Fig. 4.58A and B. Antigen–antibody immune complex-mediated tissue injury is shown in Fig. 4.59.

- Antibodies (IgG or IgM) are formed against soluble antigen (serum or drug) in large quantity and trapped in membranes of various organs (skin, kidney, lung, blood vessels, and joints) in pathologic state such as persistent infections, inhaled allergens (farmer's lung) and self-antigens (systemic lupus erythematosus).
- Immune complex activates the complement system. C3a and C5 attract neutrophils, which release hydrolytic enzymes resulting in tissue injury. Serum complement is decreased.
- Injury to vascular endothelium leads to activation of intrinsic coagulation pathway resulting in formation of microthrombi in small vessels.
- Activation of the kinin system causes vasodilation and edema. Oxygen-derived free radicals, prostaglandins and kinins to incite chronic destructive inflammatory response. Macrophages infiltration in later stages may be involved in the healing process.
- Immunofluorescence microscopy reveals immune complex deposits in biopsy specimens. Serum complement level is decreased in these patients. Immune complex formation and site of deposition are given in Table 4.63. Immune complex deposition induced organs damage is shown in Fig. 4.60.

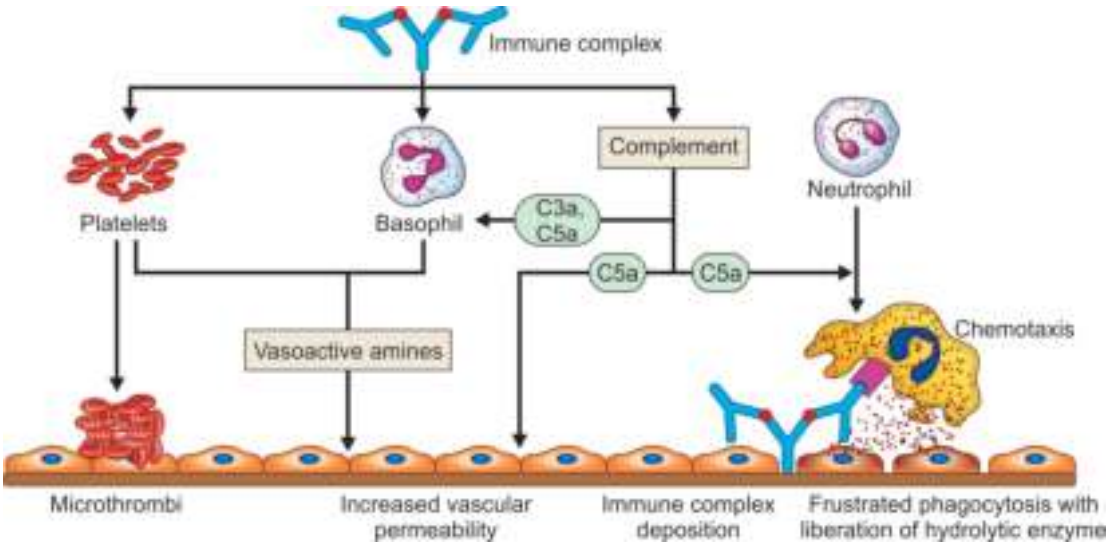


Fig. 4.59: Antigen–antibody Immune complex-mediated tissue injury. Antigen–antibody immune complexes circulating or deposited in perivascular region can damage tissues by triggering inflammation resulting in increased vascular permeability, liberation of hydrolytic enzymes and formation of microthrombi.

Table 4.63 Immune complex formation and sites of deposition

Circumstances	Etiology	Sites of Deposition
Persistent infections	Microorganism products	Blood vessels and glomeruli
Inhaled allergens (farmer's lung)	Exposure to thermophilic actinomyces in air	Lungs: IgG class, although IgM may also be involved in the formation of immune complex
Autoimmune disorders	Endogenous (self) antigens	Immune complex deposition in blood vessels, glomeruli and joints in SLE

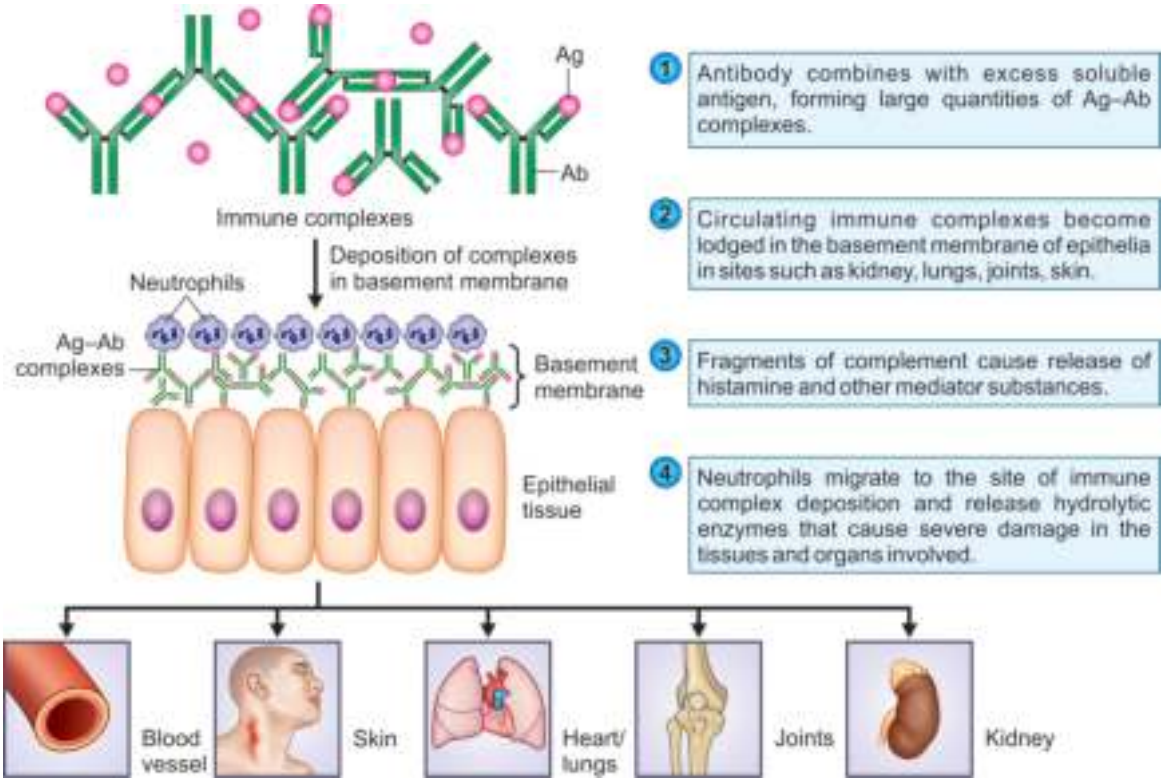


Fig. 4.60: Diseases caused by type 3 hypersensitivity reactions. In type 3 hypersensitivity, an abnormal antigen–antibody complexes, which trigger activation of complement system by classic pathway leading to damage of various tissues such as skin, joints, blood vessels.

- There are two kinds of immune complex-mediated type 3 hypersensitivity reactions, i.e. localized (Arthus reaction) and systemic immune-complex mediated type 3 hypersensitivity reactions.
- Localized (Arthus) reaction occurs at the site of injected drug or booster immunization.
  - Systemic immune-complex-mediated type 3 hypersensitivity reaction occurs when repeated antigen challenges cause systemic distribution of the immune complexes and subsequent inflammation of joints, lymph nodes and kidney. Diseases caused by type 3 hypersensitivity reactions are given in Table 4.64. Differences between Arthus reaction and serum sickness are given in Table 4.65.

Localized (Arthus Reaction) Type 3 Hypersensitivity Reaction

Arthus reaction is a localized immune complex-mediated type 3 hypersensitivity reaction that occurs when exogenous antigen is introduced, either by vaccination against tetanus and diphtheria or by organ transplant, in the presence of an excess of preformed antibodies (usually IgG, but IgM may be involved). Antigen–antibody immune complexes are deposited in the skin that activates complement system leading to local inflammation and tissue necrosis. Patient presents with localized swelling, erythema, hemorrhage, sometimes superficial skin necrosis a few hours after booster vaccination.

Table 4.64 Diseases caused by type 3 hypersensitivity reactions

Disease	Target Antigens	Organs Damage
Localized type 3 hypersensitivity reactions		
Arthus reaction	Various foreign proteins	Cutaneous vasculitis (IgG class, although IgM may also be involved)
Systemic type 3 hypersensitivity reactions		
Serum sickness	Various proteins such as foreign serum proteins (horse antithymocyte globulin)	Arthritis, vasculitis, nephritis
Systemic lupus erythematosus	Nuclear antigens (DNA to anti-DNA)	Lupus nephritis, skin lesions, arthritis
Polyarteritis nodosa	Hepatitis B virus antigen	Systemic vasculitis
Poststreptococcal glomerulonephritis	Streptococcal cell wall antigen, may be planted in glomerular basement membrane	Nephritis
Farmer's lung	Inhaled allergens (exogenous organic dust)	Lungs involvement
Reactive arthritis	Bacterial antigens (Yersinia)	Acute arthritis

Type 2 and 3 hypersensitivity reactions participate in the pathogenesis of systemic lupus erythematosus.

Table 4.65 Differences between Arthus reaction and serum sickness

Characteristics	Arthus Reaction	Serum Sickness
Disorder	Localized immune complex due to antibody excess	Systemic immune complex disorder due to antigen excess resulting to injury
Onset	4–10 days after exposure to antibody	5–7 days after entry of antigen, e.g. exogenous (horse serum, streptococcal infection) or endogenous (systemic lupus erythematosus)
Subtypes	No subtypes	Acute and chronic serum sickness
Mechanism of organ damage		Complement activation, platelet aggregation
Organs involved	Skin only	Joints, skin, heart, blood vessels, serosal surfaces
Morphology of organs	Fibrinoid necrosis of blood vessels	Immune complex-mediated necrotizing vasculitis, fibrinoid necrosis of blood vessels, proliferation of endothelial and mesangial cells
Immunofluorescence microscopy	Deposits comprising fibrinogen, complement and immunoglobulin	Granular deposits of immune complex comprising of immunoglobulin and complement
Clinical features	Dyspnea, flu-like symptoms and lung fibrosis (chronic case)	Fever, arthralgia, urticarial, lymphadenopathy and proteinuria

Systemic Immune-complex-mediated Type 3 Hypersensitivity Reaction-induced Diseases

Systemic immune-complex-mediated type 3 hypersensitivity reactions occur when repeated antigen challenges cause systemic distribution of the immune complexes and subsequent inflammation of joints, lymph nodes and kidney. Examples are serum sickness, polyarthritis nodosa, drug-induced hypersensitivity vasculitis, lupus nephritis, IgA nephropathy, membranous glomerulonephritis, post-streptococcal glomerulonephritis, hypersensitivity pneumonitis.

Serum Sickness

Serum sickness is a classic example of type 3 hypersensitivity reaction, which usually develops as a complication of antivenom or antitoxin administration therapeutically for passive immunization against microorganisms.

- Antivenom and antitoxin containing animal proteins or serum causing serum sickness include: (a) equine anti-snake venom and anti-spider venom, (b) equine or bovine antirabies antitoxin and equine botulinum antitoxin.
- Serum sickness can be induced by drugs such as penicillin or amoxycillin or cephalosporins.
- Antigen–antibody complexes are deposited in the tissues/organs that activate complement system resulting in systemic inflammation and tissues/organs damage.
  - Patient develops symptoms such as fever, skin rashes, arthralgia, edema of hand, headache, blurred vision, abdominal pain, vomiting and diarrhea within one to two weeks following exposure to antivenom or antitoxin or drug.
  - Urine analysis may show mild proteinuria.
  - Prognosis is excellent once the offending agent is withdrawal.

Polyarteritis Nodosa

Polyarteritis nodosa (PAN) is systemic vasculitis involving small- and medium-sized arteries affecting many organs especially the skin, peripheral nerve, gut, kidney and heart.

- Most cases of PAN occur in the 4th or 5th decade, although it can occur at any age. Males are more affected than women. A minority of patients with PAN have an active hepatitis B infection. In rest of the cases, the disease is idiopathic in nature.
- After several weeks or months. patient develops nonspecific symptoms such as fever, malaise, weight loss, anorexia and abdominal pain and other symptoms related to involved organs such as skin, kidney, nerves and gastrointestinal tract. Many patients develop hypertension and elevated erythrocyte sedimentation rate.

Drug-induced Hypersensitivity Vasculitis

Drug-induced hypersensitivity vasculitis is caused by use of various pharmaceutical agents such as penicillin, cephalosporin, sulphonamide, thiazide-type diuretics, phenytoin and allopurinol. Vasculitis causes changes in the walls of blood vessels, including thickening, weakening, narrowing and scarring. Withdrawal of the offending agent alone is often sufficient to induce prompt resolution of clinical manifestations, obviating the requirement for immunosuppressive and anti-inflammatory drugs.

Lupus Nephritis

Systemic lupus erythematosus (SLE) is an autoimmune disorder involving multiple systems such as blood vessels, glomeruli and joints. Anti-DNA antibodies are formed against endogenous (self) antigen.

- Lupus nephritis, one of the most serious manifestations of SLE clinically evident in 50–60% of patients usually arises within five years of diagnosis. Evaluating renal functions (blood urea nitrogen, serum creatinine urinalysis, 24 hours urine test for creatinine and protein excretion) in SLE patients are important because early detection and treatment of renal involvement can significantly improve renal outcome.
- Patients may present with fatigue, fever, skin rashes, arthritis, serositis, central nervous system disease and renal manifestations.
- Histologic examination of renal biopsy shows class I (minimal mesangial lupus nephritis), class II (mesangial proliferative lupus nephritis), class III (focal lupus nephritis) or class IV (diffuse lupus nephritis) and class V (membranous lupus nephritis). Laboratory tests for SLE disease activity include: antibodies to double-stranded DNA (dsDNA), complement protein (C3, C4), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP).

IgA Nephropathy

IgA nephropathy is an autoimmune disease arising from consequences of increased circulating levels of IgA1 with galactose-deficient hinge-region O-glycans and deposition of immune complexes in mesangial region. However, aberrant glycosylation is unable to induce glomerular injury on its own. Patients with IgA nephropathy seem to produce anti-GalNAc antibodies against defective IgA1.

- Immune complex deposition in the glomeruli leads to type 3 hypersensitivity reaction which ultimately damages the kidneys.
- Patient presents with repeated episodes of gross or microscopic hematuria, flank pain, hypertension, variable proteinuria and swelling over ankles.

Table 4.66 Different names of hypersensitivity pneumonitis according to the causative antigen

Different Name	Causative Antigen
Farmer's lung	Moldy hay containing spores of thermophilic actinomycetes
Air conditioner lung	Thermophilic bacteria
Byssinosis	Textile workers due to inhalation of cotton fibers, linen and hemp develop bronchial asthma-like presentation
Pigeon breeder's lung, also called bird fancier's disease	Proteins from serum and features, and excreta
Maple bark stippler's lung	Fungal spores
Hot tub lung	Liquid or solid droplets in the air contaminated by nontuberculous Mycobacterium or other infectious agents

- Clinical markers associated with poor prognosis include: heavy proteinuria, low serum albumin, diastolic hypertension, male sex and age below 30 years.

Membranous Glomerulonephritis

Membranous glomerulonephritis (MGN) is most common cause of nephrotic syndrome affecting mainly adults than children, which is characterized by thickening of the glomerular basement membrane marked by marked proteinuria and generalized edema.

- About 80–85% of MGN cases are idiopathic. Remaining MGN cases are secondary and caused by malignancy, infectious agents and autoimmune diseases.
- In secondary MGN, successful treatment of the underlying cause may be curative.
- Patients with primary MGN may experience spontaneous remission, persistent proteinuria of variable degree, or progression to renal failure.

Poststreptococcal Glomerulonephritis

Poststreptococcal glomerulonephritis results from the body's immune system fighting off the group A streptococcal throat and skin infections, usually in children.

- Patient presents from passage of dark, reddish-brown urine, oliguria, edema around periorbital region and face, fatigue, mild anemia, hypertension, and proteinuria in nephritic range.
- Most patients, who develop poststreptococcal glomerulonephritis recover within a few weeks without any complications. Renal failure rarely occurs more common in adults than children.

Hypersensitivity Pneumonitis

Hypersensitivity pneumonitis, also known as extrinsic allergic alveolitis, is immunologically induced inflammation of lung parenchyma and respiratory failure in response to inhalation of a large variety of natural and chemical antigens such as spores of bacteria, fungi, hay, grain, sugarcane bark, animal excreta and chemical antigens.

- Antigen exposure results in early development of type 3 immune complexes, and later followed by type 4 hypersensitivity reactions.
- Histologically, it is characterized by lung parenchymal inflammation with fibrosis and poorly formed non-necrotizing granulomas predominantly in middle to upper lobes of lung; and usually bilateral involvement.
- Disease may be diagnosed by radiographs and lung biopsy. Different names of hypersensitivity pneumonitis according to the causative antigen are given in Table 4.66.

TYPE 4 HYPERSENSITIVITY REACTION

Compared to antibody-mediated types 1, 2 and 3 hypersensitivity reactions, type 4 hypersensitivity reaction is mediated by T cells and macrophages. Specifics of type 4 hypersensitivity reactions include: T cell involvement, transplant rejection, tuberculin skin test for tuberculosis and contact dermatitis. Type 4 hypersensitivity reactions involve two major mechanisms: (a) T cell sensitization and (b) pre-sensitized T cell response.

- T cell sensitization response occurs by skin penetration by antigen, uptake of the antigen by Langerhans cell, migration to lymph nodes and formation of sensitized T cells.
- Pre-sensitized T cell response occurs after repeated contact with the antigen by T lymphocytes (CD8+ cytotoxic T cells and CD4+ helper T cells).
  - CD8+ cytotoxic T cells recognize antigens on target cells and induce cell-mediated cellular cytotoxicity resulting in direct cell destruction.
  - CD4+ helper T cells recognize antigens on antigen-presenting cells (APCs) and release of inflammatory cytokines (e.g. TNF-α and IFN-γ). Macrophages activation results in phagocytosis of target cells. Pathogenesis of type 4 hypersensitivity reactions is shown in Fig. 4.61.

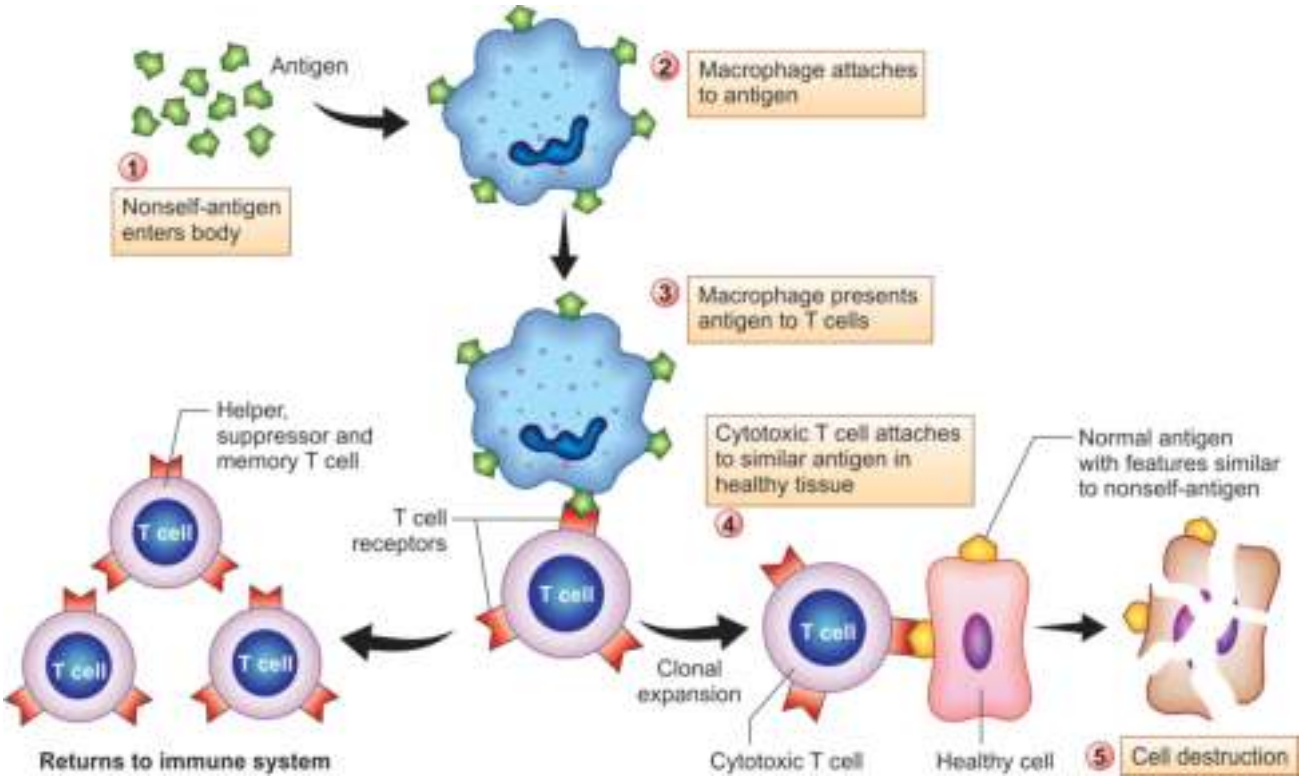


Fig. 4.61: Pathogenesis of type 4 hypersensitivity cell-mediated reaction. Macrophage capture antigen and present to T cells, thus sensitizing these T lymphocytes. Some of these lymphocytes become CD8+ cytotoxic T cells. These CD8+ cytotoxic T cells attack the antigen, where present. Other lymphocytes become T helper cells, T-suppressor cells and memory cells.

CD8+ CYTOTOXIC T CELL AND CELL-MEDIATED IMMUNE RESPONSE

CD8+ cytotoxic T cells, like CD4+ helper T cells are generated in the thymus gland and express T cell receptor (TCR). However, rather than CD4 molecule, CD8+ cytotoxic T cells express a dimeric co-receptor composed of one CD8-α and one CD8-β chain.

- CD8+ cytotoxic T cells recognize peptides presented by MHC class I molecules, found on all nucleated cells. CD8 heterodimer binds to α-region of MHC class I molecule during T cell/antigen-presenting cell interactions.
- CD8+ cytotoxic T cells play important role in immune defense against intracellular pathogens (viruses and bacteria) and tumor surveillance.

Pathophysiology

When CD8+ cytotoxic T cells recognize an antigen and become activated, which kill the intracellular virus or cancer stem cells by three mechanisms: (a) synthesis of cytokines, (b) synthesis of perforins and granzymes, and (c) Fas/FasL interactions.

- **Cytokines synthesis:** CD8+ cytotoxic T cells synthesize cytokines such as TNF-α and IFN-γ, which have antiviral, antimicrobial and antitumor effects. The antiviral activity of interferon is shown in Fig. 4.62.
- **Perforin and granzymes synthesis:** CD8+ cytotoxic T cells release cytotoxic granules containing perforins and granzymes. Perforin forms pore in the plasma membrane of the target cells, that allows entry of granzymes leading to cleaving of proteins inside the target cells, shutting down the production of viral proteins and ultimately resulting in apoptosis of the target cells.
- **Fas/FasL interaction:** CD8+ cytotoxic T cells cause destruction of target cell via Fas/FasL interactions. Activated CD8+ cytotoxic T cells express FasL on the cell surface, which binds to its Fas receptor on the surface of the target cell resulting in the activation of caspase cascade, which induce apoptosis of the target cell.

CD4+ HELPER T CELLS AND MACROPHAGES-MEDIATED TYPE 4 HYPERSENSITIVITY REACTIONS

Type 4 hypersensitivity reactions, also known as delayed-type hypersensitivity reactions (DTH), provide host defense against intracellular pathogens (e.g. Mycobacterium, fungi and certain parasites. Type 4 hypersensitivity reactions are mediated by monocyte/macrophages and CD4+ memory T cells either due to exogenous or autoantigens, rather than antibodies.

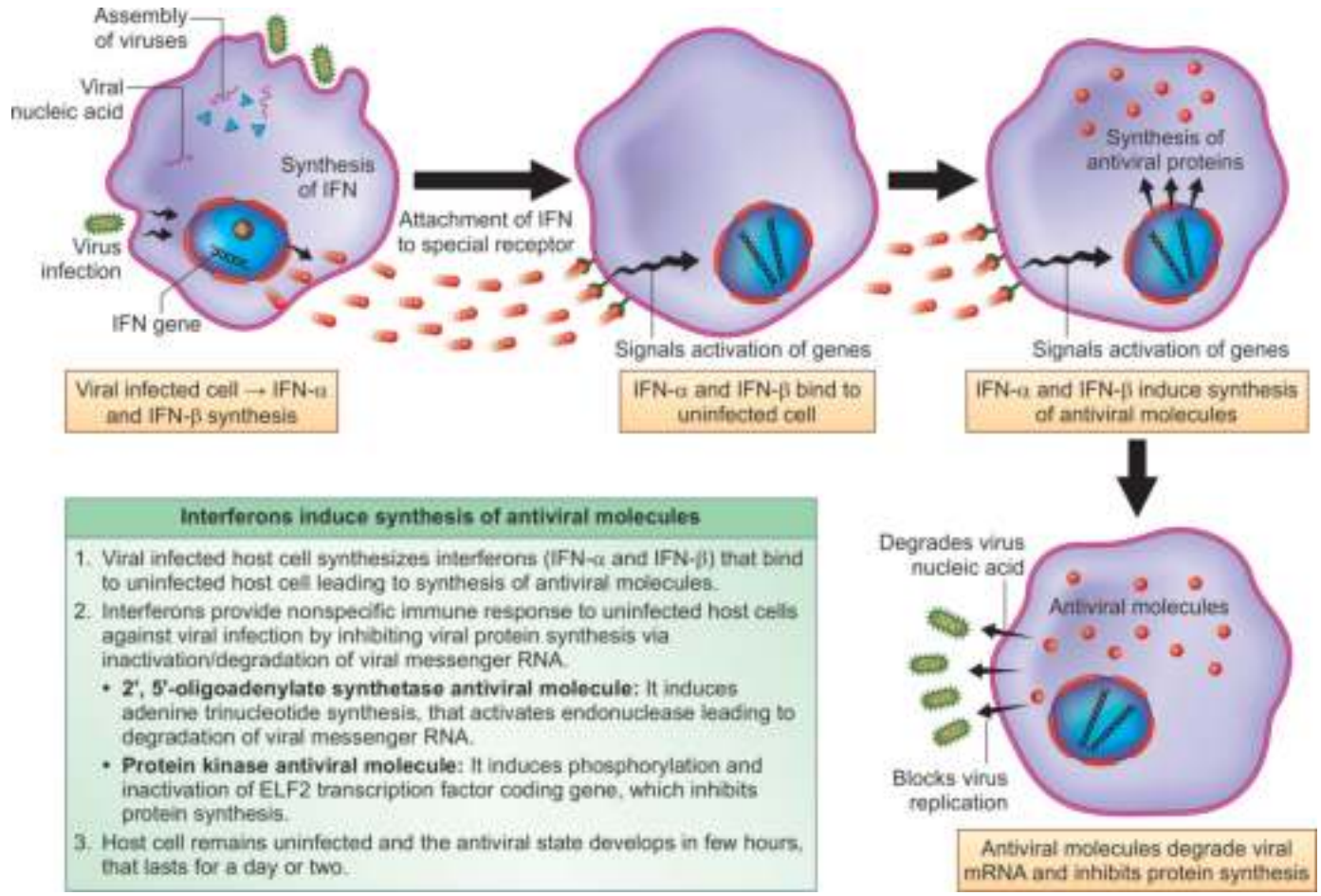
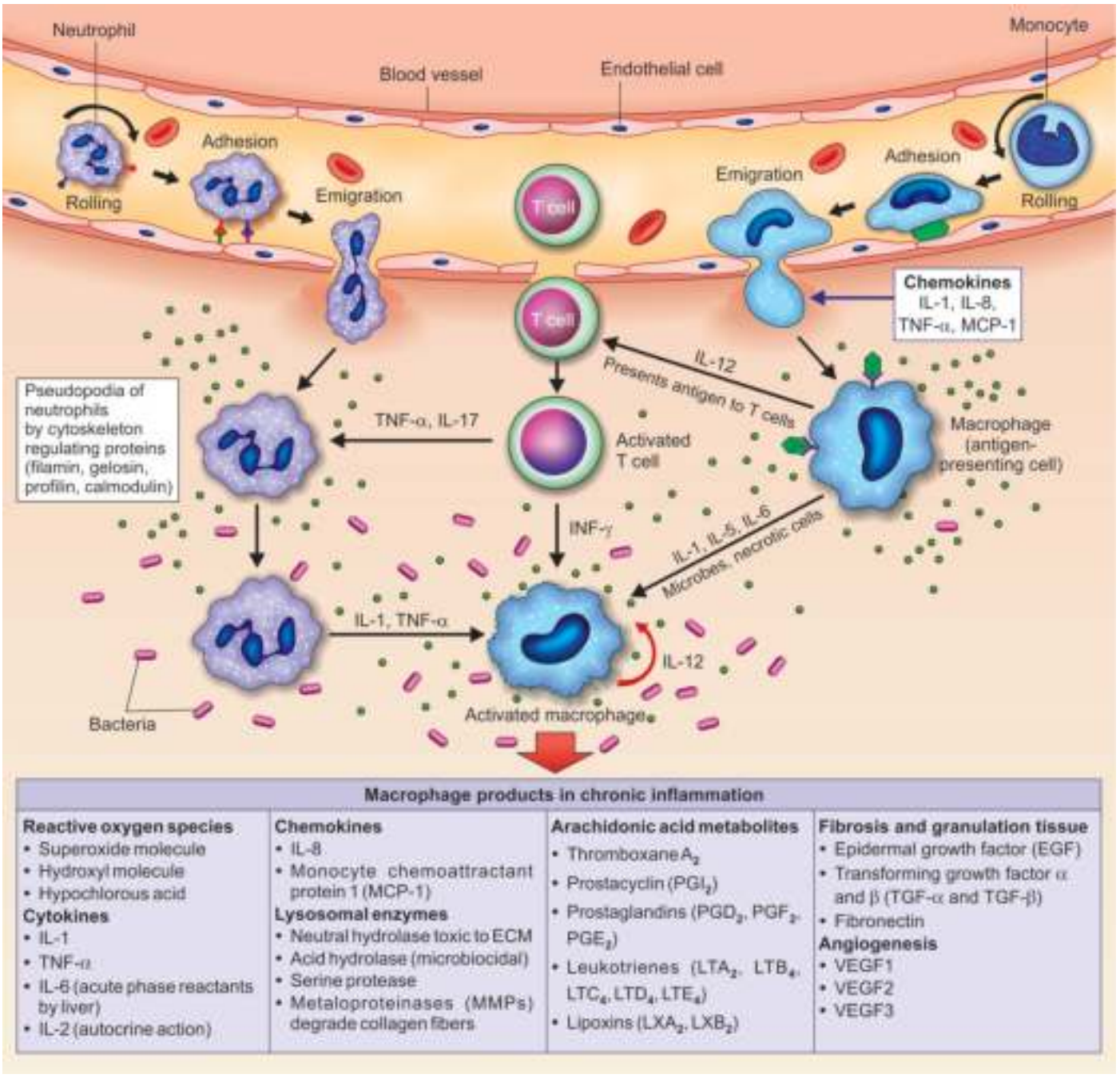


Fig. 4.62: The antiviral activity of interferon. When a cell is infected by a virus, its nucleus is triggered to transcribe interferon (IFN) gene. Interferon diffuses out of the cell and then binds to IFN receptors on nearby uninfected cells, where it induces production of proteins that eliminate viral genes and block viral replication. Note that the original cell is not protected by IFN and that does not prevent viruses from invading the protected cells.

- Delayed-type hypersensitivity reaction to exogenous antigens involves CD4+ helper T cells and antigen-presenting cells (APCs) such as macrophages, Langerhans' cells and dendritic cells, all synthesize cytokine IL-12, which activates CD4+ memory T cells and differentiate to Th1 effector cells, which synthesize various cytokines such as monocyte chemotactic factor, IL-2, interferon-γ (IFN-γ).
- IFN-γ is a central mediator of delayed hypersensitivity and powerful activator of macrophages. IFN-γ further augments the differentiation of Th1 cells. IL-2 has autocrine and paracrine action and causes proliferation of antigen-specific T cells and activation of macrophages, which accumulate and induce a local inflammatory response in a sensitized person. Role of activated macrophages in chronic inflammation is shown in Fig. 4.63.
- Delayed-type hypersensitivity reaction to autoantigens can be seen in type 1 diabetes mellitus, which is an autoimmune cell-mediated destruction of insulin secreting pancreatic β cells.
- One important cytokine is IL-1, which promotes the release of the acute phase reactants of the liver, which increases the proliferation of T cells. IL-1 acts on the hypothalamic thermoregulatory center to induce fever, which is responsible for some of the systemic symptoms of delayed type of hypersensitivity.
- Type 4 hypersensitivity reactions can be classified into three categories depending on the time of onset and clinical and histologic presentation, which include contact dermatitis, tuberculin reaction and granulomatous hypersensitivity. Categories of type 4 hypersensitivity reactions are given in Table 4.67.

Contact Dermatitis

In contact dermatitis, small antigens such as **heavy metals** (nickel, chromium), **plant** (ivy oak), **organic chemicals**, **cosmetics**, **cyanoacrylate adhesive photographic developer** and **primula paint** penetrate the skin combine with tissue protein '**hapten**' and act as a **hapten–protein complex**; which stimulate cell-mediated immune response. Subsequent exposure to these



Macrophage products in chronic inflammation

Reactive oxygen species	Chemokines	Arachidonic acid metabolites	Fibrosis and granulation tissue
• Superoxide molecule • Hydroxyl molecule • Hypochlorous acid **Cytokines** • IL-1 • TNF-α • IL-6 (acute phase reactants by liver) • IL-2 (autocrine action)	• IL-8 • Monocyte chemoattractant protein 1 (MCP-1) **Lysosomal enzymes** • Neutral hydrolase toxic to ECM • Acid hydrolase (microbiocidal) • Serine protease • Metaloproteinases (MMPs) degrade collagen fibers	• Thromboxane A_2 • Prostacyclin (PGI_2) • Prostaglandins (PGD_2, PGF_2, PGE_2) • Leukotrienes (LTA_2, LTB_4, LTC_4, LTD_4, LTE_4) • Lipoxins (LXA_2, LXB_2)	• Epidermal growth factor (EGF) • Transforming growth factor α and β (TGF-α and TGF-β) • Fibronectin **Angiogenesis** • VEGF1 • VEGF2 • VEGF3

Fig. 4.63: Role of activated macrophages in chronic inflammation. Macrophages are activated by immunological or nonimmunological mechanisms. Various products synthesized by activated macrophages participate in tissue injury and fibrosis.

Table 4.67 Categories of type 4 hypersensitivity reactions

Type	Antigen and Site	Reaction Time	Clinical Appearance	Histology
Contact dermatitis	Epidermal contact with organic chemicals, poison ivy, heavy metals	48–72 hours	Eczema	Lymphocytes. Followed by macrophages; edema of epidermis
Tuberculin Test	Intradermal route (tuberculin or lepromin test)	48–72 hours	Local induration, swelling and swelling with or without fever	Lymphocytes and macrophages
Granulomatous inflammation	Persistent antigen or foreign body presence, *Mycobacterium tuberculosis* and leprosy	21–28 days	Hardening	Epithelioid cell granuloma, giant cells with or without necrosis

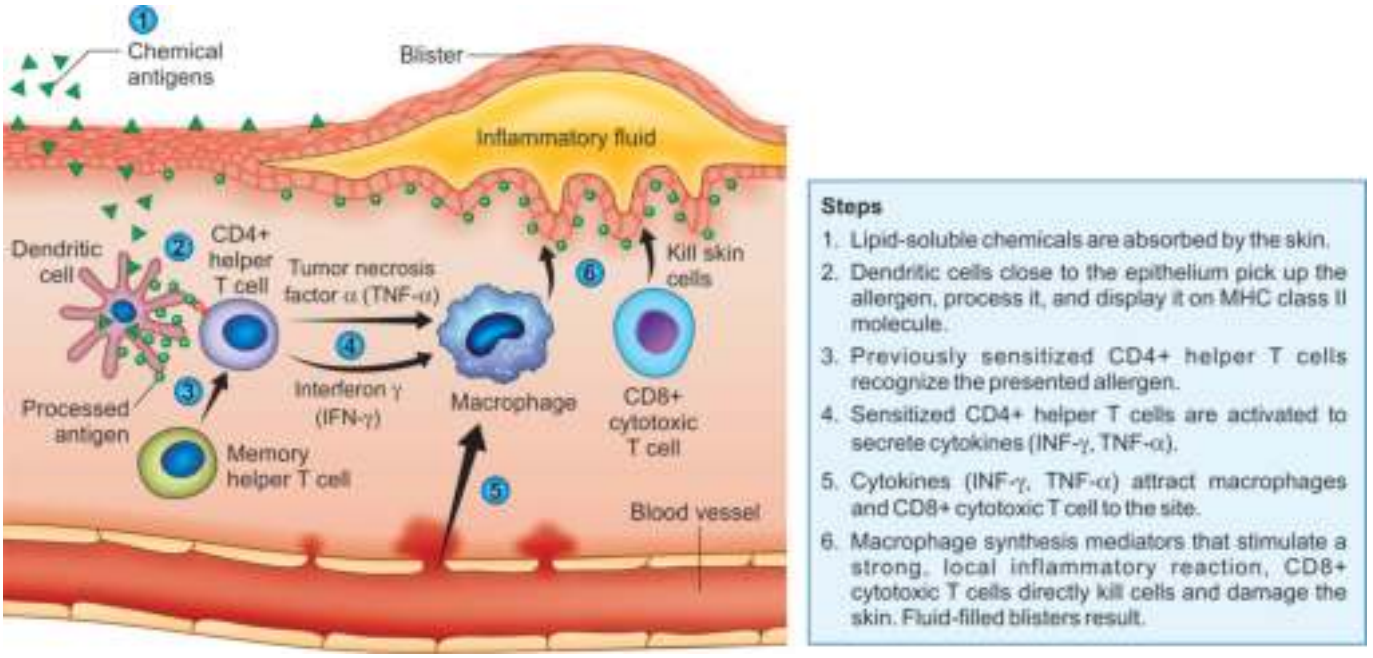


Fig. 4.64: Genesis of contact dermatitis. Contact dermatitis from poison oak, is showing various stages of involvement: blisters, scales and thickened patches. Lipid-soluble chemicals are absorbed by the skin. Dendritic cells close to epidermis pick up the allergen, process it.

substances induces eczema within 48–72 hours. Also, keratinocytes, which express MHC class II molecules and intercellular adhesion molecule 1 (ICAM-1), produce cytokines (IL-1, IL-6, IL-8), that help in the establishment of the contact hypersensitivity reaction. Histologic examination reveals lymphocytic infiltration around dermal blood vessels, together with dermal and epidermal edema leading to vesicle formation. Contact dermatitis is shown in Fig. 4.64.

Tuberculin Skin Test: Type 4 Hypersensitivity Reaction

Tuberculin skin test—type 4 hypersensitivity reaction is a localized inflammatory reaction develops within 48–72 hours in patients with tuberculosis (TB) or vaccinated against tuberculosis. Patients are injected tuberculin (purified protein derivative of tubercle bacillus) via intracutaneous route. Tuberculin skin test induces skin induration, swelling and redness resulting from fibrin deposition is marked by accumulation of lymphocytes, monocytes, and small numbers of neutrophils around small blood vessels (i.e. perivascular cuffing). Other antigens from *Mycobacterium leprae* and *Leishmania tropica* can also cause a similar reaction.

Granulomatous Inflammation Type 4 Hypersensitivity Reactions

Granulomatous inflammation type 4 hypersensitivity reaction results from the persistence of intracellular pathogens or other antigens including silica, beryllium, talc within macrophages, which are unable to process and digest, that leads to development of granulomas as occur in tuberculosis cases in more than 3 weeks.

- Granuloma is defined as an aggregation of modified macrophages (epithelioid cells) surrounded by lymphocytes and fibroblasts. Epithelioid cells may differentiate into multinucleate giant cells. Langhans' giant cells show peripherally arranged nuclei seen in tuberculosis. Epithelioid cell granulomas in tubercular lymphadenitis are shown in Fig. 4.65.
- Examples of granulomatous hypersensitivity include tuberculosis, leprosy, syphilis, blastomycosis, histoplasmosis, toxoplasmosis, leishmaniasis, sarcoidosis, Crohn's disease, rheumatoid arthritis, inorganic antigens (zirconium, inert minerals like silica), autoimmune diseases (e.g. type 1 diabetes mellitus, multiple sclerosis, rheumatoid arthritis and Guillain-Barré syndrome).
- Corticosteroids and other immunosuppressive agents are used in treatment. Examples of granulomatous inflammation type 4 hypersensitivity reactions are given in Table 4.68.

Type 1 Diabetes Mellitus

Type 1 diabetes mellitus results from the autoimmune destruction of islets of Langerhans β cells of the endocrine, pancreas, that lead to insulin deficiency. The process of autoimmune destruction of pancreatic β cells results in granuloma formation in genetically susceptible persons under the triggering effect of one or more environmental factors and usually progresses over a period of many months to years.

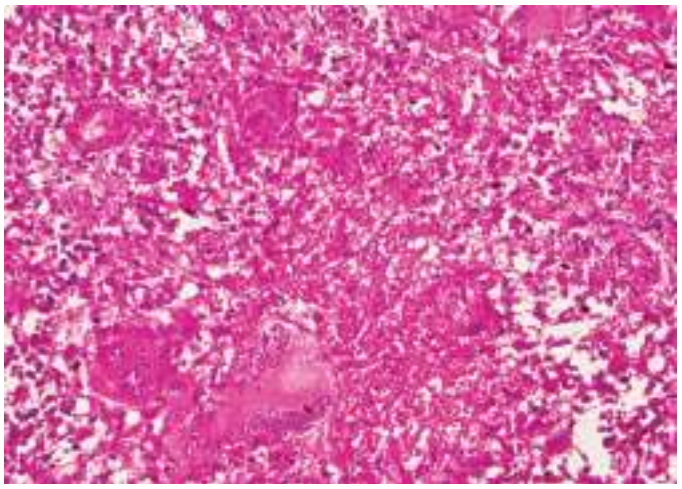


Fig. 4.65: Tubercular lymphadenitis. Hematoxylin and eosin-stained section of involved lymph node shows epithelioid cell granulomas, caseous necrosis and Langhans' cells. Epithelioid cell granuloma can be defined as specifically and structurally organized collection of epithelioid cells, macrophages, lymphocytes and dendritic cells. Dendritic cells are important because these cells present antigens to CD4+ helper T cells in the lymph nodes, in which activated T cell immune response can subsequently be developed. The signaling events lead to the formation of epithelioid cell granuloma, caseous necrosis and Langhans cells, the hallmark of tuberculosis (100X).

Multiple Sclerosis

Multiple sclerosis is a chronic neurodegenerative disease targeting the central nervous system (CNS), which is believed to be autoimmune in nature. Disease is mediated by autoreactive lymphocytes that crosses the blood–brain barrier and enter the CNS, where they cause local perivascular inflammation and tissue damage that results in demyelination, gliotic scarring, axonal loss and paralysis. Patient can develop granulomatous panuveitis.

Rheumatoid Arthritis

Human parvovirus B19 is a causative agent of rheumatoid arthritis, which is characterized by the presence of autoantibodies known as rheumatoid factor (RF) and anticitrullinated peptide antibodies (ACPA), which includes the anticyclic citrullinated peptide antibody or anti-CCP.

- Rheumatoid factor has been recognized as a feature of many persons with rheumatoid arthritis.
- T cells and macrophages are recognized as two critical cellular components involved in rheumatoid arthritis by inducing chronic arthritis with granulomatous inflammation, destruction of articular cartilage and bones.
- Signs and symptoms of rheumatoid arthritis may include tender, warm, swollen joints. Joint stiffness that is usually worse in the morning hours and after inactivity, fever, fatigue and anorexia.

Crohn's Disease

Crohn's disease is a type of inflammatory bowel disease (IBD) that causes inflammation of ileum and colon, often with granulomas, fibrosis, and stricture formation, which leads to abdominal pain, severe diarrhea, fatigue, weight loss and malnutrition.

Guillain-Barré Syndrome

Guillain-Barré syndrome is an autoimmune progressive granulomatous disease that affects the nerves (polyneuritis) causing skeletal muscle weakness and loss of sensation, sometimes progressive to complete paralysis, skeletal muscle weakness begins in the legs and spreads to the arms and body.

Tissues/Organs Transplant Rejection

The four classes of transplants or grafts are determined by the degree of MHC class molecules similarity between graft and host, which include: (a) autografts (one part of body to another), (b) isografts (between identical twins), (c) allografts (between two members of same species), and (d) xenografts (between two different species).

Table 4.68 Granulomatous inflammation type 4 hypersensitivity reactions

Disease	Specificity of Pathogenic T Cells	Clinicopathological Manifestations
Type 1 diabetes mellitus	Antigen of pancreatic islets β cells (insulin, glutamic acid decarboxylase, others)	Insulitis (chronic inflammation in islets destruction of β cells, diabetes)
Multiple sclerosis	Protein antigens in CNS myelin (basic proteins, proteolytic proteins)	Demyelination in CNS with perivesicular inflammation, paralysis, ocular lesions
Rheumatoid arthritis	Unknown antigens in joint synovium (type 2 collagen)	Chronic arthritis with inflammation, destruction of articular cartilage and bones
Peripheral neuropathy (Guillain-Barré syndrome)	Protein antigens of peripheral nerve myelin	Neuritis, paralysis
Crohn's disease (inflammatory bowel disease)	Unknown antigen may be derived from intestinal microbes	Chronic inflammation of ileum and colon, often with granulomas, fibrosis, stricture formation

- All major organs may be successfully transplanted. Allografts require tissue match (MHC class molecules must correspond); rejection is controlled with drugs.
- CD4+ cytotoxic T cells directed against foreign cells of a grafted tissue; involves recognition of foreign major histocompatibility complex (MHC) class molecules and rejection of tissue/organ.
- Graft rejection can be minimized by tissue matching procedures, immunosuppressive drugs, and use of tissues that do not provoke a type 4 hypersensitivity reaction.

TYPE 5 HYPERSENSITIVITY REACTION

Type 5 hypersensitivity reaction is sometimes considered as a subtype of type 2 hypersensitivity reaction, in which antibodies combine with target receptors present on a cell surface, that induces cells to proliferate, differentiate and enhances overactivity of the cells.

- Graves' disease is autoimmune disease named after Robert J Graves, and an example of type 5 hypersensitivity reaction due to circulating autoantibodies, in which excess synthesis of thyroid hormones results in hyperthyroidism.
- It is postulated that long-acting thyroid stimulating autoantibodies mimic TSH and bind to thyroid stimulating hormone (TSH) receptor present on thyroid follicular cell surface and activate excess production and secretion of thyroid hormone and thyroglobulin that is mediated via-3′, 5′-cycle adenosine monophosphate (cyclic AMP), resulting in excess iodine uptake, protein synthesis, and thyroid gland growth, which is responsible for Graves' disease.

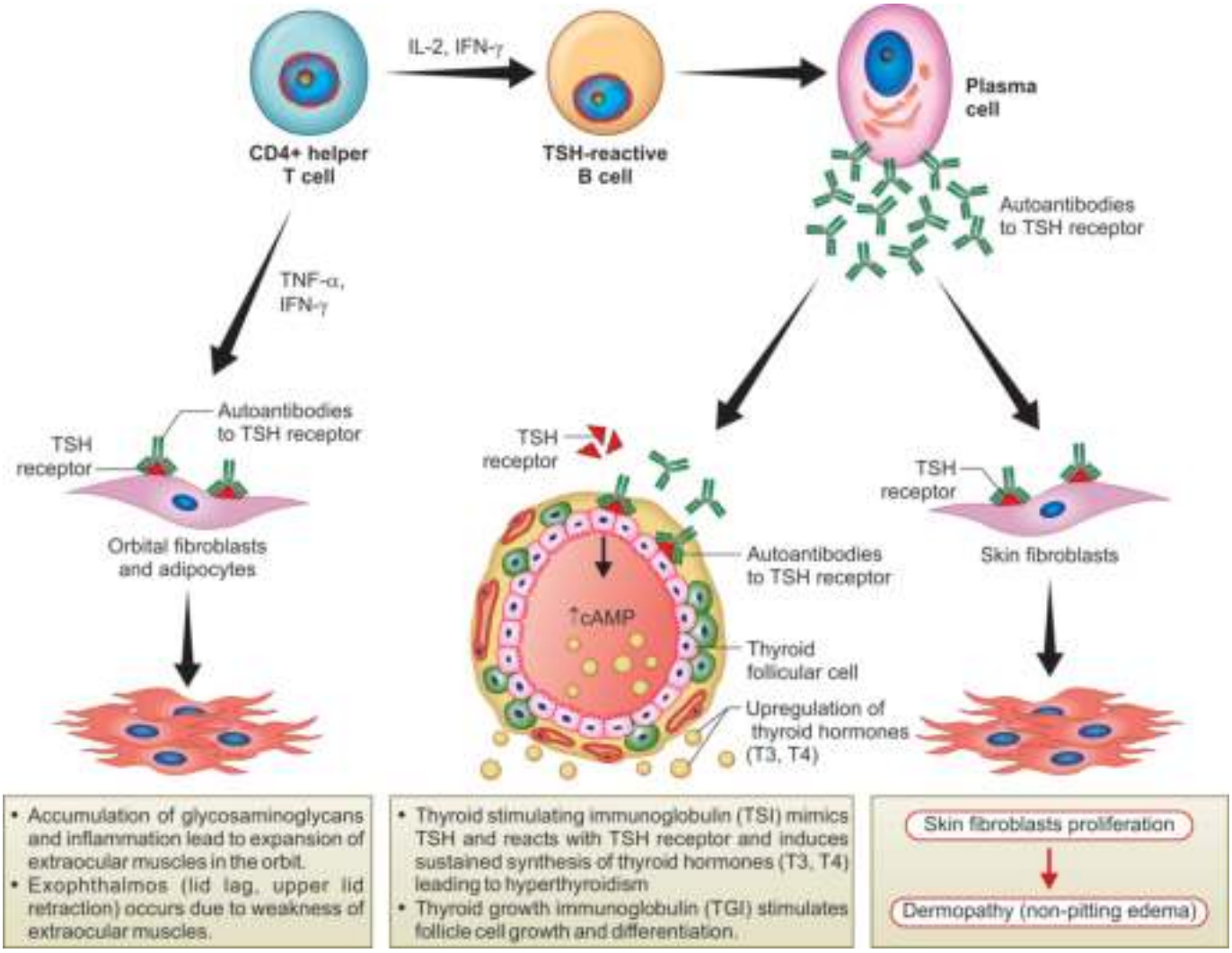


Fig. 4.66: Pathogenesis of Graves' disease. Graves' disease is an autoimmune disorder that results in the overproduction of thyroid hormones. Women are more affected than men in the age group 30–50 years. Graves' disease can cause features of hyperthyroidism, ophthalmopathy, dermopathy, combination of genes and virus trigger the immune system to produce excess of thyroid stimulating immunoglobulin (TSI) and thyroid growth immunoglobulin (TGI), TSI mimics TSH and reacts with TSH receptors and induces sustained synthesis of thyroid hormones. TGI stimulates thyroid follicle growth and differentiation.

GRAVES' DISEASE

Graves' disease is usually a disease of young usually female patients, who present with heat intolerance, tachycardia, tremors, weight loss and orbitopathy. Pathophysiology of thyroid-associated orbitopathy (TAO) is thought to an autoantibody-mediated reaction that can affect orbital and periorbital soft tissue. Long standing thyroid-associated orbitopathy features lid retraction, proptosis and vertical diplopia. Pathogenesis of Graves' disease is shown in Fig. 4.66.

IMMUNOLOGIC TOLERANCE

Thymus gland and bone marrow are sites of maturation for T and B cells respectively, which play important role in humoral and cell-mediated immunity against pathogens.

- Immunologic tolerance is a state of unresponsiveness to various environmental allergens and gut microbes in which T and B cells remain alive but cannot exert effector functions against a particular antigen, hence it provides protection against invading pathogens.
- Immunologic tolerance is classified into central and peripheral tolerance depending on where the state is originally induced in central lymphoid organs such as bone marrow and thymus gland, and peripheral lymphoid organs such as lymph nodes, spleen, mucosa-associated lymphoid tissues, tonsils, lymphoid tissues and organs. B cells and T cells become either immune competent or tolerant towards encountered antigens.
  - Central immunologic tolerance develops very early in life, in which immune system learns to discriminate self- from nonself-antigens, that prevents development of autoimmune diseases in normal persons.
  - Peripheral immunologic tolerance is the key to prevent over-reactivity of the immune system to various environmental antigens. CD4+ regulatory T cells (Treg cells) that inhibit CD4+ helper T cells or CD8+ cytotoxic T cells are involved in the development of peripheral immunologic tolerance.
- Central immunologic tolerance occurs by clonal deletion of autoreactive lymphocyte clones before these cells develop into fully immunocompetent B cells in bone marrow and T cells in thymus gland. It is the main, way the immune system learns to discriminate self- from nonself-antigens.
- T cell development involves positive or negative selection and lineage commitment, which depends on expression of TCR repertoire and exposure to MHC class molecules. T cells possess high affinity receptors for self-antigens.
  - Positive selection of T cells occurs first when naïve T cells are exposed to antigens in the thymus gland. T cells, which possess receptors with sufficient affinity for self-MHC class molecules are selected. Other T cells that do not express sufficient affinity to self-antigens will undergo a deletion process known as death by negligence, which involves apoptosis of the cells.
  - Negative selection of T cells with a very high affinity for self-MHC class molecules is induced to anergy or lineage divergence to form CD4+ regulatory T cells.
- When immunologic tolerance breaks down, individuals can develop autoimmune disease. In genetically predisposed individuals, autoimmunity may be triggered as a result of the failure of intrinsic tolerance mechanisms and/or environmental exposure triggers such as infection. To prevent self-tissue destruction, T cells and B cells are eliminated in primary and secondary lymphoid organs. This process is called central and peripheral immunologic tolerance.
- Immunologic tolerance depends on the concerned action of a variety of mechanisms that operate at different sites and stages of T cell and B cell development. The different ways in which the immune system prevents activation of autoreactive lymphocytes causing tissue damage are listed, along with specific mechanism and where such immunologic tolerance predominantly occurs.
- Factors influencing immunologic tolerance comprise structure of molecule, stage of differentiation when lymphocyte first encounter the epitopes, site of the encounter and nature of the cell presenting the epitopes.
- Central and peripheral immunologic tolerance of T cells and B cells is shown in Fig. 4.67. Positive and negative selection of T cells during development in thymus gland is shown in Fig. 4.68. Central and peripheral immunological; tolerance of B cells is shown in Fig. 4.69. Layers of immunologic tolerance are given in Table 4.69. Factors influencing immunologic tolerance are given in Table 4.70.

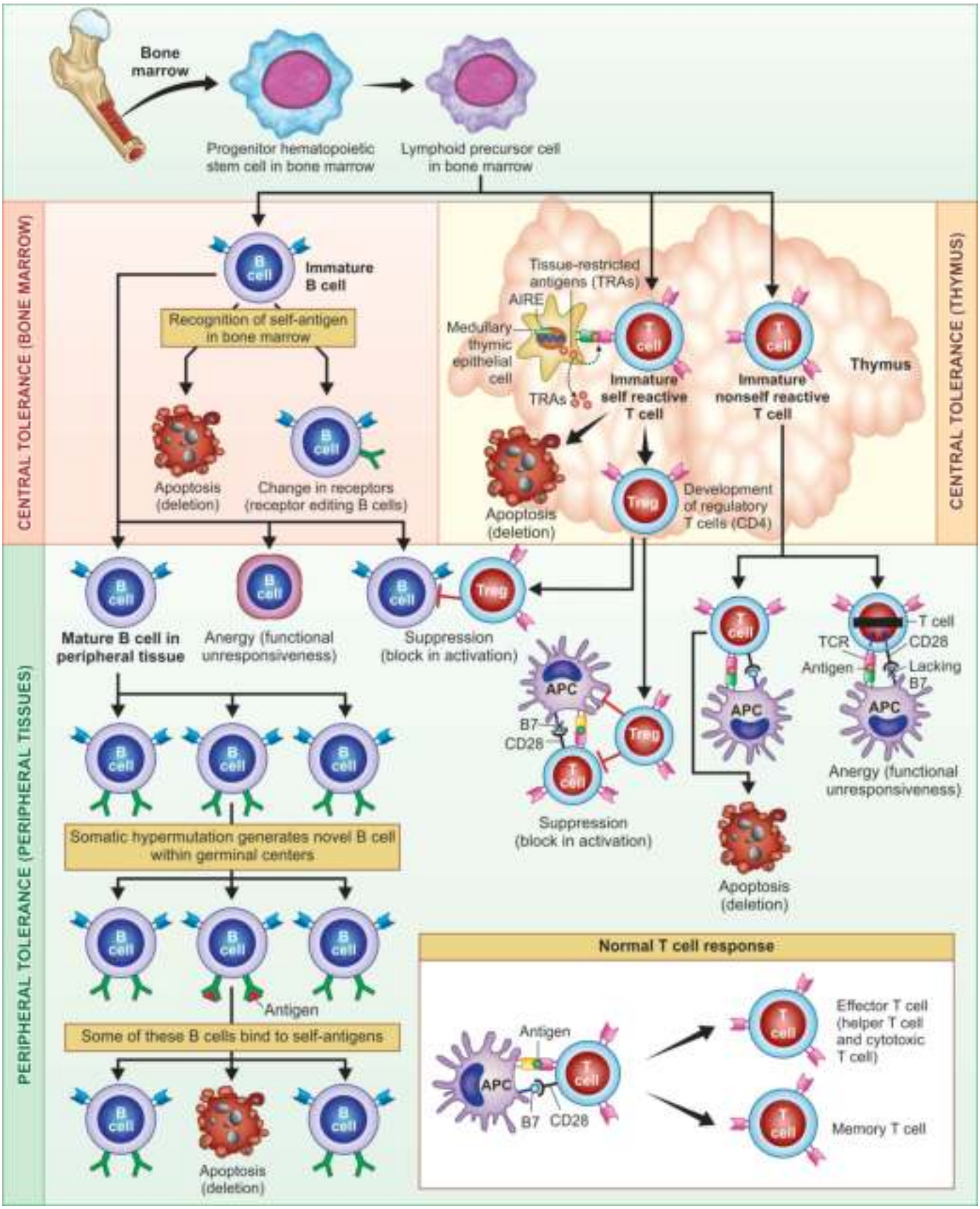


Fig. 4.67: Central and peripheral immunologic tolerance of T cells and B cells. Induction of immunologic tolerance refers to the regulatory education of both B cells and T cells, when occurs in both central organs (bone marrow and thymus gland) and peripheral organs (lymph nodes, spleen and mucosa-associated lymphoid tissue).

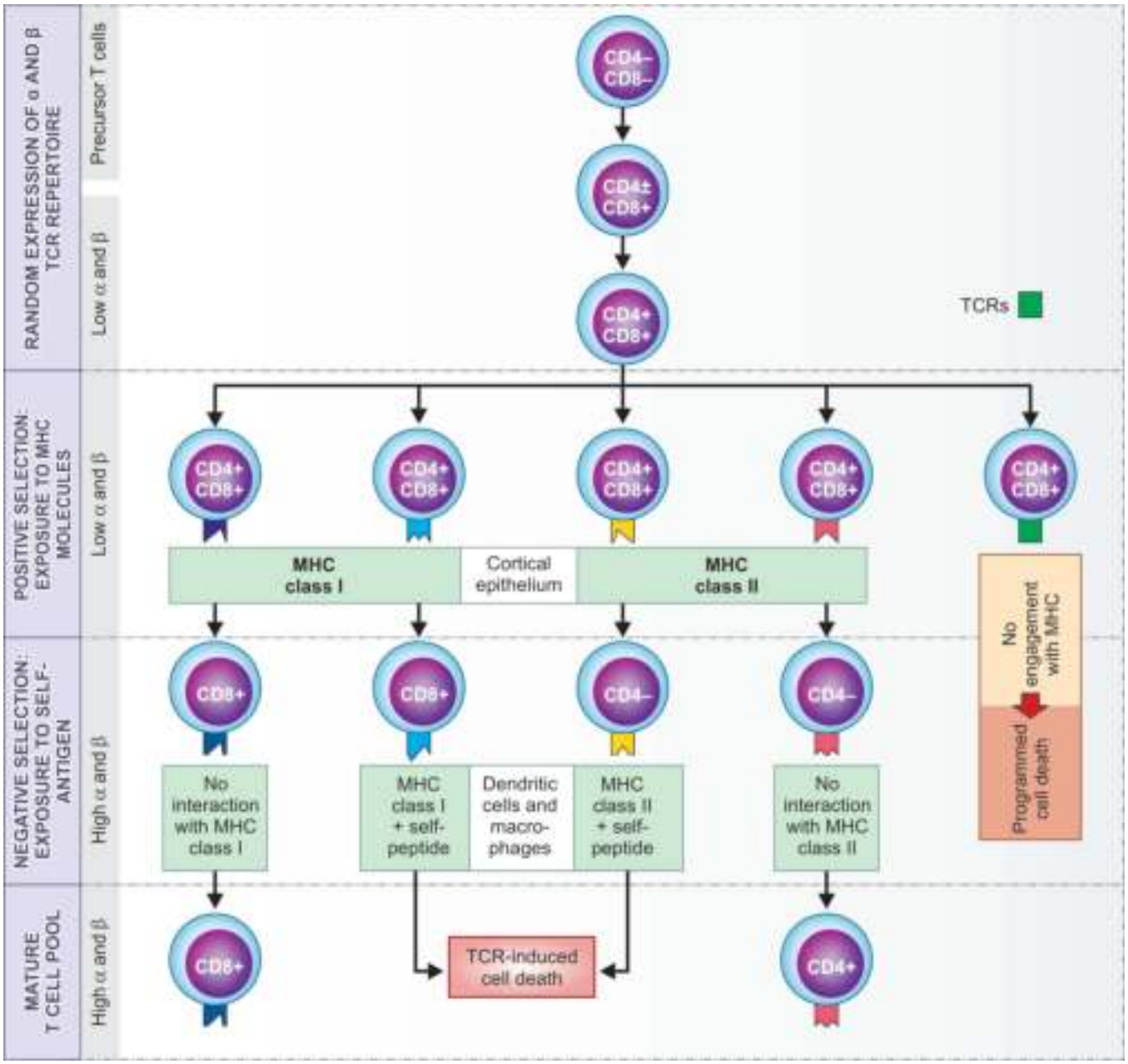


Fig. 4.68: Positive and negative selection of T cells during development in thymus gland. T cell progenitors develop in the bone marrow migrate to thymus gland. Early T cells are CD4 negative and CD8 negative (double negative thymocytes). After TCR rearrangement in the thymus gland, double negative thymocytes differentiate into CD4+ and CD8+ (double-positive thymocytes). Following interaction with self-peptide MHC complex class I molecules, thymocytes become CD8+ cytotoxic T cells. Thymocytes interacting with self-peptide MHC class II molecules become CD4+ helper T cells.

T CELL IMMUNOLOGIC TOLERANCE

T cell immunologic tolerance is a state of unresponsiveness of T cells towards specific self- or nonself-antigens. T cells and B cells with receptors specific for self-antigens are deleted in lymphoid cell development in bone marrow and thymus gland during fetal and early neonatal periods. This process is called central immunologic tolerance that allows elimination of self-reactive B cells and T cells in the tissues by negative selection. Lymphocytes that do not receive survival signals undergo apoptosis.

- T cells function as effector cells as well as regulator cells of the immune system. T cells become educated in the thymus gland and dependent on self-MHC class molecules for survival.
- The subset of T cell receptors that receive the correct antigen signals will leave the thymus gland and circulate in the peripheral lymphoid tissues. Some CD4+ helper T cells receive signals in the thymus

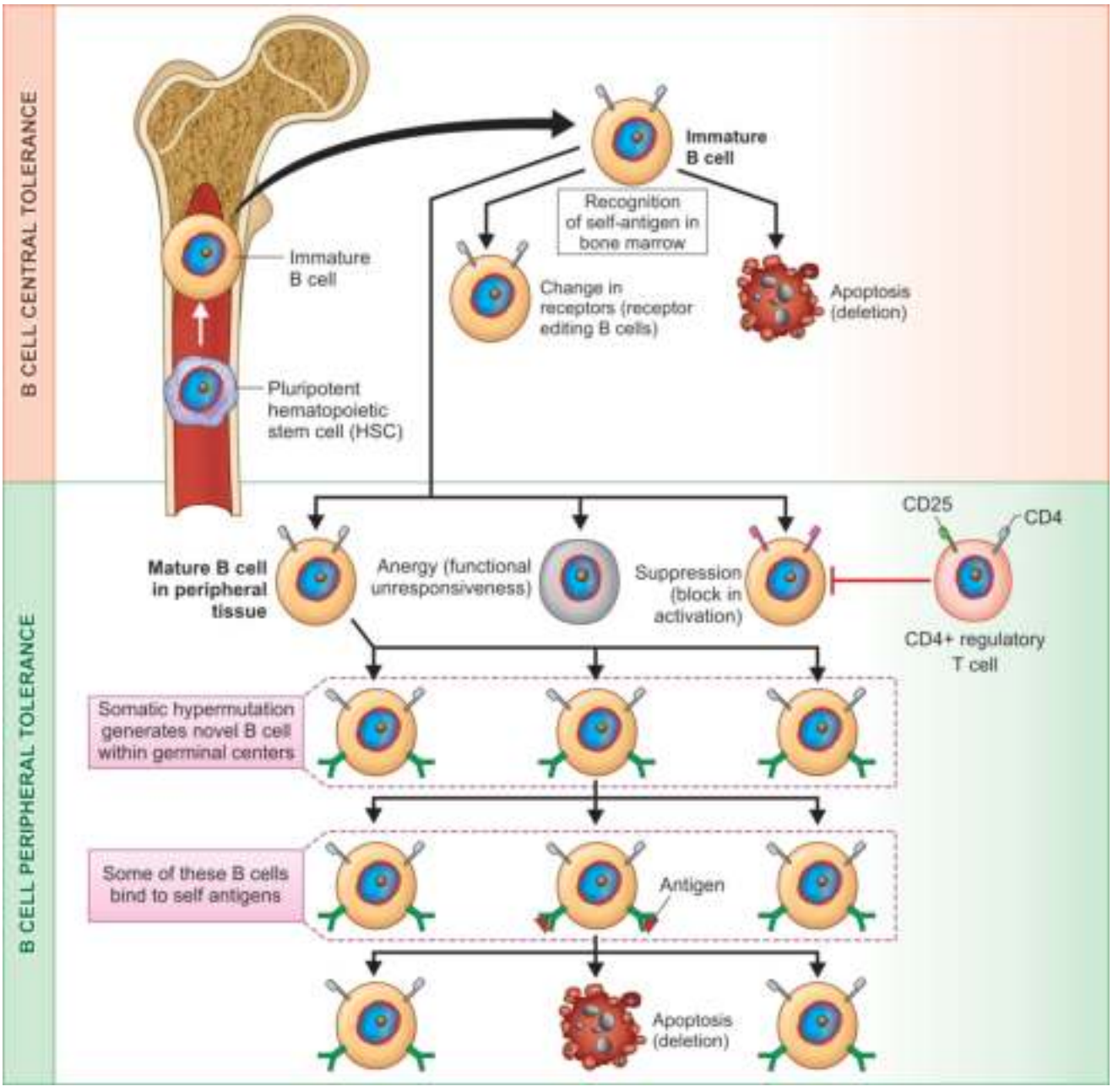


Fig. 4.69: Central and peripheral immunologic tolerance of B cells. Peripheral tolerance occurs after central tolerance. It takes place in the periphery after B cells egress from primary lymphoid tissues/organs. Its main purpose is to ensure that self-reactive B cells which have escaped central tolerance do not cause autoimmune diseases.

gland that select them to differentiate into CD4+ regulatory T cells, which express FOXP3 transcription factor and suppress the immune response by direct and indirect mechanisms.

- Through elimination of autoreactive T cells and B cells, central immunologic tolerance ensures that the immune system does not attack self-peptides hence prevent development to autoimmune diseases.
- Normal individuals are tolerant to their own antigens (self-antigen). Immunologically privileged sites are brain, eye, testis, uterus (fetus). Immunologic tolerance in pregnancy allows mother to gestate a genetically distinct offspring with an alloimmune response mutated enough to prevent miscarriage. Central and peripheral immunologic tolerance of T cells is shown in Fig. 4.70.

T CELL IMMUNOLOGIC TOLERANCE IN CENTRAL LYMPHOID ORGANS

Double negative (CD4 and CD8 negative) T cell progenitors migrate from bone marrow and enter the thymic cortex and rearrange their receptors to

Table 4.69 Layers of immunologic tolerance

Type of Tolerance	Mechanism	Site of Action
Central tolerance	▪ Deletion ▪ Editing	▪ Thymus gland (T cells) ▪ Bone marrow (B cells)
Antigen segregation	Physical barrier to self-antigen access to lymphoid system	Peripheral organs (e.g. thyroid gland, pancreas)
Peripheral anergy	Cellular inactivation by weak signaling without co-stimulus	Secondary lymphoid organs
Regulatory T cells	Suppression by cytokines intercellular signals	Secondary lymphoid tissue and sites of inflammation; multiple tissues in steady state
Functional deviation	Differentiation of CD4+ regulatory T cells that limits inflammatory cytokine secretion	Secondary lymphoid tissue and sites of inflammation
Activation-induced cell death	Apoptosis	Secondary lymphoid tissue/organs and sites of inflammation

Table 4.70 Factors influencing immunologic tolerance

Factors Affecting Response	Favoring Immune Response	Favoring Tolerance
Physical form of antigen	Large aggregated complex molecules properly processed	Soluble aggregates-free simple small molecules not processed
Route of administration of antigen	Subcutaneous and sometimes intramuscular route	Oral or sometimes intravenous route
Dose of antigen	Optimum dose	Ranging from small to large dose

Immunologic tolerance of host depends on heredity, age, gender and health.

become CD4+ and CD8+ (double positive thymocytes) independent on MHC class molecules.

- T cells expressing an αβ complex receptor must interact with self-peptide MHC class molecules to survive. Cells are instructed to repress expression of either CD4 or CD8 and to develop into single positive T cells. Cells that interact strongly with MHC class molecules on thymic epithelia receive a survival signal (positive selection). Depending on which MHC class molecules are recognized, T cells will display (single positivity) either CD4 (CD4+ helper T cells) or CD8 (CD8+ cytotoxic T cells).
- CD4+ helper T cells that recognize self-antigens expressed on thymocytes in the context of MHC class II molecules undergo apoptosis. The key factor in determining positive and negative selection is the strength of the antigen recognition by the maturing T cell.
- T cells with a receptor that bind with high-avidity to autoantigens on thymic epithelia undergo apoptosis leading to negative selection. T cells with a receptor that bind with low-avidity to autoantigens lead to positive selection. CD4+ regulatory T cells (Treg CD4+ and CD25+) that are autoantigen-specific are generated by intermediate degrees of binding.
- The autoantigens are host tissue proteins expressed on thymic epithelia under regulation of the transcription factor autoimmune regulator (AIRE). Many T cells are deleted in the thymus gland, only a fraction is present in peripheral lymphoid tissues or organs.
- The autoimmune regulator (AIRE) protein is part of a complex that regulates the expression of tissue-restricted antigens (TRAs) in medullary thymic epithelial cells (MTECs). Peptides derived from these antigens are displayed on the cells in the absence of functional autoimmune regulator (AIRE). These self-reactive T cells are not eliminated; which can enter tissues where the antigens continue to be produced and cause organ-specific autoimmunity including autoimmune polyglandular syndrome type 1 (APS1), which cause damage to parathyroid and adrenal glands.

T CELL IMMUNOLOGIC TOLERANCE IN PERIPHERAL LYMPHOID ORGANS

When self-reactive T cells leave the central lymphoid organs and settle in peripheral lymphoid tissue such as lymph nodes, spleen, MALT (mucosa-associated lymphoid tissue) and SALT (skin-associated lymphoid tissue), peripheral immunologic tolerance ensures that these self-reactive T cells are eliminated, and become anergic (functionally unresponsiveness to antigen).

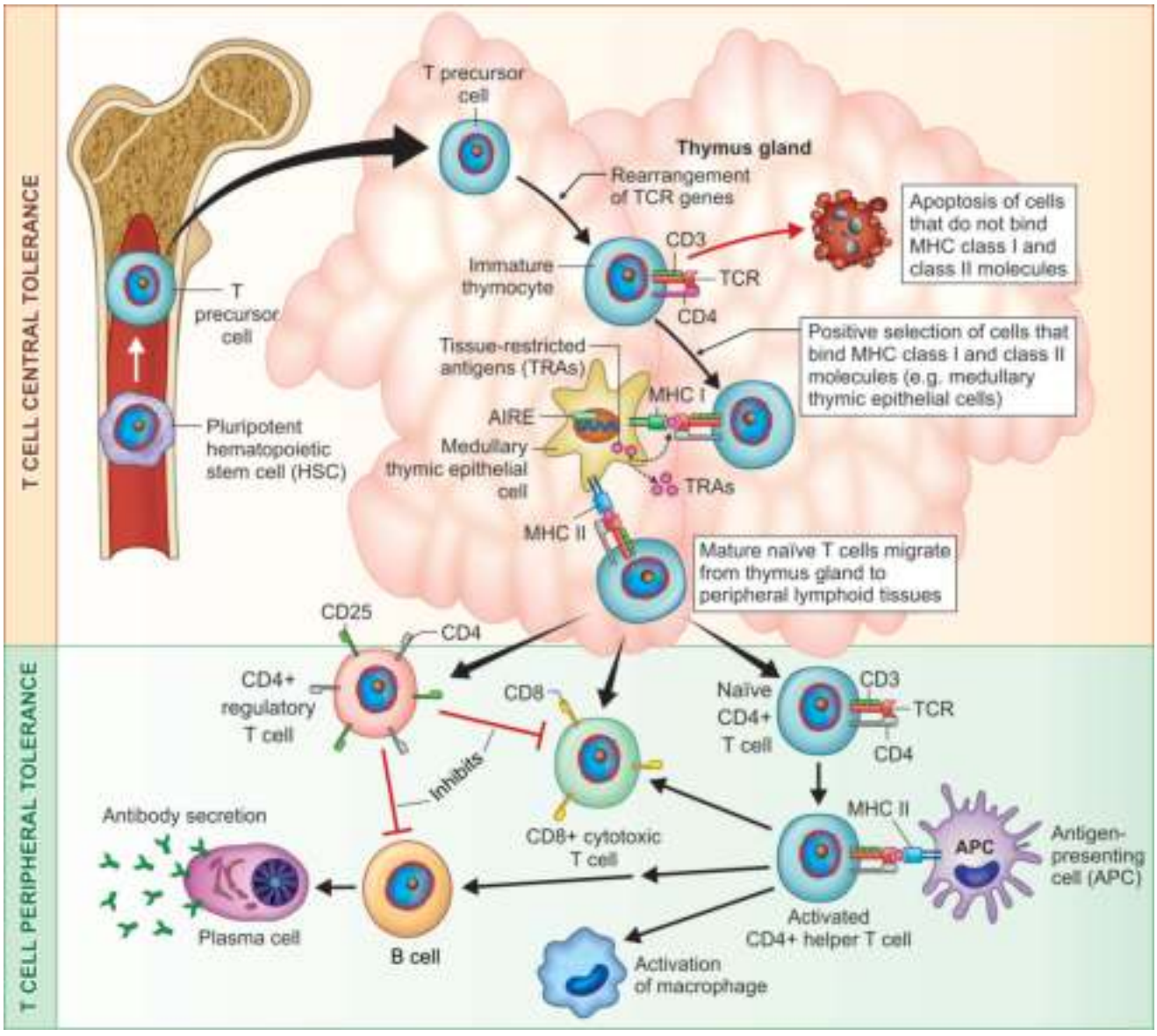


Fig. 4.70: Central and peripheral immunologic tolerance of T cells. After positive or negative selection three types of mature T cells are left: CD4+ helper T cells, CD8+ cytotoxic T cells and CD4+ regulatory cells. CD4+ helper T cells are involved in activating CD8+ cytotoxic T cells, and macrophages (particularly via the secretion of the cytokine interferon-γ) and obligatory for most B cell response.

- Several autoreactive clones of T cells are found in the peripheral blood of healthy persons. Autoreactive clones of T cells potentially activated, undergo proliferation in the setting of subacute bacterial endocarditis, which can lead to emergence of self-reactive clones of T cells that cause damage to kidneys.
- Peripheral immunologic tolerance occurs through one of these mechanisms: clonal deletion, ignorance, anergy and immune regulation.

Clonal Deletion

In fetal life, all T cells and B cells that recognize 'self'-antigens are eliminated by apoptosis. Fas-mediated apoptosis plays a critical role in the removal of mature autoreactive B cells and T cells.

- Fas-mediated apoptosis plays a key role in immune-privileged sites (e.g. central nervous system, eyes and testes). FasL is expressed on antigen-presenting cells, activated T cells and some other cells. Even foreign antigens accessing these tissues do not generally trigger immune responses.
- IL-2 is a cytokine predominantly secreted by activated CD4+ helper T cells essential for T cell homeostasis. IL-2 cytokine suppresses excessive T cell response by inducing activation-induced cell death (AICD) via caspase pathway and elicits the function of CD4+ FOXP3+ T regulatory cells (Tregs).

- Insufficient production of key transcription activators (e.g. NF-κB, AP-1, NFAT-1) during T cell activation reduces IL-2 production. Transcription suppressors (e.g. **CREM**) can also reduce the transcriptional activity of the IL-2 gene promoter resulting in T cell anergy.

Antigen Sequestration (Ignorance)

Autoreactive T cells may remain unresponsive by ignoring self-antigens located in immunologically privileged sites such as brain, eye (vitreous humor), testis, uterus (fetus), because these organs have low immunogenicity (i.e. low-binding affinity). These autoreactive T cells have the potential to be activated under certain circumstances and therefore pose a threat to the host.

- Sympathetic autoimmune ophthalmia is a severe inflammatory damage to both eyes, caused by release of sequestrated ocular self-antigens into the circulation, where these self-antigens eventually activate peripheral autoreactive T cells.
- Immune system is not normally exposed to ocular antigens, but trauma to a single eye releases autologous antigen that activate immune cells resulting in severe granulomatous inflammation of both eyes.

Clonal Anergy

Normally, both B cells and T cells require two signals for activation: (a) one set of signal for recognition of peptide antigen in association of MHC class molecule on the surface of antigen-presenting cells, and (b) second set of costimulatory signals provided by antigen-presenting cells. Lack of second signal switches the cell off.

- Certain T cell associated molecules such as CD28 must bind to their ligands B7–1 (CD80) and B7–2 (CD86). Anergy is a state of unresponsiveness induced by activation upon self-antigen recognition.
- Autoreactive T cells can be made unresponsive to antigens if the T cells engage MHC class molecule on an antigen-presenting cell (signal 1) without engagement of costimulatory molecules such as B7–1 (CD80), B7–2 (CD86) and IL-2 (signal 2).

Immune Regulation Achieved by the Action of Regulatory T Cells (Tregs)

'Induced' CD4+ and CD25+ regulatory T cells (iTregs) play an important role in maintaining immunological unresponsiveness to self-antigens and in suppressing immune responses deleterious to the host.

- 'Induced' regulatory T cells are also produced in the peripheral lymphoid organs rather than in the thymus gland, which have similar effector functions as natural regulatory CD4+ regulatory T cells.
- Patient with genetic CD4+ regulatory T cells (Tregs) dysfunction develops lymphadenopathy and inflammatory infiltrates consisting of autoreactive T cells in multiple organs. During active inflammation, CD4+ regulatory T cells do not provide protection to immune system function.

Peripheral Suppression by CD4+ Regulatory T Cells

Cells which recognize self-antigens develop into CD4+ regulatory T cells, which also express CD25+, α-chain of IL-2 receptor and transcription factor of the family FOXP3.

- CD4+ regulatory T cells synthesize IL-4, IL-10 and TGF-β, which have ability to downregulate function of autoreactive T cells.
- IPEX (immune dysregulation, polyneuropathy, enteropathy) is X-linked syndrome of systemic autoimmunity caused by mutations in FOXP3 gene, which codes for a member of the forkhead transcription factor family, expressed primarily in CD4+ and CD25+ regulatory T cells.

Pathology Pearls: Clinical Significance of Immunologic Tolerance

- **Induction of immunologic tolerance** is done to prevent allogenic grafts, treatment of autoimmune diseases and allergic diseases; and limiting tumor growth.
- **Termination of immunologic tolerance** is done to treat malignant tumor and infectious diseases by enhancing first and second signals.
- **Rheumatoid arthritis** is an autoimmune disease mainly affecting the joints. Clinical significance of cytotoxic T lymphocytes-associated antigen 4 (CTLA-4) is used a biologic response modifier to treat these patients significantly reducing joint inflammation.
- **CTLA-4** is a molecule expressed on activated T cells that firmly binds to costimulatory molecules on antigen-presenting cells (APCs), which decreases the function of T cells.
  - A fusion protein consisting of CTLA-4 using monoclonal antibodies (abatacept or belatacept) is used in rheumatoid arthritis and other transplantation in clinical practice.
  - **CTLA-4** is constitutively expressed on cancer stem cells and can trigger apoptosis upon ligand interaction. There is also an interest in blocking CTLA-4 using monoclonal antibodies (e.g. ipilimumab) to inhibit tumor tolerance.

B CELL IMMUNOLOGIC TOLERANCE

Immunologic tolerance for B cells is a state in which B cells do not initiate an immune response to an antigen. Central immunologic tolerance is the main way by which immune system learns to discriminate self- from

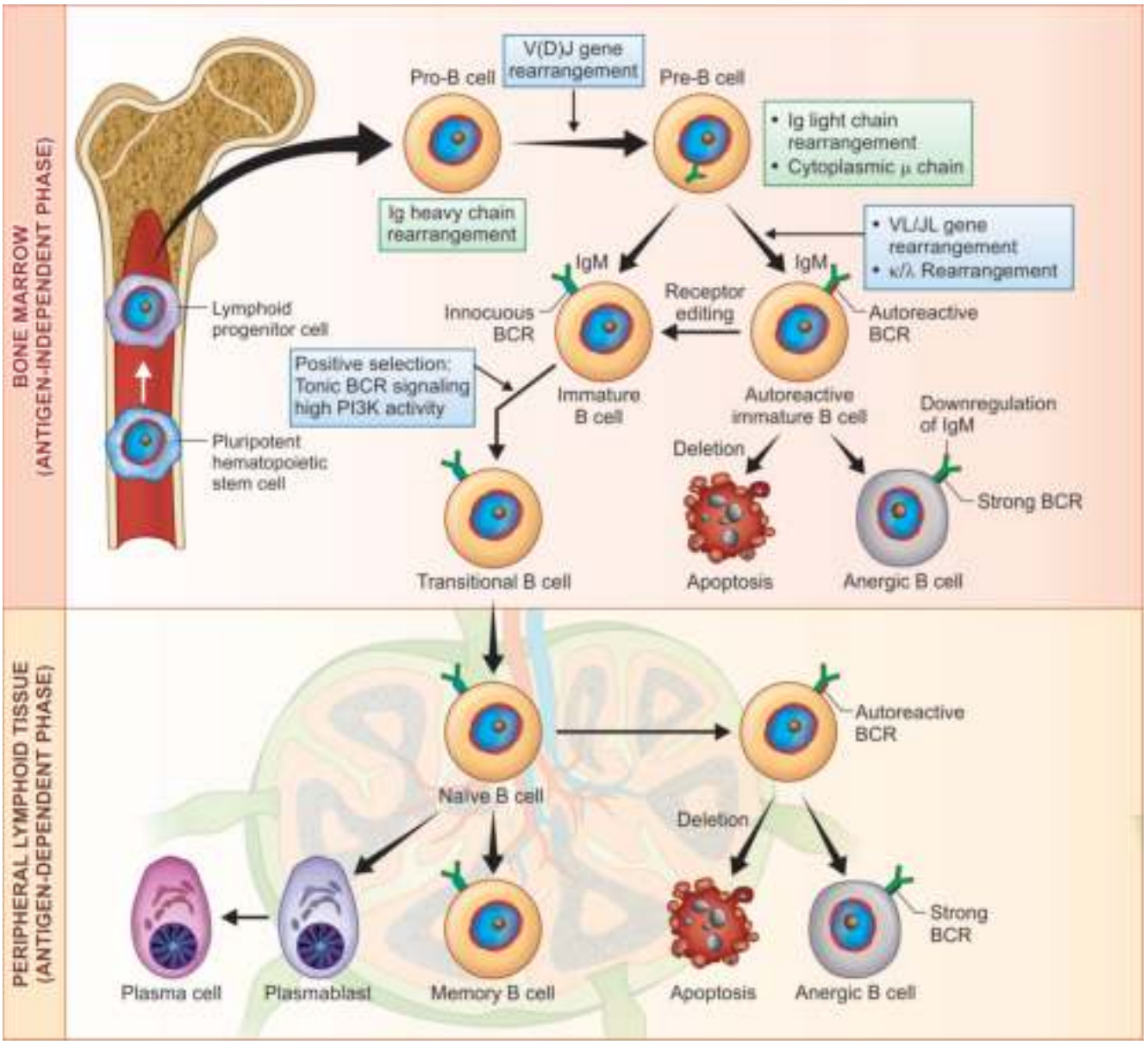


Fig. 4.71: Central and peripheral immunologic tolerance of B cells. Both clonal and anergy are mechanisms utilized in the central as well as peripheral lymphoid organs. Strength of BCR signaling affects the fate of the B cells undergoing tolerance. Receptor editing occurs primarily in the bone marrow. Transitional B cells are found in the bone marrow and spleen. Peripheral immunologic tolerance is primarily found in splenic transitional B cells, which depends on tonic BCR signaling thresholds and survival signals from BAFF.

nonself-antigens. Peripheral immunologic tolerance is key to prevent over-reactivity of the immune system to various environmental antigens (e.g. allergens and gut microbes). Central and peripheral immunologic tolerance of B cells is shown in Fig. 4.71.

B CELL IMMUNOLOGIC TOLERANCE IN CENTRAL LYMPHOID ORGANS

During normal B cell development in the bone marrow, set of processes assist in induction of B cell central immunologic tolerance.

- Education of B cells and elimination of autoreactive B cell clones is somewhat different from that of T cell central immunologic tolerance.
- B cells are still immature when they relocate from bone marrow to spleen T cell zones.
- Autoreactive B cells are not necessarily deleted during negative selection in the bone marrow. B cells that recognize autologous antigens, which are eliminated via apoptosis or become unresponsive (anergic).
- Autoreactive B cells that escape negative selection become part of a maximally-diverse immune system.

Pathology Pearls: B Cells Immunologic Tolerance in Central Lymphoid Organs

- Immunologic tolerance inhibits the potentially destructive immune responses of lymphocytes to host tissues. B cell immunologic tolerance is regulated at the stage of immature B cell development by receptor editing and apoptosis mechanisms.
- Receptor editing involves recombination of immunoglobulin light chain gene leading to secondary rearrangements that can alter antigen receptor specificity by replacing one light chain with another.
- B cell receptor (BCR) signaling in immature B cells promotes the positive selection of B cells with unligated receptors through a phosphoinositide-3 kinase (PI3K)-dependent pathway, whereas BCR ligation promotes negative selection in part through inhibiting this pathway and probably also by downregulating B cell receptor (BCR) levels.
- Increasing number of single gene defects have been described that inhibit central tolerance for B cells in human beings leading to human autoimmune diseases and in several primary immunodeficiencies caused by single gene mutations.

B CELL IMMUNOLOGIC TOLERANCE IN PERIPHERAL LYMPHOID ORGANS

B cell immunologic tolerance in peripheral lymphoid tissues normally occurs when B cells encounter cognate antigen in a way that leads to 'unproductive' BCR (B cell receptor) signaling and induce anergy. Its main purpose is to ensure that autoreactive B cells which have escaped central immunologic tolerance do not cause autoimmune disease.

- Imperfect T cell peripheral immunologic tolerance causes autoimmune disease. T cell-dependent B cell activation requires help from preactivated cognate autoreactive T cells. T cell-independent B cell activation can be achieved by autologous antigens.
- Microbial antigens structurally similar to autologous antigens can lead B cells to produce cross-reactive antibodies in a phenomenon known as molecular mimicry. B cells hypermutate their receptors on activation, so there is a second chance that B cells may become autoreactive to induce autoimmune disease.

AUTOIMMUNE DISEASES

Autoimmune reactions cause disease, when immune system is not able to distinguish self- from nonself-antigens. Autoantibodies or host T cells attack against self-antigens in tissues or organs, which may be systemic or organ-specific. Susceptibility to autoimmune diseases appears to be influenced by gender and genes in the MHC. Autoimmune diseases are mediated by types 2, 3 and 4 hypersensitivity reactions, which commonly affect females. Autoimmune diseases include systemic lupus erythematosus, rheumatoid arthritis, Hashimoto thyroiditis, type 1 diabetes mellitus, myasthenia gravis, autoimmune hemolytic anemia, Sjögren syndrome, and multiple sclerosis.

Pathology Pearls: Pathogenesis of Autoimmune Diseases

- Mechanism of autoimmune disorders occurs by breakdown of peripheral tolerance.
- There is no evidence of breakdown of central tolerance. A number of possible mechanisms may mediate autoimmunity. Pathogenesis of autoimmune diseases is shown in Fig. 4.72.

Genetic Predisposition

- Susceptibility to autoimmune disease is influenced by genes in the major histocompatibility complex (MHC).
- Some HLA antigens are associated with increased incidence of certain autoimmune disorders. For example, there is increased incidence of **Hashimoto's thyroiditis** in HLA-DR5 and HLA-B5 positive individuals.

Environmental Factors

Some viruses may initiate autoimmune reactions in genetically susceptible persons. Some viruses trigger autoimmune islet cell destruction result in **type 1 diabetes mellitus**.

Exposure of Cryptic Self and Epitope Spreading

- Cryptic self means hidden epitopes or protein that has not been exposed during embryonic life.
- Generally, each self-protein in the body expresses few epitopes to T cells during embryonic development, thus, T cells are either deleted in the thymus or undergo anergy in the peripheral organs. But sometimes during adult life, the protein may present some uncommon epitopes, which may lead to autoimmune reaction.

Host Antigens Recognized as Nonself

- Host antigens are recognized as nonself, if modified by viral infection, inflammation, and trauma or forming complex with a drug.
- Examples of host antigens include thyroglobulin, lens protein, and spermatozoa. A foreign antigen may share a common structure with a host antigen.

Release of Sequestered Antigens

Spermatozoa and ocular antigens are completely sequestrated during development, which act as foreign bodies, when these come in contact with systemic circulation resulting immune response.

Autoantibodies

Many autoimmune disorders show presence of autoantibodies directed against host tissue. The demonstration of autoantibodies is presumptive and not entirely conclusive in the diagnosis of autoimmune nature of disorders.

Dysfunctional Immunoregulation

- Autoimmune disorders are caused by following mechanisms: (a) there may be increased in CD4+ helper T cells function, or decreased in CD8+ cytotoxic T cells function. (b) EB virus may activate B cells resulting in polyclonal antibody synthesis.
- Superantigens (TSST, Staphylococcus) activate a large pool of CD4+ helper T cells in antigen-independent manner without relation to their epitope's specificity. (c) There may be thymic defects of T cell.

Breakdown of T Cells Anergy

Antigen-presenting cells express B7 ligands that synthesize IL-2 that stimulates Th1 cells as in case of **multiple sclerosis**.

Failure of Apoptosis

Failure of apoptosis allows persistence and proliferation of autoreactive T cells in peripheral tissues.

SPECIFIC AUTOIMMUNE DISEASES

Autoimmune diseases encompass a group of loosely related conditions, most of which feature fibrinoid necrosis in connective tissue due to antinuclear antibodies (ANAs) and various other autoantibodies. Selected

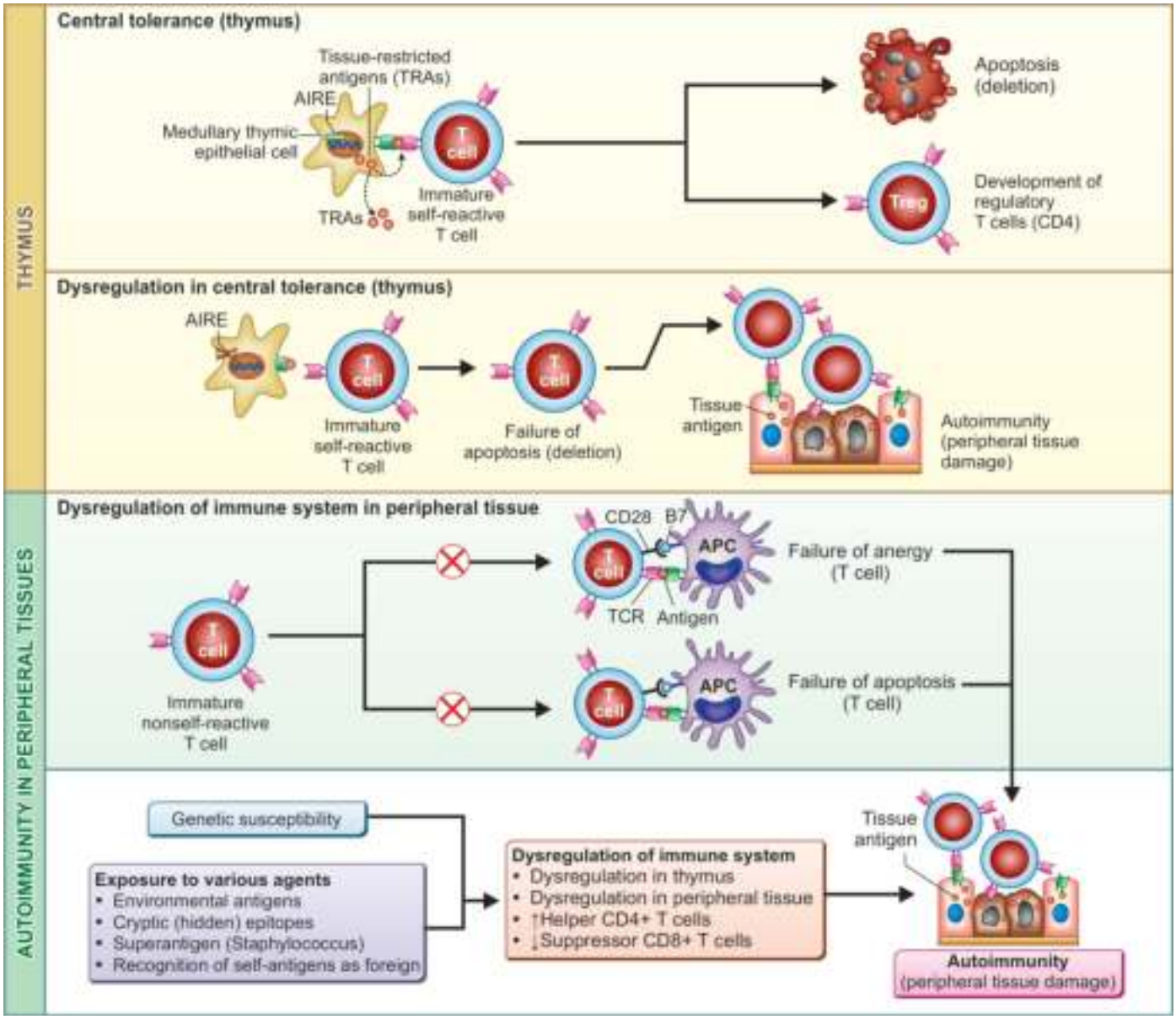


Fig. 4.72: Pathogenesis of autoimmune diseases. Autoimmune diseases result from susceptibility of genes that may interfere with self-immune tolerance and injurious stimuli leading to influx of lymphocytes into the tissue. Activation of lymphocytes causes tissue damage.

autoimmune diseases with autoantibodies reacting only with antigens in the affected organs are given in Table 4.71. Therapeutic strategies in autoimmune diseases are given in Table 4.72.

Table 4.71 Selected autoimmune diseases with autoantibodies reacting only with antigens in the affected organs

Disease	Organ or Tissue	Type of Hypersensitivity	Characteristics
Endocrine system			
Hashimoto's thyroiditis	Thyroid gland	2	Antibodies against thyroid peroxidase and thyroglobulin causing destruction of thyroid follicles
Graves' disease	Thyroid gland	2	Antibodies against thyroid-stimulating hormone receptor (TSH receptor) on thyroid follicular cells resulting in excess of T3 and T4 hormones and thus hyperthyroidism
Primary myxedema	Thyroid gland	2	Antibodies against thyroid peroxidase
Type 1 diabetes mellitus	Pancreas	2	Antibodies (glutamic acid decarboxylase 65, insulin, insulinoma-associated 2[IA-2] and zinc transporter 8[ZnT8]) against islet cell cytoplasmic antigen; causing destruction of insulin secreting β cells of islets of Langerhans
Addison disease	Adrenal gland	2	Antibodies against surface antigens on steroid producing cells; microsomal antigens
Male infertility	Testes	2	Antibodies against surface antigens on spermatozoa
Skin			
Pemphigus vulgaris	Skin	2	Antibody-mediated activation of proteases cause disruption of intercellular junction cadherin adhesion protein in prickle layers of epidermis results in skin vesicles (i.e. bullae)
Bullous pemphigoid	Skin	2	Antibodies against epidermal-dermal basement membrane
Vitiligo	Skin	2	Antibodies against surface antigens on melanocyte
Discoid lupus erythematosus	Skin	2	Antibodies against nuclear antigen
Connective tissue			
Rheumatoid arthritis	Joints	3, 4	Antibodies formed against collagen IgG (rheumatoid factor)
Ankylosing spondylitis	Joints	2	Antibodies (multiple connective tissue proteins) against sacroiliac and spinal apophyseal joints
Systemic lupus erythematosus	Multiple organs involvement	2, 3	Antibodies formed against nuclear antigens (DNA-anti DNA)
Scleroderma (excess collagen deposition in organs)	Systemic involvement	2	Antibodies formed against many intracellular organelles such as DNA topoisomerase, centromeres
Dermatomyositis	Dermis and skeletal muscle	2	Antibodies against extractable nuclear antigen
Neuromuscular system			
Myasthenia gravis	Neuromuscular junction	2	Antibody formed against acetylcholine receptors at neuromuscular junction resulting in inhibiting acetylcholine binding and downregulation of receptors
Multiple sclerosis	Neural tissue	2, 4	T cells and antibodies (myelin basic protein) sensitized to surface antigens on myelin sheath resulting in destroy neurons

Contd...

Table 4.71 Selected autoimmune diseases with autoantibodies reacting only with antigens in the affected organs (*Contd...*)

Disease	Organ or Tissue	Type of Hypersensitivity	Characteristics
Eyes			
Sjögren syndrome	Lacrimal gland, salivary gland and thyroid gland	2	Antibodies (SS-A, SS-B) against antigens on lacrimal gland, salivary gland, thyroid gland and nuclei of cells
Phacogenic uveitis	Lens	2	Antibodies against lens
Sympathetic ophthalmia	Uveal tract	2	Antibodies against uveal tract antigen
Hepatobiliary system			
Primary biliary cirrhosis	Liver	2	Antinuclear antibodies formed against bile duct cells
Cardiovascular system			
Antinuclear cytoplasmic antibody (ANCA)-mediated vasculitis	Blood vessels	2	ANCA-mediated vasculitis resulting in neutrophil degranulation and inflammation of vessels
Rheumatic fever	Heart	2	Antibodies to group A streptococci cross react with heart tissue and major joints (molecular mimicry)
Renal system			
Immune complex-mediated glomerulonephritis	Kidneys	2, 3	Immune complexes formed in excess and deposition in glomerular basement membrane
Goodpasture syndrome	Kidney glomeruli and lung alveoli	2	Antibodies to Goodpasture antigen in noncollagenous proteins in basement membrane of glomeruli and alveoli
Hematological system			
Idiopathic neutropenia	Neutrophils	2	Antibodies formed against surface antigen present on neutrophils
Idiopathic lymphopenia	Lymphocytes	2	Antibodies formed against surface antigen present on lymphocytes
Autoimmune hemolytic anemia	Red blood cells	2	Antibodies formed against surface antigen present on RBCs resulting in opsonization and phagocytosis of RBCs and thus hemolysis, anemia
Autoimmune thrombocytopenic purpura	Platelets	2	Antibodies formed against platelet membrane proteins resulting in opsonization and phagocytosis of platelets and thus thrombocytopenia
Pernicious anemia	Gastric parietal cells		Antibodies against intrinsic factor synthesized by parietal cells prevent transport of vitamin B_{12}

Table 4.72 Therapeutic strategies in autoimmune diseases

Target	Therapeutic Strategies
Self-reactive lymphocytes	▪ Inhibition of lymphocyte function (cytotoxic drugs, cyclosporine A, corticosteroids) ▪ Reinduction of anergy (peptide therapy) ▪ Removal of co-stimulation (anti-CD28 antibodies) ▪ Induction of inhibitory T cells (oral feeding of antigen)
Tissue damage	Anti-inflammatory drugs (corticosteroids)
Organ dysfunction	▪ Replacement therapy (joint replacement) ▪ Renal dialysis ▪ Thyroxine administration in hypothyroidism ▪ Insulin administration in diabetes mellitus

SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is an autoimmune chronic inflammatory disease characterized by a generalized dysregulation and hyperactivity of B cells, with production of autoantibodies to individual's own DNA, RNA, and autologous proteins, leads to immune complex formation and deposition in glomeruli, skin, lungs, joints synovium, mesothelium (serous membranes) and other organs. Renal involvement is most common complication of SLE, which can occur at all ages, but is more common in young women during childbearing age.

Pathogenesis

Autoantibodies are formed against individual' own double-stranded DNA and soluble nuclear Sm (Smith) antigen part of nucleosome. High titers of these autoantibodies are nearly pathognomonic for systemic lupus erythematosus.

- Insoluble aggregates of immune complex are deposited in vessel walls, serosal surfaces, and other extravascular sites, and bound to complement.
- The antigen–antibody complement complexes are highly chemotactic for neutrophils, which release lysosomal enzymes and other chemical mediators (prostaglandins, kinins, and oxygen-derived free radicals) leading to tissues/organs damage.

Clinical Features

Patient with systemic lupus erythematosus (SLE) presents with fever, malaise, lymphadenopathy, and weight loss. Recent clinical manifestations in systemic lupus erythematosus are shown in Fig. 4.73. Clinical and immunologic domains of systemic lupus erythematosus are given in Table 4.73.

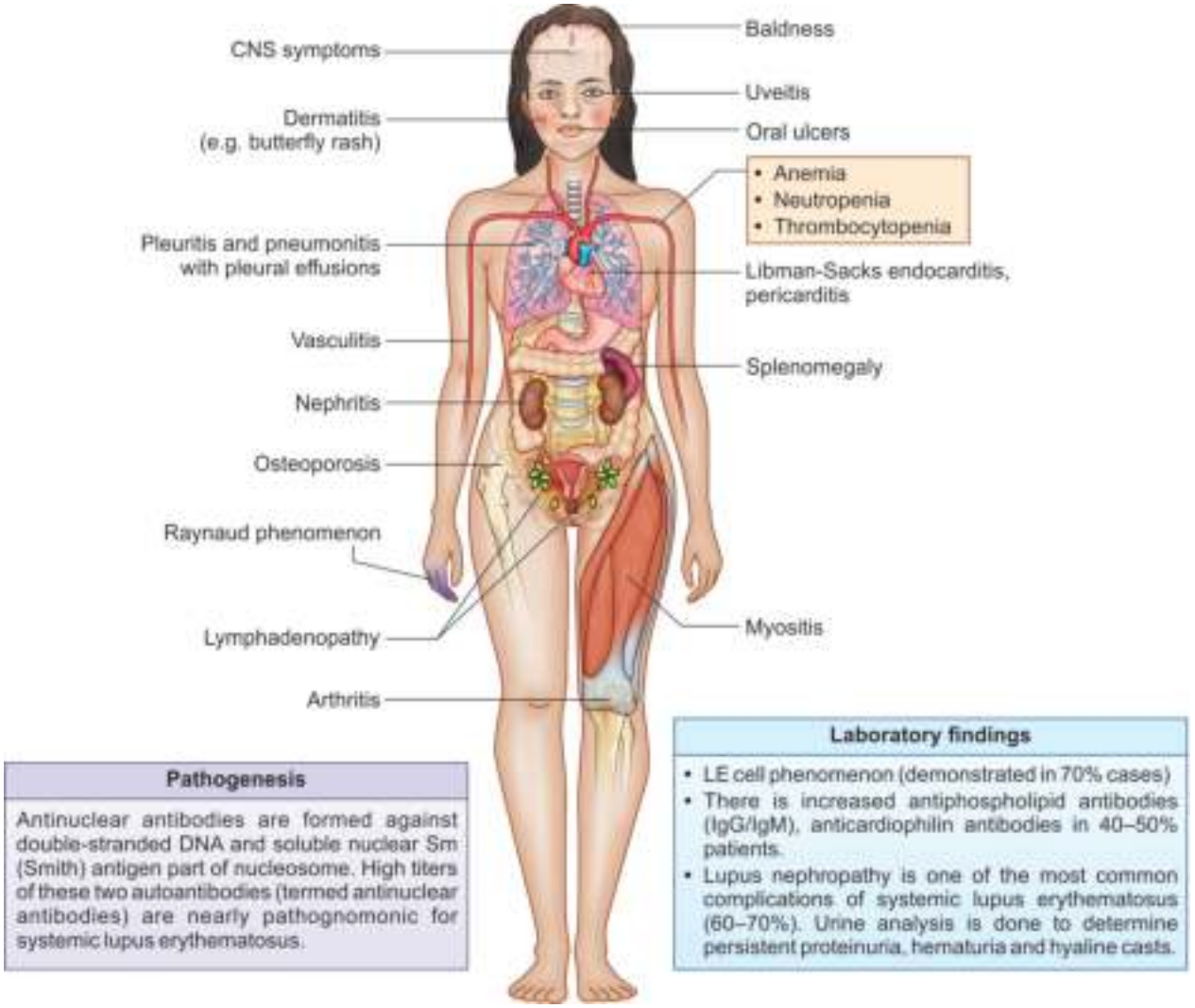


Fig. 4.73: Clinical systemic manifestations and systemic lupus erythematosus (SLE). Patients with systemic lupus erythematosus present with fever, malaise, arthralgias, headache, loss of appetite, weight loss. Red, butterfly-shaped rash over cheeks and nose often following exposure to sunlight. Signs and symptoms may develop suddenly or slowly, that may be mild or severe; temporary or permanent. An antinuclear antibody (ANA) blood test measures the presence of antibodies that are directed against body's cells, a sign of systemic lupus erythematosus.

Table 4.73 Recent clinical and immunologic domains of systemic lupus erythematosus

Domain	Criteria
Clinical domains	
Constitutional symptoms	Fever >38.3°C
Hematologic manifestations	▪ Leukopenia ▪ Thrombocytopenia ▪ Autoimmune hemolytic anemia
Neuropsychiatric manifestations	▪ Delirium ▪ Psychosis ▪ Seizures
Ophthalmic fundus examination	Yellowish, cotton wool-like lesions (cytoid bodies)
Skin	▪ Butterfly rash over the base of the nose and malar eminences ▪ Inflammation of superficial blood vessels responsible for telangiectasis (spider vein), red lines and painful bumps
Cervical lymph nodes	Lymphadenopathy
Spleen	Splenomegaly
Mucocutaneous manifestations	▪ Alopecia ▪ Ulcers in mucous membranes of nose, throat, and mouth ▪ Subacute cutaneous lupus erythematosus (SCLE)/discoid lupus erythematosus (DLE) ▪ Acute cutaneous lupus erythematosus (ACLE)
Cardiovascular manifestations	▪ Acute pericarditis ▪ Pericardial effusion ▪ Libman-Sacks endocarditis ▪ Immune complex-mediated vasculitis ▪ Vasospasm of small blood vessels of fingers (Raynaud's phenomenon)
Pulmonary manifestations	▪ Pleuritis ▪ Pleural effusion ▪ Diffuse interstitial pulmonary fibrosis
Musculoskeletal system manifestations	Painful, swollen, but nonerosive arthritis of hands, knees and seldom spine, tenderness and stiffness in two or more joints for at least 30 minutes in the morning
Renal manifestations including renal biopsy histology finding depending on damage to the glomeruli	▪ Urine analysis: Presence of >0.5 g/24 hours by 24-hour urine or equivalent spot urine protein to creatinine ratio ▪ Class II: Mesangial proliferative lupus nephritis ▪ Class III: Focal lupus nephritis/IV (diffuse lupus nephritis) ▪ Class IV: Diffuse lupus nephritis ▪ Class V: Membranous lupus nephritis
Female genital system	Repeated spontaneous miscarriages attributable to antibody-mediated inhibition of tPA activity necessary for trophoblastic invasion of the uterus
Immunologic domains	
Antiphospholipid antibody syndrome (IgG)	▪ Anticardiophilin antibodies (IgA, IgG, or IgM) at medium or high titer (>40 APL, GPL, or MPL) or anti-β_2-GP1 antibodies (IgA, IgG, or IgM) ▪ Lupus anticoagulant positive ▪ Spontaneous abortion ▪ Recurrent arterial or venous thromboses (deep veins, portal vein, pulmonary artery) ▪ Prolonged activated partial thromboplastin time (APTT)
Complement system proteins	▪ C3 or C4 low ▪ C3 and C4 low
Systemic lupus erythematosus specific antibodies	▪ Anti-Sm antibodies ▪ Anti-Ds antibodies in an immunoassay with demonstrated ≥90% specificity for systemic lupus erythematosus

- **Skin manifestations:** Patient develops butterfly skin rash over the base of the nose and malar eminences, often is accentuated by sun exposure. When superficial blood vessels become inflamed, patient may develop telangiectasis (spider vein), red lines and painful bumps. A biopsy of sun exposed skin that is not involved with a rash will demonstrate immune complex deposition in systemic lupus erythematosus (SLE).
- **Mucocutaneous manifestations:** Patient can develop ulcers in the mucous membranes particularly in the nose, throat, and mouth.
- **Musculoskeletal manifestations:** Most people develop painful, swollen, but nonerosive arthritis. Joints are asymmetrically involved such as hands, knees and seldom spine. Nonspecific muscle pain is another common symptom.
- **Raynaud phenomenon:** Raynaud phenomenon is manifested by vasospasm of small vessels, most often of the fingers.
- **Pulmonary manifestations:** Patient develops diffuse interstitial pulmonary fibrosis, manifested as interstitial pneumonitis or diffuse fibrosing alveolitis.
- **Serous membranes involvement:** Patient develops inflammation and effusion of pericardium and pleura. Pleurisy leads to pain during inspiration.
- **Heart:** Nonbacterial cardiac vegetations are present on both sides of the mitral valve leaflet (most common) and tricuspid valve (less frequent), which are known as 'Libman-Sacks' endocarditis.
- **Blood vessels:** Immune complex vasculitis can occur in any organ.
- **Spleen:** In the spleen, perivascular fibrosis with concentric rings of collagen around splenic arterioles results in a characteristic onion-skin appearance.
- **Reproductive system manifestations:** Repeated spontaneous miscarriages are sometimes the first sign of the disease, which is attributable to antibody-mediated inhibition of tissue plasminogen activator (tPA) activity necessary for trophoblastic invasion of the uterus.
- **Antiphospholipid antibody syndrome:** One-third of patients with systemic lupus erythematosus (SLE) possess elevated concentrations of antiphospholipid antibodies.
  - Prolonged activated partial thromboplastin time (APTT), recurrent arterial or venous thromboses (deep veins, portal vein, pulmonary artery), and spontaneous abortion is highly suggestive of the antiphospholipid antibody syndrome.
  - Current treatment includes anticoagulation therapy (aspirin, heparin, and warfarin) and immunosuppression in refractory cases.
- **Central nervous system and ocular manifestations:** Patient develops neurologic and psychiatric manifestations. Ophthalmic fundus examination shows yellowish, cotton wool-like lesions (cytoid bodies).
- **Lupus nephropathy:** Lupus nephritis is one of the most common complications of systemic lupus erythematosus (60–70%). Immune complexes (DNA-anti-DNA) may localize in glomeruli by deposition from the circulation, formation *in situ*, or both, which cause activation of complement system by classical pathway resulting in damage to tubules and interstitium. Decreased serum C3 level correlates with disease activity. In general, the more immune complex deposition and the more cellular proliferation, the worse the renal disease. Renal changes are highly variable.

Laboratory Diagnosis

In diagnostic pathology, hematoxylin body or 'LE body' is a dense homogenous, basophilic structure easily stained with hematoxylin. Hematoxylin body or LE body consists of degraded nuclear material from injured glomerular cells into the urinary space and phagocytosed by renal tubular epithelial cell, along with autoantibodies and a limited amount of cytoplasm. Hematoxylin body also called LE body in the involved glomeruli in systemic lupus erythematosus is shown in Fig. 4.74.

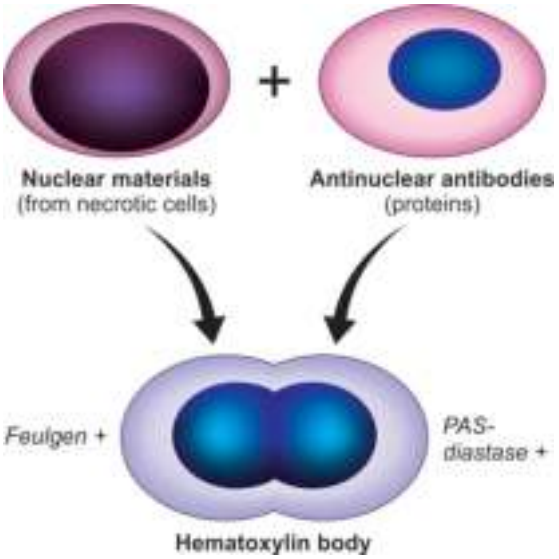


Fig. 4.74: Hematoxylin body also called LE body in the involved glomeruli in systemic lupus erythematosus. In diagnostic pathology, hematoxylin body or 'LE body' is a dense homogenous, basophilic structure easily stained with hematoxylin. It consists of degrades nuclear material from injured glomerular cells into the urinary space and phagocytosed by renal tubular epithelial cell, along with autoantibodies and a limited amount of cytoplasm.

- **LE cell phenomenon:** LE cell is performed *in vitro*, which is possibly mediated by an ANA known as antinucleosome-specific autoantibody. LE cells are formed in a mixture of mechanically damaged neutrophils and autoantibody-containing patient serum. LE test is positive in about 70% of SLE cases and has now been largely replaced by more sensitive determinations.
- **Antiphospholipid antibodies:** Antiphospholipid antibodies are present in 40–50% patients. There is an increase level of IgG/IgM anticardiophilin antibodies. Lupus anticoagulant antibodies interfere with blood coagulation resulting in increased clotting time.
- **Serum complement concentration:** Serum complement level is often greatly decreased, especially in association with active renal involvement. Immune complexes at dermal–epidermal junction are demonstrable in skin biopsies.
- **Urine analysis:** Urine analysis is done to determine persistent proteinuria, hematuria and hyaline casts in lupus nephritis.
- **Hematologic tests:** Reticulocytes count is increased as the patient may develop autoimmune hemolytic anemia. Peripheral blood smear shows leukopenia (lymphopenia), and thrombocytopenia. Bleeding time is prolonged.
- **Immunofluorescence microscopy:** Immunofluorescence microscopy is done to demonstrate antinuclear antibodies against DNA, histone proteins, non-histone proteins bound to RNA, nucleolar antigens. Anti-Smith (Sm) antigen antibodies are formed against dsDNA-pathognomonic of SLE.

SCLERODERMA

Scleroderma is an autoimmune disease of connective tissue characterized by progressive systemic sclerosis. Inflammation stimulates fibroblasts to synthesize excessive collagen fibers, which frequently occurs in skin, hence the name scleroderma: Greek word *sclero* meaning hard and Greek word *derma* meaning skin. Scleroderma occurs most frequently in young women (25 to 50 years), which involves widespread fibrosis in skin, gastrointestinal tract (especially the esophagus), heart, muscle, lung and kidney.

Clinical Pearls: Local and Systemic Scleroderma

There are two main categories of scleroderma: localized and generalized. Each category is comprised of several conditions.

Local Scleroderma

- Local scleroderma is restricted to skin. The initial edema may last for several weeks or months. Skin thickening develops over a course of 3 years, and then the symptoms gradually stabilize or even reverse.
- Local scleroderma occurs in two forms: morphea scleroderma and linear scleroderma.
  - **Morphea scleroderma:** Patient presents with discrete oval patches on trunk, face, or extremities, where skin is dry and thick. These lesions become pale in center and purplish around the edges.
  - **Linear scleroderma:** Patient presents with discolored line or band on legs, arms or forehead.

Systemic Scleroderma

- Systemic sclerosis has a slow-onset that begins as CREST syndrome, but it may eventually involve internal organs.
- Tissues most at risk are digestive tract, the heart and circulatory system, kidneys, lungs, musculocutaneous system, especially synovial membranes in joints and around tendons. It can be fatal. It has three major subtypes.
  - **Limited scleroderma:** Limited scleroderma begins in CREST syndrome, and it is only progressive.
  - **Systemic scleroderma:** Systemic scleroderma is more sudden onset and earlier involvement of internal organs.
  - **Sine scleroderma:** Sine scleroderma does not involve the skin at all. It only involves organs.

Clinical Features

Patient initially presents with skin changes (fixed facial appearance), painful joints, and dysphagia (esophagus involvement).

- **Facial expression:** Skin involvement in scleroderma results in fixed facial expression. There is thickening, tightening and rigidity of facial skin, with small constricted mouth and narrow lips, in atrophic phase of scleroderma.
- **Sclerodactyly:** Sclerodactyly is present (claw-like appearance of the hand). Raynaud phenomenon is seen in approximately 75% of patients. Fingers are partially fixed in significant position, terminal phalanges become atrophied, fingers are pointed and ulcerated.
- **Esophageal fibrosis:** Esophageal fibrosis in scleroderma results in dysphagia. Interstitial pulmonary fibrosis is a serious complication.
- **Renal involvement:** The kidneys are involved in more than half of patients with scleroderma, which show marked vascular changes, often with focal hemorrhage and cortical infarcts. Among the most severely affected vessels are the interlobular arteries, arcuate arteries and afferent arterioles showing marked thickening consisting of dense laminated matrix, rich in elastic fibers and small amount of collagen fibers. Early fibromuscular thickening of the subintima causes luminal narrowing, followed by fibrosis. Hypertension often occurs in scleroderma.
- **Pulmonary involvement:** Pulmonary interstitial fibrosis is a serious complication in scleroderma.

Laboratory Diagnosis

Autoantibodies virtually specific for scleroderma include: (a) Scl70 nonhistone nuclear protein (topoisomerase) in 70% cases, (b) nucleolar autoantibodies (primarily against RNA polymerase) and (c) anticentromere antibodies, which are also associated with the "CREST" variant of the disease. CREST syndrome is a less severe variant of systemic sclerosis (scleroderma) is characterized by calcinosis, Raynaud phenomenon, esophageal dysfunction, sclerodactyly, and telangiectasia.

Clinical Pearls: CREST Syndrome

- **C stands for calcinosis:** It refers to accumulation of calcium deposits in the skin especially in fingers.
- **R stands for Raynaud's phenomenon:** It is a result of impaired circulation and vascular spasm in the hands.
- **E stands for esophageal dysmotility:** It refers sluggishness of the digestive tract and chronic gastroesophageal reflux.
- **S stands for sclerodactyly:** It is hardening of fingers, a result of the accumulation of scar tissue in the hands.
- **T stands for telangiectasis:** It is discoloration of the skin caused by permanently stretched and damaged capillaries. It is also known as 'spider veins.

SJÖGREN SYNDROME

Sjögren syndrome is an autoimmune disorder, characterized by keratoconjunctivitis sicca and xerostomia in the absence of other connective tissue disease. Sjögren syndrome most often affects women in their late middle age.

Pathogenesis

Antinuclear autoantibodies formed are directed against soluble nuclear nonhistone proteins, especially the antigens SS-A and SS-B in patients with Sjögren syndrome. Autoantibodies to DNA or histones or salivary gland are rare.

Clinical Features

Patient with Sjögren syndrome presents with triad of xerostomia (dry mouth), keratoconjunctivitis sicca (dry eyes) and any one of autoimmune disorders such as rheumatoid arthritis (most common), scleroderma, Hashimoto's thyroiditis or polymyositis. Bilateral salivary glands show enlargement. Salivary gland shows diffuse infiltration by lymphocytes and plasma cells obscuring the parenchyma, which may mimic lymphoma. In some cases, it can lead to malignant lymphoma of salivary gland.

Laboratory Diagnosis

Serum electrophoresis in Sjögren syndrome reveals significant polyclonal hypergammaglobulinemia (a broad-based elevation of serum γ-globulins). Antinuclear autoantibodies against soluble nuclear nonhistone proteins SS-B is highly specific, however against SS-A is less specific.

POLYMYOSITIS

Polymyositis is related to direct muscle cell damage produced by CD8+ cytotoxic T cells, which involves the proximal muscles of the extremities. Initially, healthy muscle fibers are surrounded by CD8+ cytotoxic T cells and macrophages, followed by degeneration of muscle fibers. Increased serum creatine kinase and frequent presence of ANAs (anti-Jo-1, an antibody against histidyl-tRNA synthetase) are characteristic. Histologic examination of muscle biopsy reveals necrotic muscle cells along with lymphocytic infiltrate.

DERMATOMYOSITIS

Dermatomyositis is an immune-mediated disorder involving capillaries that results in obliteration of capillaries supplying skin and skeletal muscle leading to ischemic injury. Involvement of capillaries of skin leads to reddish-purple rash over exposed areas of the face and neck, hence condition is called dermatomyositis in a middle-aged man. There is increased risk of development of squamous cell lung carcinoma. Immunofluorescence microscopy demonstrates C5b–9 proteins (i.e. membrane attack complex) in the walls of capillaries.

MIXED CONNECTIVE TISSUE DISEASE

Mixed connective tissue disease (MCTD) shares many clinical features with other connective tissue disorders, but renal involvement is uncommon, which most often occurs in women (peak 35–40 years). Patient presents with joints pain, myositis, Raynaud phenomenon and esophageal hypomotility. There is increased titer of specific ANAs (anti-nRNP). Immunofluorescence microscopy reveals speckled nuclear appearance on morphologic ANA analysis.

POLYARTERITIS NODOSA

Polyarteritis nodosa is an immune complex-mediated vasculitis and characterized by segmental fibrinoid necrosis of small and medium arteries of many organs, which most often occurs in men. Small and medium arteries undergo ulceration and thrombosis resulting in aneurysms and infarction of organs. Histopathologic changes at different stages of disease process may show acute or chronic or healing stage.

Pathogenesis

Polyarteritis nodosa is mediated via type 3 hypersensitivity reaction, through antigen–antibody complexes, in

which antineutrophilic cytoplasmic autoantibodies are formed resulting in liberation of hydrolytic enzymes and damage to blood vessels. Hepatitis B virus (30–40%) and intravenous amphetamines play important role in its pathogenesis. Drugs, such as sulfonamides and penicillin, may form immunogenic hapten–protein complexes.

Organs Involved

Organs involved in polyarteritis nodosa include renal arteries (85%), coronaries (75%), hepatic arteries (75%), mesenteric arteries (60%), musculocutaneous arteries (40%), arteries of central nervous system, peripheral nervous system, retina, pancreas and skin.

Clinical Features

Patient presents with abdominal pain, hypertension, uremia, polyneuritis, allergic asthma, urticaria or skin rash, splenomegaly, fever, leukocytosis, and proteinuria. Involvement of lungs results in chest pain, cough, dyspnea, and hemoptysis. Severe dyspnea and eosinophilia occur in 20% of patients.

RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a chronic inflammatory autoimmune disorder that primarily attacks synovium of peripheral joints (interphalangeal and metacarpophalangeal joints) and the surrounding muscles, tendons, ligaments, and blood vessels. Knee joints are also involved with formation of subcutaneous nodules. Partial remissions and unpredictable exacerbations mark the course of this potentially crippling disease.

- Subcutaneous nodules are raised, firm, nontender present in the olecranon bursa and along extensor surface of arm. The skin slides freely over subcutaneous nodules. Rheumatoid arthritis is three times more common in women than men, which can occur at any age, but the peak onset is ages 30–60 most often in HLA-DR4 positive individuals.
- Approximately 80% of patients with classic rheumatoid arthritis are positive for rheumatoid factor (**RF**) demonstrated in serum, which represents multiple antibodies, principally IgM, but sometimes IgG or IgA, directed against the Fc fragment of IgG. Rheumatoid factor is useful in diagnosis. Significant titers of RF are also found in patients with related collagen vascular diseases, such as systemic lupus erythematosus, scleroderma, and dermatomyositis. Clinical manifestations of rheumatoid arthritis are shown in Fig. 4.75.

Morphologic Changes in Joints

Progressive changes in rheumatoid arthritis include synovial inflammation, hyperplasia, hypertrophy,

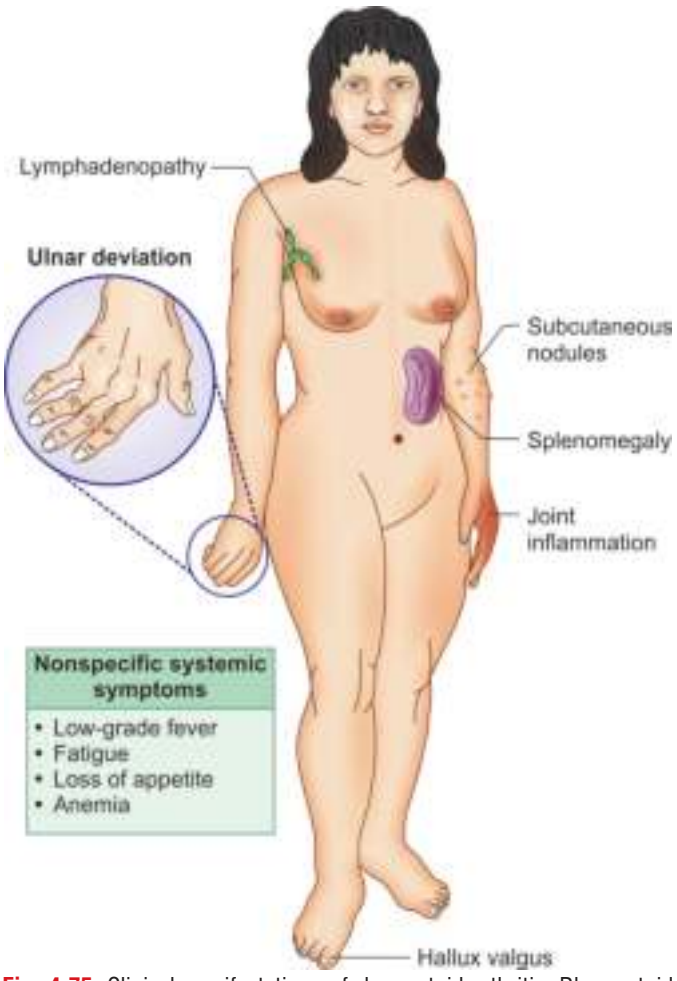


Fig. 4.75: Clinical manifestations of rheumatoid arthritis. Rheumatoid arthritis is a chronic inflammatory autoimmune disorder that primarily attacks synovium of interphalangeal and metacarpophalangeal joints and surrounding muscles, tendons, ligaments, and blood vessels with formation of subcutaneous nodules present in the olecranon bursa and along extensor surface of arms. Patient presents with nonspecific systemic symptoms, lymphadenopathy, ulnar deviation, subcutaneous nodules and painful joints.

formation of numerous villi (fronds), formation of pannus (granulation tissue) invading articular cartilage, subchondral bone results in destruction of joint capsule and bone, which is followed by fibrous ankylosis and calcification.

- **Stage I of rheumatoid arthritis:** Synovitis develops from congestion and edema of the synovial membrane and joint capsule. Initially, synovium shows acute inflammation followed by infiltration by lymphocytes and plasma cells. Synovial cells undergo hyperplasia and hypertrophy resulting in formation of numerous villi and frond-like folds.
- **Stage II of rheumatoid arthritis:** Formation of pannus (thickened layers of granulation tissue), which covers and invades articular cartilage and subchondral bone, which eventually erodes and destroys the joint capsule and bone.

- **Stage III of rheumatoid arthritis:** Fibrous ankylosis (fibrous invasion of the pannus and scar formation that occludes the joint space) occurs. Bone atrophy and misalignment cause visible deformities and restrict movement, leading to muscle atrophy, imbalance and, possibly, partial dislocations.
- **Stage IV of rheumatoid arthritis:** Fibrous tissue calcifies, resulting in bony ankylosis (fixation of a joint) and total immobility. Pain associated with restricted joint movement may restrict active joint use and cause fibrous or bony ankylosis, soft tissue contractures, and joint deformities. Subcutaneous nodules develop in approximately one-third of patients of rheumatoid arthritis.

Clinical Features

Patient of rheumatoid arthritis presents with fatigue, malaise, anorexia, weight loss, fever, and myalgia, bilateral symmetrical swelling and stiffness of joints especially in the morning or after inactivity.

- Chronic inflammatory changes occur in proximal interphalangeal and metacarpophalangeal joints of the hands.
- Patient develops ulnar deviation of fingers resulting from synovitis of ligaments. Minimal radial deviation of the wrist may occur.
- Extra-articular manifestations include effusions (pleura, pericardium), vasculitis, and anemia of chronic disease, neurologic manifestations, lymphadenopathy and secondary reactive amyloidosis.

GOODPASTURE SYNDROME

Goodpasture syndrome is life-threatening autoimmune disorder characterized by nephritic syndrome and pulmonary hemorrhage (hemoptysis). Goodpasture antigen is normally present in glomerular basement membrane and lung alveoli. Autoantibodies cross react with antigens located in glomerular basement membrane and pulmonary alveoli resulting in renal failure and pulmonary hemorrhage. Immunofluorescence study reveals smooth and linear staining pattern due to antibody and complement in Goodpasture syndrome (renal and lung basement membrane).

GRAVES' DISEASE

Graves' disease is type 5 hypersensitivity reaction (anti-receptor mediated) also considered type 2 hypersensitivity reaction. Antibodies to the TSH receptors are observed in patients with Graves' disease, an autoimmune disorder that mimics the action of TSH, but is not regulated by natural negative feedback controls, which leads to follicular glandular hyperplasia and increased synthesis of thyroid hormones resulting in hyperthyroidism. Patient also develops exophthalmos due to infiltration by lymphocytes in retrobulbar ocular muscles in 60–90%.

MYASTHENIA GRAVIS

Myasthenia gravis is an antibody mediated type 2 hypersensitivity reaction disorder. Antibodies bind to the postsynaptic acetylcholine receptors in the motor end plates of skeletal muscles and block neurotransmission resulting to progressive muscle weakness involving particularly the external ocular, eyelids and proximal limb muscles, which may cause death by respiratory muscles paralysis.

PEMPHIGUS VULGARIS

Pemphigus vulgaris is an acantholytic disease caused by autoantibodies against various epidermal cell proteins commonly presenting with flaccid blisters, mucosal erosions in oral cavity and genitalia and scaling. Average age of onset is 40–60 years. IgG autoantibodies to desmoglein-1 (skin) or desmoglein-3 (mucosa) disrupt intercellular junctions in epidermis found in patients with pemphigus vulgaris. Histologic examination of lesion shows intradermal acantholysis with intact basal layer (**tombstone sign**) and intradermal eosinophils. Immunofluorescence study reveals intercellular IgG and C3 as smooth and linear staining pattern in pemphigus (skin intercellular protein, desmosomes).

TISSUE/ORGAN TRANSPLANTATION IMMUNOLOGY

In clinical practice, the term transplantation generally refers to the transfer or replacement of cells, tissues, or organs from one individual to another. Organ transplantation is the treatment of choice for end stage organ failure.

- Transplants are broadly classified as solid organ or hematopoietic stem cell (**HSC**) transplants and within these classifications are categorized according to the relationship of the donor and the recipient.
- Adverse immune responses can be suppressed by immunosuppressant drugs, radiation, or recipient T cell depletion. These processes, however, can result in clinically significant immunodeficiency state.
- Immune system poses a significant barrier to successful organ transplantation when tissue/organ is transferred from donor to the recipient.
- Transplant rejection occurs as result of triggering immune response to destroy the transplanted tissue/

organ. Long-term survival of the transplant can be maintained by manipulating the immune system to reduce the risk of transplant rejection.

- For a successful transplant, recipient and donor must be properly matched for HLA antigens and blood group to minimize the risk of transplant rejection. Tissue typing is done to know how the recipient's blood serum reacts to donor cells. Immunosuppressive drugs are administered to damp immune system and to prevent and treat transplant rejection.

Pathology Pearls: Several Types of Tissues/Organs Transplantation
Autograft
One's own tissue is grafted to another part, e.g. skin autograft in the individual.
Isograft
Graft between identical twins is known as isograft. Donor and recipient are genetically identical.
Allograft (Allogenic Graft)
▪ Transplantation of tissues/organs from a donor to a non-genetically identical individual of the same species. ▪ Allografts are most common type of transplants.
Xenograft
Graft between animals of different species is known as xenograft (e.g. monkey to human).
Hematopoietic Stem Cell Transplant
▪ Hematopoietic stem cells (HSCs) are used as transplants to treat leukemias and other hematological disorders. ▪ HSCs can be harvested either directly from the bone marrow or from the preserved umbilical cord from consenting mothers following childbirth.

IMMUNOLOGY OF TRANSPLANT REJECTION

Transplant rejection response is initiated mainly by host cells that recognize the foreign HLA antigens of the graft, either directly on antigen-presenting cells (APCs) in the transplant or indirectly after uptake and presentation by APCs.

- There are three mechanisms of graft rejection: hyperacute rejection, acute vascular rejection and chronic rejection.
- Preformed and *de novo* donor-specific antibodies (**DSAs**) are central in pathogenesis of hyperacute, acute and chronic transplant rejection.

DISTINGUISHING SELF- AND NONSELF-ANTIGEN

When the immune system encounters a foreign pathogen, it mounts an attack against pathogen to protect the body from infection. To prevent an attack on our own cells and tissues (autoimmunity), the immune system is able to differentiate between our own healthy tissues and foreign invaders.

- Foreign invaders are presented to the immune system in the form of small molecules called **antigens**. Detection of these nonself-antigens will trigger the immune response and stimulate the production of antigen-specific antibodies that mark the infected cells for destruction by immune system and help in amplification of immune response.
- The human leukocyte antigen (**HLA**) system is a complex of genes on chromosome 6 in humans, which encode cell surface proteins responsible for the regulation of the immune system. These cell–surface proteins are found on the surface of all cells and act as 'self-markers' instructing the immune system not to trigger an immune response.
- Each person has own specific set of HLA proteins, based upon their unique genetic makeup that the immune system has learnt not to react. Any cell that does not display these HLA proteins can be identified as nonself by the immune system and will be treated as foreign invader.

PATHOPHYSIOLOGY OF TRANSPLANT REJECTION

Transplant rejection occurs when the recipient's immune system attacks the donor's transplant and begins destroying the transplanted tissue/organ. The transplant rejection can be divided into two phases: (a) initial sensitization phase in which antigen reactive lymphocytes in the recipient's lymph node proliferate in response to the donor's alloantigens and (b) later an effector phase in which the recipient's sensitized effector cells mediate immune destruction of the transplant.

- The immune system is triggered by the presence of donor's own unique set of HLA proteins, which the recipient's immune system will identify as foreign invader.
- The degree of similarity between the HLA genes of the recipient and donor is known as histocompatibility; the more genetically compatible the recipient and the donor, and the more tolerant the recipient's immune system should be to the transplant.
- However, unless the recipient and the donor are genetically similar as in identical twins, there is always be some degree of **transplant rejection**. Non-self HLA proteins, other surface proteins on

Table 4.74 Comparison of acute and chronic transplant rejection

Characteristics	Acute Transplant Rejection	Chronic Transplant Rejection
Stage of rejection	Acute transplant rejection is second stage of rejection	Chronic transplant rejection is third stage of rejection
Onset	Acute transplant rejection occurs within days or suddenly after cessation of immunosuppressive drugs	Chronic transplant rejection occurs over months to years
Components	Acute transplant rejection consists of two components: (a) Acute cellular component comprises CD4+ helper T cells and CD8+ cytotoxic T cells. (b) Antibodies-mediated vasculitis	Chronic transplant rejection does not consist of CD4+ helper T cells and CD8+ cytotoxic T cells. It is characterized by progressive dysfunction of the organs
Mechanism	Acute transplant rejection is mediated by CD4+ helper T cells, CD8+ cytotoxic T cells and antibodies	Chronic transplant rejection shows presence of macrophages, lymphocytes, plasma cells and eosinophils
Morphology	Acute transplant rejection of kidney transplant shows injury to tubules and vascular endothelium resulting in exfoliation of tubular cells in lumen and necrotizing vasculitis. The blood vessels of kidneys are thrombosed resulting in ischemic changes	Arterioles undergo fibrosis of renal vessels leading to ischemic injury to organ

the donor's transplant can also be identified as a foreign antigen and induce immune response. Some patients may develop graft-versus-host disease (GVHD).

CLINICAL STAGES OF TRANSPLANT REJECTION

Transplant rejection can be classified into three types: hyperacute, acute and chronic, which are differentiated by how quickly the recipient's immune system is activated and the specific aspect of immune system involved. Comparison of acute and chronic transplant rejection is given in Table 4.74.

Pathology Pearls: Patterns of Renal Transplant Rejection

Hyperacute Transplant Rejection

- Hyperacute transplant rejection is mediated by preformed antibodies in recipient's plasma as a result of ABO incompatibility between donor and recipient.
- Classic type 2 hypersensitivity reaction results in immune complex formation locally and occluding small blood vessels in transplant.

Acute Vascular Transplant Rejection

- Acute early transplant rejection is antibody-mediated partly by destruction of renal tubules by CD8+ cytotoxic T cells.
- Acute late-onset transplant rejection occurs within 11 days and 6 weeks after transplant, which occurs in patients, who have been immunosuppressed with prednisolone and azathioprine. Florid vascular damage occurs mediated by antibody-complement system.

Chronic Transplant Rejection

Fibromuscular hyperplasia of small and medium-sized arteries with continuing endothelial injury resulting in ischemic necrosis of glomeruli (? immune mechanism).

HYPERACUTE TRANSPLANT REJECTION

Hyperacute transplant rejection occurs within minutes or hours after a transplantation due to preformed antibodies in the recipient that match foreign antigens of the donor.

- The preformed antibodies could have been generated as a result of multiple pregnancies and prior blood transfusions before transplantation. Preformed antibodies react with vascular endothelial cells of blood vessels of the transplant, causing neutrophilic vasculitis, fibrinoid necrosis, extensive thrombosis of small vessels resulting in ischemic damage and rapid transplant rejection.
- Patient presents with sudden cessation of urine output, along with fever and pain in the area of the transplant site. Hyperacute solid organ transplant rejection is shown in Fig. 4.76.

ACUTE VASCULAR TRANSPLANT REJECTION

Acute transplant rejection occurs within six months after transplantation. Some degree of acute vascular transplant rejection occurs in all transplantations, except between identical twins. Recipients are at higher risk within first three months of transplant.

- Acute vascular transplant rejection is caused by formation of antibodies following the detection of nonself-antigens in the donated transplant. If diagnosed early enough, acute transplant rejection can be treated by immunosuppressive therapy to prevent permanent damage to the transplant in some cases.
- Acute transplant glomerulopathy develops in 4–10% of allogeneic transplant. Blood vessels of transplant are destroyed by CD8+ cytotoxic T cells and antibodies resulting in acute vascular transplant rejection. Acute vascular transplant rejection is a complication that

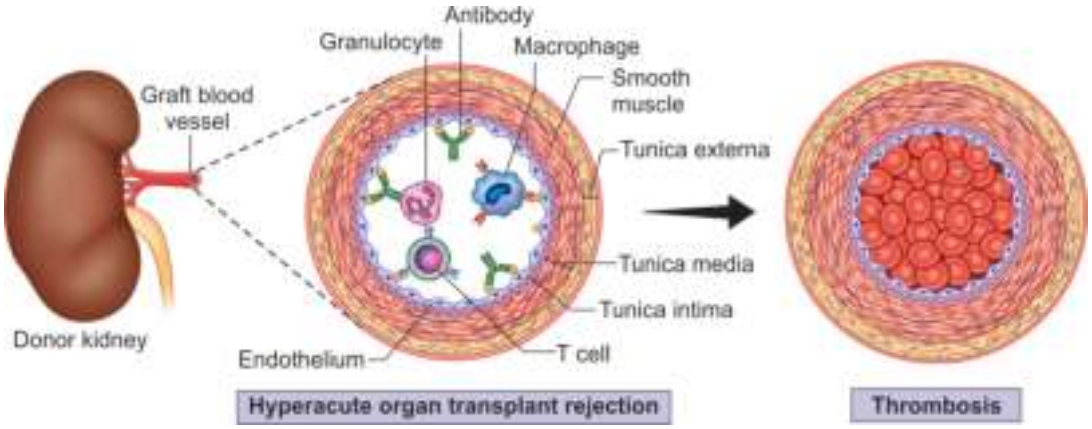


Fig. 4.76: Hyperacute solid organ transplant rejection. Hyperacute rejection occurs before vascularization of the graft, which happens a few minutes or first few days after the organ transplant when antigens are completely unmatched or recipient. The chance of hyperacute rejection is greatest when a recipient possesses preformed anti-ABO blood group antibodies. The transplanted kidney becomes grossly mottled and cyanotic. Capsule bulges out due to marked edema and rupture of the graft may occur. Histologically, renal biopsy evaluation shows endothelial damage with associated sparse polymorphonuclear cells infiltrate followed by widespread capillary thrombosis and necrosis, interstitial hemorrhage, The organ must be removed right way so the recipient does not have fatal outcome. Plasmapheresis may be used to remove circulating antibodies from the blood.

occurs after renal transplantation and is sometimes resistant to anti-rejection therapy resulting in poor prognosis. Recognition of alloantigens in organ graft occurs by direct and indirect pathways described as under: Pathogenesis of renal graft rejection by direct and indirect pathways is shown in Fig. 4.77A and B.

Acute Transplant Rejection by Direct Pathway

Donor's MHC class I and class II are recognized by host (recipient) CD8+ cytotoxic T cells and CD4+ helper T cells. CD4+ helper T cells undergo proliferation and synthesize cytokine IFN-γ, which activates macrophages leading to damage to renal tubules. T cells responding to transplant antigens differentiate to CD8+ cytotoxic T cells that kill renal transplant blood vessels and tubules. Acute cellular transplant rejection occurs within days to weeks to months after transplantation. Pathogenesis of acute transplant rejection by direct pathway is shown in Fig. 4.78.

Clinical Features

Patient presents with abrupt onset of azotemia and oliguria, which may be associated with fever and graft tenderness.

Histologic Examination

Histologic examination of rejected transplant shows edema, interstitial infiltrates of lymphocytes (CD4+ helper T cells and CD8+ cytotoxic T cells) and macrophages. Tubules show lymphocytic infiltration and tubular necrosis.

Acute Transplant Rejection by Indirect Pathway

When antibody-mediated mechanisms are prominent, it may show evidence of arteritis with thrombosis and cortical necrosis. Antibody-mediated rejection of solid transplant is a significant cause of morbidity and mortality. It is difficult to treat these cases. Acute transplant rejection by indirect pathway affects recipients sensitized with pre-transplant donor-specific antibodies (DSAs) to human leukocyte antigen (HLAs) or post-transplant *de novo* HLA or non-HLA antibodies.

Risk Factors

Risk factors for acute transplant rejection include younger age, female gender, prior sensitization to orthoclone (OKT3) monoclonal antithymocyte antibody used for induction therapy in renal transplantation, cytomegalovirus seropositivity, pregnancy, previous blood transfusions, surgical procedures, pre-transplant cardiac support with ventricular assist device and pretransplant or post-transplant hemodialysis.

Pathogenesis

Recipient antigen-presenting cells (APCs) recognize and pick-up graft antigens and activate CD4+ helper T cells, which synthesize IFN-γ, resulting in damage to renal blood vessels. CD4+ helper T cells also stimulate B cells to synthesize antibodies. Antibody binding to antigen activates complement system via classical pathway which cause vascular endothelial injury of renal vessels. Immunological detection of C4d deposition (specific marker for the presence of antidonor recipients) in allografts has evolved as a sensitive and specific diagnostic tool for antibody-mediated transplant rejection.

Diagnosis

Gold standard tissue staining technique for C4d (specific marker for the presence of antidonor recipients) is immunofluorescence (IF) using a monoclonal antibody.

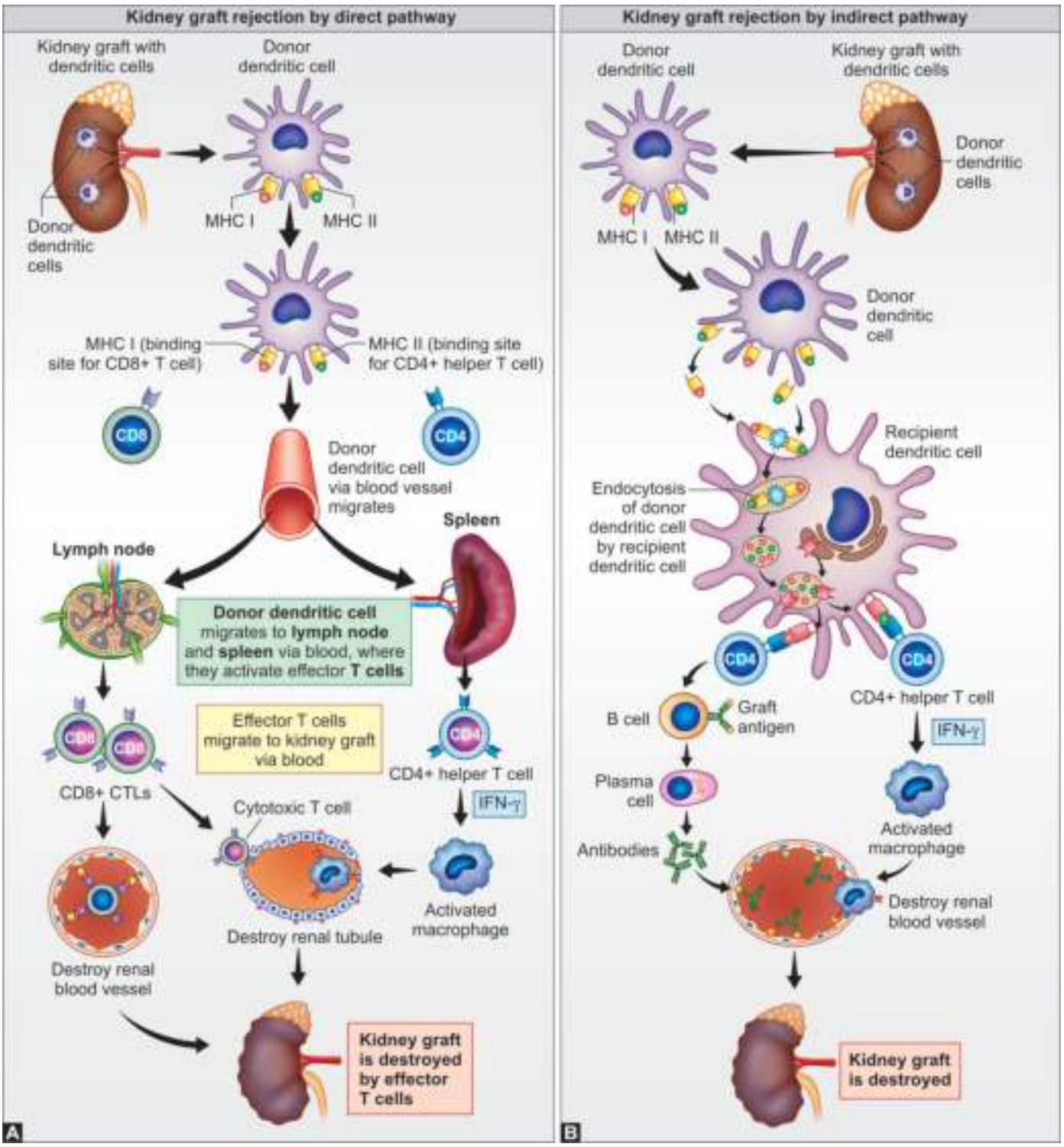


Fig. 4.77: Pathogenesis of renal graft rejection by direct and indirect pathways. Graft rejection occurs when the recipient's immune system attacks the donated renal graft leading to graft destruction. Immune response is triggered by the presence of the donor's own unique set of HLA class molecules, which are recipient's immune system will identify as foreign. The main difference between direct and indirect alloantigen recognition stems from the origin of the antigen-presenting cell (APC). (A) In direct alloantigen recognition, involved antigen-presenting cells are derived from the donor renal graft, (B) in indirect alloantigen recognition, the antigen-presenting cells of recipient are involved.

CHRONIC TRANSPLANT REJECTION

Chronic transplant rejection is primarily caused by antibody-mediated vascular damage within months to years after successful transplantation. It commonly manifests as scarring of the transplant, which can occur months to years after acute transplant rejection has subsided.

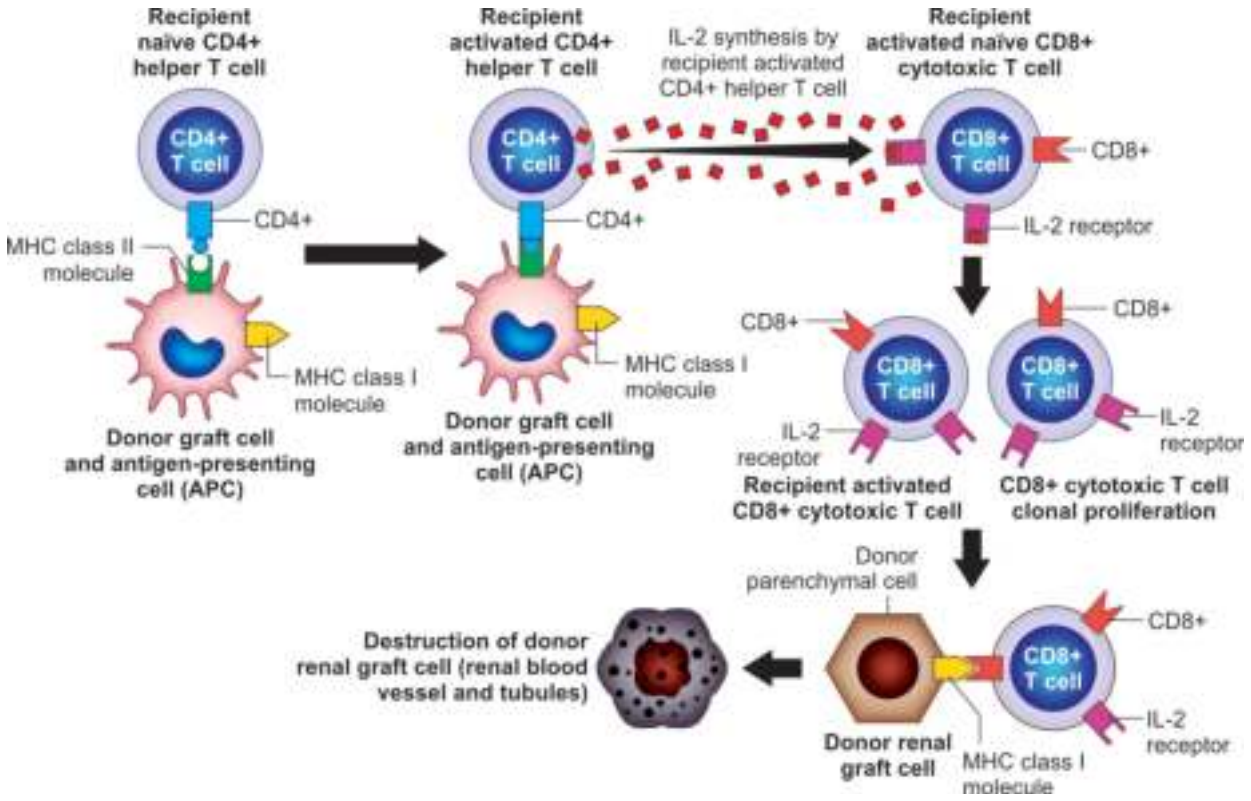


Fig. 4.78: Direct pathway of acute graft rejection of kidney graft. Donor organ cell and antigen-presenting cell (APC) interacts with CD4+ helper T cell of recipient leading to activation of CD4+ helper T cell and synthesis of IL-2 cytokine that activates CD8+ cytotoxic T cell of recipient. It is followed by interaction of CD8+ cytotoxic T cell of recipient with transplanted donor kidney organ resulting in destruction of renal blood vessels and tubules in the recipient.

- Chronic transplant rejection is characterized by arteriosclerosis, glomerulosclerosis, and tubular atrophy-cell reaction and synthesis of cytokines induce proliferation of vascular smooth muscle cells especially kidney transplantation.
- At present, there is no cure for chronic transplant rejection other than removal of the transplant.

Pathogenesis of chronic solid organ transplant rejection is shown in Fig. 4.79.

GRAFT-VERSUS-HOST DISEASE

Graft-versus-host disease (GVHD) is a significant problem, when hematopoietic stem cell (HSC)

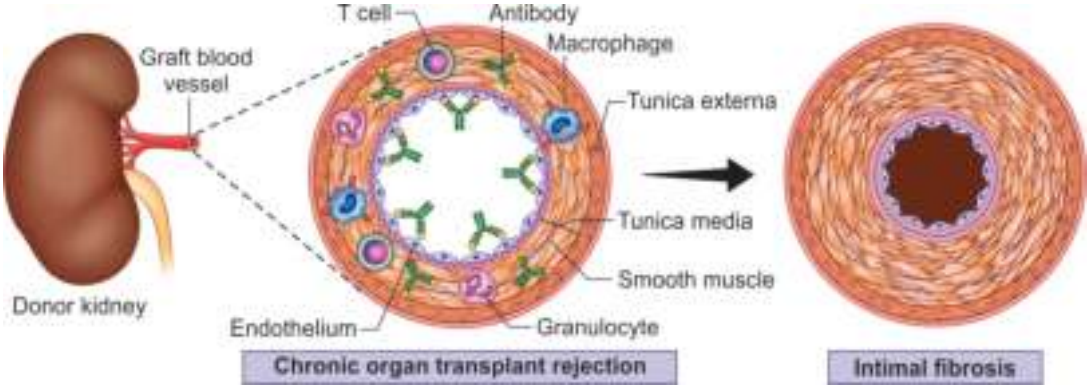


Fig. 4.79: Pathogenesis of chronic solid organ transplant rejection. Chronic graft rejection of solid organs is defined as the loss of allograft function several months after transplantation. The transplanted organ may still be in its place, but persistent immune system attacks on the allo-MHC class molecules expressed by its component cells have gradually caused the organ to lose functioning. The main histological finding in biopsies of rejected solid organs is arteriosclerosis that induces a progressive narrowing of blood vessels of solid graft. This is typically referred to as a vasculopathy or organ graft vascular disease. This entity is usually accompanied by parenchymal fibrosis of graft.

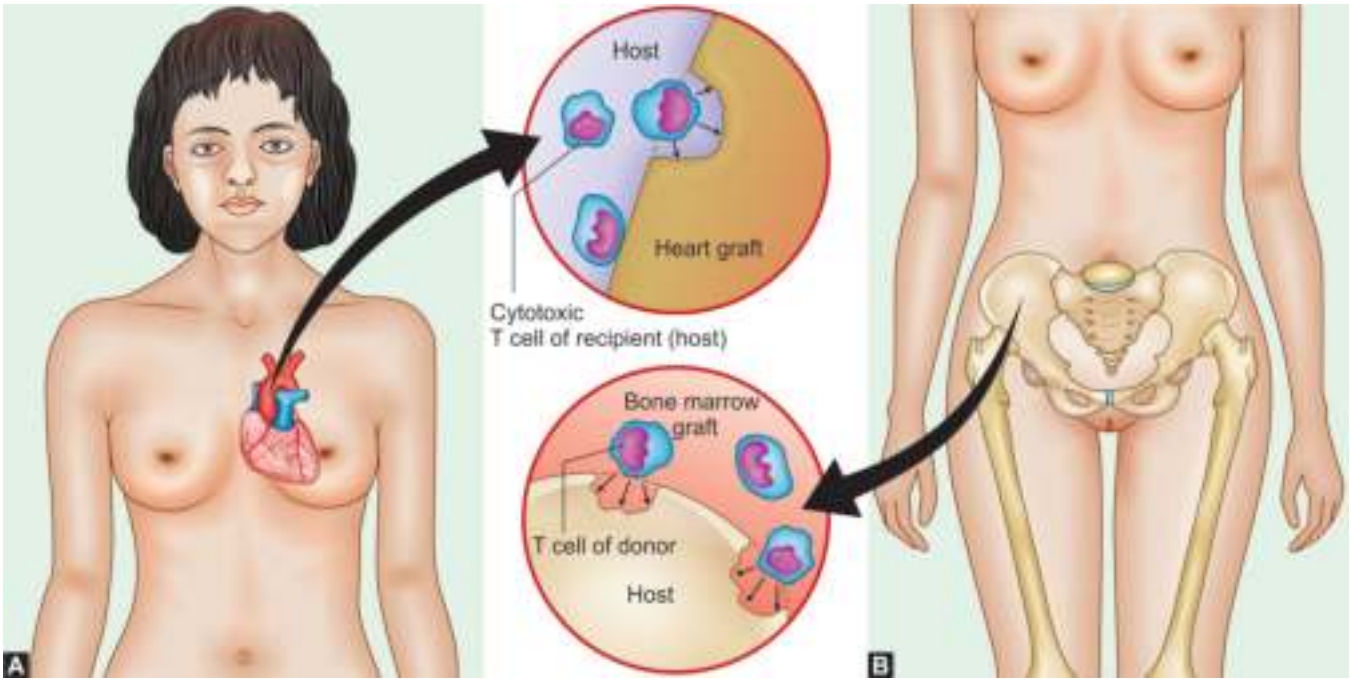


Fig. 4.80: Potential reactions in organ transplantation. (A) CD8+ cytotoxic T cells of host immune system encounter the cells of donated organ (heart) and mediate the organ rejection, (B) grafted bone marrow contains endogenous T cells that recognize host's tissues as foreign and mount an attack on many tissues and organs. The recipient will develop symptoms of graft-versus-host disease.

transplantation is performed in patients of aplastic anemia, acute leukemia and immunological deficiency. CD8+ cytotoxic T cells from transplant directly damage recipient host cells. GVHD can also occur when a patient with severe combined immunodeficiency disease (SCID) is transfused blood containing HLA-incompatible lymphocytes.

- **Billingham's criteria for transplant rejection:** Billingham's described criteria for graft-versus-host disease. A donor graft has immunocompetent cells. Recipient possesses antigens foreign to graft. A recipient who is unable to mount immune response.
- **Classification of GVHD:** There are two types of graft-versus-host disease: acute GVHD occurs in <100 days and chronic GVHD occurs in >100 days. Potential reactions in organ transplantation are shown in Fig. 4.80A and B.

PATHOGENESIS

CD8+ cytotoxic T cells from graft directly damage host epithelial cells of skin, gastrointestinal mucosa and liver. Cytokines from graft CD4+ helper T cells recruit macrophages, which damage host epithelial cells of skin, gastrointestinal mucosa (duodenum, ileum and colon) and liver. Clinically, GVHD manifests as pruritic rash, diarrhea, abdominal cramps, anemia, hepatosplenomegaly and liver dysfunction. Elevation of serum bilirubin and liver enzymes signals the hepatic involvement.

Acute Graft-versus-host Disease (GVHD)

Acute GVHD develops by priming of immune response, activation and expansion of donor; and effector phase.

- **Priming of immune cells:** Immunosuppression damage occurs in several tissues including liver and gut. Gut damage is very important in the pathogenesis. Mucosa of gut becomes permeable resulting in synthesis of IL-1 and TNF-α. There is upregulation of adhesion molecules, MHC antigens and increased recognition of host antigens by T cells. Entry of bacterial product, i.e. lipopolysaccharide (LPS) from systemic circulation aggrevates acute graft-versus-host disease.
- **Donor's cells activation and expansion:** T cells recognize the antigens presented by APCs in the presence of costimulatory molecules (CTLA-4: B7, CD28). Both CD4+ helper T cells and CD8+ cytotoxic T cells can initiate acute GVHD reaction. This depends on allogenic component of host.
- **Effector phase:** Along with T cells, natural killer cells (NK cells) secrete cytokines that continue to stimulate other cells and cause damage tissue. The Fas/FasL pathway and perforin/granzyme pathway play important role in this effector phase to induce acute GVHD.

Chronic Graft-versus-host Disease (GVHD)

Pathogenesis of chronic GVHD remains obscure. Role of development of autoantibodies is still not clear. Synthesis of cytokines by Th2 cells play role in the

pathogenesis of chronic GVHD. Immune dysregulation causing immunodeficiency and autoantibodies to several cell surface and intracellular proteins lead to autoimmunity. Various theories have been proposed in its pathogenesis.

- **Breakage of immune tolerance to self-antigens:** Damage to thymus epithelium dysregulates central immunologic tolerance by generation of CD4+ helper T cells from donor stem cells.
- **CD4+ and CD25+ regulatory T cells reduction:** These cells are reported to be reduced in chronic GVHD via synthesis of cytokines such as TGF-β and IL-10.
- **Role of B cells and their antibodies:** Increased levels of BAFF drive B cell autoimmunity. There is development of antibodies to minor histocompatibility proteins encoded by Y chromosome in a male recipient receiving transplant from a female. There may be role of amplification of autoimmune responses and in epitope spreading of autoreactive T cell and B cell.
- **Fibrotic change:** Synthesis of TGF-β and IL-13 produce fibrotic change. Recruitment of macrophages and other cells to the site causes tissue damage. There is C3 deposition at the dermoepidermal junction in skin.

Pathology Pearls: Organs Involved in Acute GVHD

- Acute GVHD occurs within 100 days of transplant.
- Most commonly seen is a triad involvement of liver (hepatitis), skin (dermatitis) and duodenum (enteritis).
- Other systems may be involved causing pneumonitis, hemorrhagic cystitis, thrombocytopenia and anemia.

Skin Manifestations

- Patient presents with maculopapular rash, erythema, bullae, and desquamation.
- Skin epidermal cells are infiltrated by lymphocytes.
- Duodenal mucosa shows lymphocytic infiltration.

Liver Manifestations

- Patient presents with symptoms related to hepatitis.
- Liver shows lymphoid cells in portal triad invading and attacking hepatocytes, bile ducts epithelial cells and vascular endothelial cells.

Duodenum Manifestations

- Patient presents with diarrhea (green, water, mucoid stool; may contain cells and fecal casts), intestinal bleeding and crampy abdominal pain.
- Barium studies show increased transit time and loss of haustral folds.
- Light microscopy reveals single cell necrosis/apoptotic bodies at the base of the crypts or glands.

Pathology Pearls: Organs Involved in Chronic GVHD

- Chronic GVHD may follow acute GVHD or *de novo.* It is one of the problems affecting the long-term survivors of allogenic graft. GVHD affects liver and colon.
- Liver biopsy shows lymphocytic infiltration in portal triad, fibrosis and loss of bile ducts. Histology of colonic biopsy shows lymphocytic infiltration, glandular degeneration and mucosal atrophy with loss of glands.
- Other organs may be involved. Patient may develop cholestasis, oesophageal reflux and strictures, dryness and stenosis of vagina, polymyositis, fasciitis, myasthenia gravis, nephrotic syndrome and positive autoimmune serology. Organs involved and clinical manifestations in chronic GVHD are given in Table 4.75.

Diagnostic Modalities

Demonstration of donor derived lymphocytes in the areas of tissue destruction in biopsy specimens, viz. dermoepidermal junction, crypts of gut epithelium, bone marrow, etc.

- DNA polymorphism studies are done using VNTR analysis fingerprinting.

Table 4.75 Organs involved and clinical manifestations in chronic GVHD

Organs	Clinical Manifestations
Gastrointestinal tract	Dysphagia, pain, vomiting, diarrhea, abdominal pain
Skin	Skin rash (lichenoid, sclerodermatous, hyper-/hypopigmented, flaky) and alopecia
Oral and ocular organs	Sjögren's syndrome related to lacrimal gland destruction and reduced tear flow, dry and painful eyes, conjunctivitis, corneal ulcers
Liver	Increased transaminases, hyperbilirubinemia, cirrhosis
Lungs	Bronchiolitis obliterans, organizing pneumonia
Hematologic/immune system	Cytopenias, dysfunction
Serous membrane	Pericarditis and pleuritis

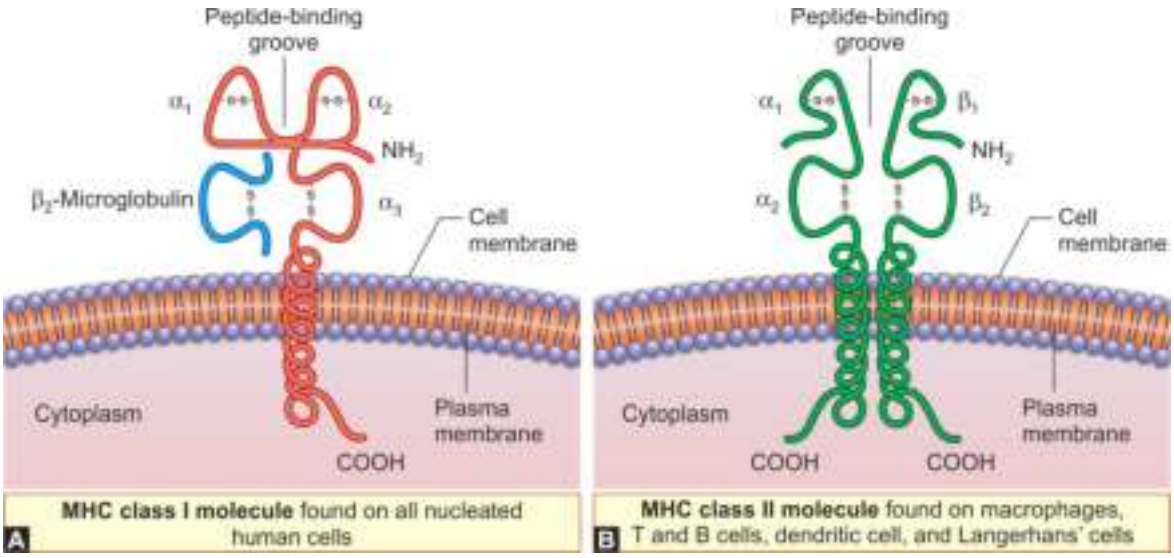


Fig. 4.81: Structure of major histocompatibility complex (MHC or HLA) class I and class II molecules. MHC class I and class II molecules play a pivotal role in the adaptive immune response. Both classes of MHC molecules share the task of presenting peptides on the cell surface for recognition by naïve CD4+ helper T cells and CD8+ cytotoxic T cells.

- **Polymerase chain reaction (PCR)** with donor HLA-DR sequence is specific primers.
- **Fluorescence *in situ* hybridization (FISH)** using Y-chromosome specific DNA probe combined with IHC using monoclonal antibodies define cellular phenotypes.
- **Demonstration of one-way HLA** match between the donor and the recipient is performed.

FINDING ELIGIBLE DONOR–RECIPIENT MATCH

Transplant rejection can be minimized by matching the donor and recipient for compatibility prior to transplantation. Transplantation is successful in properly matched donor and recipient. Compatibility between donor and recipient is assessed using a panel of tests.

Pathology Pearls: Renal Transplantation

- The likelihood of transplant rejection is increased with increasing MHC disparity.
- In addition to **MHC matching**, two additional compatibility tests are performed for renal transplant recipients. Kidneys are also matched for **ABO blood** group antigens.
- For renal transplants, overall rate of **transplant failure** in the first year is 10%, and overall rate of transplant failure at 5 years is 35%.
- The last level of matching is performed by cross-matching assay, which determines whether there is pre-existing antibody in the recipient to donor histocompatibility antigens.
- The determination of donor MHC antigens and antibody to ABO blood group antigens is important for preventing renal transplant rejection.

ABO BLOOD GROUP COMPATIBILITY

The donor and recipient are tested for compatible blood groups prior to organ transplantation. This is the first test to be performed as the organ transplant will be rapidly rejected if the blood groups do not match. ABO compatibility is not necessary in young children undergoing transplantation and also hematopoietic stem cell (HSC) transplantation. When kidneys are transplanted into ABO-incompatible recipients, only 50% of these cases develop impaired renal function one year post-transplant period.

TISSUE TYPING

Tissue typing is carried out to match major histocompatibility complex (MHC or HLA) proteins of donor and recipient on the surface of cells. When HLA types are matched properly, the survival of transplanted organs increases dramatically. Family members, in particular siblings, are often the best major histocompatibility complex (MHC or HLA) matches due to genetic similarity. Structure of major histocompatibility complex (MHC or HLA) class I and class II molecules is shown in Fig. 4.81A and B.

Pathology Pearls: Major Histocompatibility Complex

- Success of organ transplants and skin grafts depends on a proper matching of histocompatibility antigens that occur on all cells of the body.
- Chromosome 6 of mouse contains a cluster of genes known as the major histocompatibility complex, which in human is called human leukocyte antigen (HLA) complex. The alleles of HLA genes are codominant.

- The products of these genes determine histocompatibility, i.e. compatibility between donor and recipient tissues in transplants. The array of HLA alleles on a homologue of our chromosome 6 is known as a haplotype.
- An individual inherits one HLA haplotype from each parent. The large number of alleles at this locus ensures that only identical twins have the identical haplotype.
- The best HLA matches would occur within a family.
- Therefore, the preference order for transplants is as follows: identical twin > sibling parent > unrelated donor.

CROSS-MATCHING

Blood samples are taken from both the donor and the recipient for cross-matching, in which the cells of the donor are mixed with the blood serum of the recipient. If the recipient's antibodies attack donor cells, they are considered a **positive crossmatch** and the transplantation will not be suitable due to increased risk of hyperacute transplant rejection.

PANEL OF REACTIVE ANTIBODY TEST

The blood serum of patients awaiting transplantation are analyzed for **reactive antibodies** against a random panel of cells.

- Previous exposure to foreign tissue by multiple pregnancies or blood transfusion or prior transplantations, are likely to increase the number of HLA antibodies in the bloodstream.
- The more the HLA antibodies present, the higher the panel reactive antibody level denoted to the patient, and greater chance of transplant rejection.
- If the panel reactive antibody levels are high, it may be more difficult to find a match and a higher dosage of immunosuppressive drugs may be required by the recipient.

SEROLOGICAL SCREENING

Pre-transplantation serological screening of donor and recipient is essential for patients undergoing hematopoietic stem cell (HSC) transplantation. Serological testing is performed to know the immune status of both donor and recipient against a number of clinically significant infectious organism including viruses like human immunodeficiency virus (HIV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV), thus determining potential for reinfection or reactivation upon immunosuppressive therapy. Donor and recipient are often matched according to the CMV and Epstein-Barr virus status.

PREVENTION OF TRANSPLANT REJECTION

The chances of transplant rejection may be reduced by good matching between donor and recipient, and suppression of immune response of the recipient.

IMMUNOSUPPRESSIVE DRUGS

Solid organ transplantation immunosuppressive medications have revolutionized the field of transplantation. Generally, immunosuppressive drugs damp the immune system either globally by suppression of T cell reactivity or causing death of reactive T cells to prevent transplant rejection. Immunosuppressive drugs are required for any form of transplantation across MHC barriers. The likelihood of transplant rejection is increased with increasing MHC disparity.

- Various immunosuppressive drugs are given to the recipient including azathioprine, cyclosporine A, tacrolimus (FK506), rapamycin and biological immunosuppression monoclonal antibodies.
- Immunosuppressive drugs are administered in two phases: (a) induction phase involves high dose, and (b) maintenance phase involves using a drug in the long-term at a lower dose.

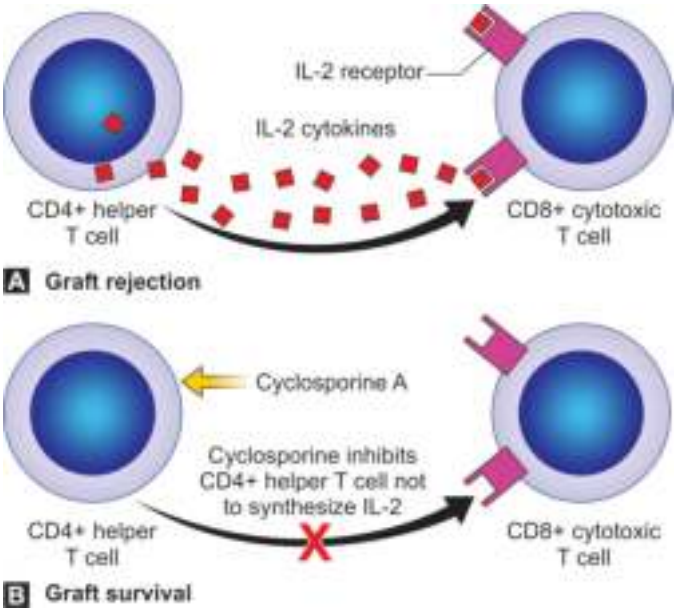


Fig. 4.82. The action of cyclosporine A in preventing transplant rejection. Cyclosporine A drug administration to prevent organ graft rejection. (A) CD4+ helper T cell synthesizes IL-2 that binds to IL-2 receptor on CD8+ cytotoxic T cell leading to graft rejection. (B) Cyclosporine A is administered to prevent graft rejection. Cyclosporine A inhibits CD4+ helper T cells not to synthesis IL-2 that fails to stimulate CD8+ cytotoxic T cell resulting in graft survival.

- The combination of immunosuppressive drugs, and dosage administered, will vary depending on the type of transplant and the chosen treatment regime. If a patient experiences an episode of acute transplant rejection by using immunosuppressive drugs, the dosage is also likely to be increased. Side effects of drugs can also cause alternative drugs to be used.
- The action of cyclosporine A in preventing transplant rejection is shown in Fig. 4.82A and B. Immunosuppressive drugs used in transplant recipient are given in Table 4.76.

ADVERSE EFFECTS OF IMMUNOSUPPRESSIVE DRUGS

Adverse effects of immunosuppressive treatment comprise **nephrotoxicity**, **infection**, **hyperlipidemia**, **hypertension**, and neoplasia risk. Adverse effects of immunosuppressive drugs in recipient of transplant are given in Table 4.77.

Table 4.76 Immunosuppressive drugs used in transplant recipient

Drug	Product	Mechanism
Azathioprine	Azathioprine metabolized to 6-mercaptopurine	▪ Inhibits T cell-mediated transplant rejection ▪ Interferes with enzyme involved in nucleic acid synthesis ▪ Inhibiting proliferation of T cells
Cyclosporine A	Fungal product first-line of drug used in prevention of allograft rejection	▪ Has no effect on the replicating cells of bone marrow ▪ Penetrates antigen-sensitive cells in the G0 and G1 phase ▪ Inhibits RNA polymerase leading to blockage of synthesis of IL-2 by T helper cells
Tacrolimus (FK506)	Fungal product	Inhibits synthesis of lymphokines by recipient T helper cells
Rapamycin	Fungal macrolide	Interferes with the intracellular signaling pathways of the IL-2 receptor
Biological immunosuppression	▪ Monoclonal antibodies ▪ Toxin subunit of riacin conjugated with monoclonal antibodies ▪ Lymphokine (IL-2) complexed with diphtheria toxin	▪ Monoclonal antibodies directed against CD4+ helper T cells (anti-CD4), CD25 molecule and IL-2 receptors expressed on CD4+ regulatory T cells ▪ Targeted against CD25 molecule expressed on activated CD4+ regulatory T cells ▪ Locks IL-2 receptors on activated CD4+ regulatory T cells and kills the cells

Table 4.77 Adverse effects of immunosuppressive drugs in recipient of transplant

Adverse Effects	Findings
Acute nephrotoxicity (cyclosporine A induced)	▪ Vacuoles formation in proximal tubules and hyalinization of arterioles ▪ Interstitial fibrosis and tubular atrophy
Infections risk	Cytomegalovirus infection common
Hypertension (cyclosporine A induced)	Hypertension following renal transplant (50% cases) associated with vasoconstriction
Hyperlipidemia	Present in 60% post-transplant cases increasing risk of cardiovascular deaths in 30% cases
Post-transplant neoplasia	Squamous cell carcinoma, B cell lymphoma especially associated with Epstein-Barr virus, Kaposi's sarcoma and cervical carcinoma

AMYLOIDOSIS

Amyloidosis is fundamentally a disorder of abnormal folding of normal soluble proteins, formation of insoluble fibrils having an antiparallel, β-pleated sheet tertiary structure, and deposition in the extracellular tissue in one or many body organs, systems and soft tissues. Amyloidosis may be localized or systemic. It is not a single disease entity but rather a diverse group of inherited or acquired disorders.

- Amyloid material is resistant to digestion, hence accumulated within tissue, interferes with function and destroys vital organs. Amyloid is deposited between the endocardium and the basement

membrane of blood vessel walls that involves microvasculature of any organ including great blood vessels of the brain. Initial clinical manifestations of amyloidosis include weakness, fatigue, and weight loss. Later life-threatening manifestations depend on organ involved.

- Amyloid deposits in spleen can cause splenic rupture and severe hemorrhage.
  - Amyloid deposition in the liver can cause decreased synthesis of coagulation factors in patients with advanced liver disease.
  - Patient with renal involvement develops nephrotic syndrome progressing to renal failure.
  - Amyloid deposition in subendocardial region produces restrictive cardiomyopathy resulting in cardiac arrhythmias associated with fatal outcome.
- Sometimes, amyloid deposits are limited to a single organ. Amyloid deposits are nodular and encountered in the lungs, larynx, skin, urinary bladder, tongue, and the region about the eyes may be involved. In some of these cases, infiltrates of plasma cells may be found, and the amyloid is of AL type.
- Several factors participate in the misfolding of proteins, which include: (a) increased concentration soluble proteins in chronic inflammation, (b) impaired excretion of soluble proteins in long-term hemodialysis, (c) genetic predisposition due to gene mutations, and (d) limited proteolysis may generate a protein that forms amyloid fibrils (amyloid associated Alzheimer's disease).
- Amyloid is composed of fibrillar proteins, and nonfibrillary glycoproteins [amyloid P component also called serum amyloid P (SAP), glycosaminoglycans and apolipoprotein E]. Serum amyloid P component may contribute to stability of amyloid deposits.
- Amyloid material is demonstrated by Congo red staining and apple green birefringence under polarized light microscope. Radioactive iodine labeled SAP is used to assess amyloid deposition in nuclear medicine studies. Irrespective of molecular composition, amyloid always has the same characteristic histologic and ultrastructural appearance.

Pathology Pearls: Physical Nature of Amyloid

Amyloid deposits typically contain three components: amyloid protein fibrils, amyloid P component and sulfated glycosaminoglycans (GAGs).

Amyloid Protein Nonbranching Fibrils

- Amyloid protein fibrils constitute 90% of the amyloid structure. Regardless of their derivation, all amyloid deposits are composed of nonbranching fibrils, measuring 7.5–10 nm in diameter.
- Each of these nonbranching fibrils of amyloid is made up of two filaments of polypeptide chains, which are folded in ribbon-like twisted "**β-pleated sheet fibrils**" extending over the length of the fibril.
- Amyloid protein nonbranching fibrils bind to proteoglycans, glycosaminoglycans, heparan sulphate, dermatan sulphate and plasma proteins.
- **Congo red** binds to these nonbranching fibrils produces a red-green dichromatism (birefringence) under polarized light, which is commonly used to demonstrate amyloid in tissues.

Amyloid P Component

- In addition, amyloid protein fibrils deposits are intimately associated with the amyloid P (pentagonal globular rod-like structure) component (AP), a glycoprotein related to normal serum amyloid P (SAP).
- P component is very similar to serum C reactive protein, which constitutes 10% of amyloid material.

Sulfated Glycosaminoglycans

- Sulfated glycosaminoglycans (GAGs) are small but constant component.
- Amyloid fibrils and amyloid AP are closely associated with GAGs, complex carbohydrates of connective tissue.
- The presence of abundant charged sugar groups in these adsorbed proteins give the deposits staining characteristics resembling starch (amylose).

CHEMICAL NATURE OF AMYLOID

Out of more than 20 biochemically distinct forms of amyloid proteins identified, there are two, chemically distinct types of amyloid protein fibrils designated amyloid light chain (AL), and amyloid associated (AA). There are several minor types unrelated to AL or AA such as amyloid-β, transthyretin, β_2-microglobulin, procalcitonin. Classification of amyloidosis is given in Table 4.78. Differences between primary and secondary amyloidosis are given in Table 4.79.

AMYLOID LIGHT CHAIN AMYLOIDOSIS

Amyloid light chain (AL) amyloidosis results from aggregation of misfolded proteins of either kappa (κ) or lambda (λ) light chains synthesized by monoclonal plasma cells in multiple myeloma that are deposited in tissues as amyloid fibrils. The light chains (either κ or λ) undergo aggregation, probably because they contain amino acid residue that destabilize the domain structure. Pathogenesis of amyloidosis (AL type) is shown in Fig. 4.83. Bronchopulmonary amyloidosis is shown in Table 4.80.

- In the immunocyte-associated form, perivascular and vascular localizations are common. Amyloid

Table 4.78 Classification of amyloidosis

Associated Disorders	Major Amyloid Protein	Precursor Protein	Organs Involved
Primary amyloidosis			
Multiple myeloma, immunoblastic lymphoma	AL (immunoglobulin light chains or fragments)	Ig light chain, κ- or λ-chain	Systemic amyloidosis (kidney, heart, tongue, bone marrow and peripheral nerves)
Heavy chain disease	AH	Ig heavy chain	Systemic amyloidosis (kidney, heart, tongue, bone marrow and peripheral nerves)
Reactive (secondary) amyloidosis			
Chronic inflammation, autoimmune diseases and some malignancies	AA (serum amyloid protein, an acute phase reactant)	Serum AA (Apo)	Systemic amyloidosis (kidney, liver and spleen)
Hemodialysis associated amyloidosis			
Chronic renal failure	$A\beta_2M$	β_2-Microglobulin	Systemic amyloidosis (synovium, carpel tunnel syndrome and tongue)
Hereditary and familial associated amyloidosis			
Familial amyloid nephropathy (FAN) with urticaria and deafness, familial amyloidotic cardiomyopathy	ATTR	Transthyretin	Systemic amyloidosis (peripheral nerves, autonomic nerves, heart, and kidney)
Familial amyloidotic polyneuropathy	AApoAI	Apolipoprotein AI	Systemic amyloidosis (heart, skin, kidney, nerves, liver, larynx, and blood vessels)
Familial amyloidotic neuropathy	AApoAII	Apolipoprotein AII	Localized amyloidosis (kidney)
Hereditary cerebral hemorrhage with amyloid	ACys	Cystatin C	Localized amyloidosis (cranial blood vessels)
Familial amyloidosis with cranial neuropathy and corneal lattice dystrophy	AGel	Gelsolin	Systemic amyloidosis (cranial nerves, peripheral nerves, cornea, and kidney)
Endocrine system associated amyloidosis			
Medullary thyroid carcinoma	ACal (calcitonin amyloid)	Procalcitonin	Localized amyloidosis (thyroid gland)
Senile changes associated amyloidosis			
Alzheimer's disease	Aβ	Aβ protein precursor (AβPP)	Localized amyloidosis (nervous system)
Alzheimer disease	Aτ	τ (tau)	Localized amyloidosis (brain)
Amyloid cardiomyopathy	TTR	Transthyretin	Localized amyloidosis (myo-cardium)
Atrial amyloid of aging	AANF	Atrial natriuretic factor	Localized amyloidosis (cardiac atria)
Prion disorder associated amyloidosis			
Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease, fatal familial insomnia with amyloid	AprP	Prior protein	Localized amyloidosis (central nervous system)

In addition to major amyloid substance listed, all amyloid deposits also comprise amyloid P glycoprotein as a common constituent.

Table 4.79 Differences between primary and secondary amyloidosis

Parameters	Primary Amyloidosis	Secondary Amyloidosis
Etiology	Multiple myeloma, immunoblastic lymphoma	▪ Chronic inflammation (tuberculosis, chronic osteomyelitis, bronchiectasis) ▪ Autoimmune disorders (rheumatoid arthritis, ulcerative colitis, regional enteritis, dermatomyositis, scleroderma, ankylosing spondylitis) ▪ Cancers (Hodgkin's disease and renal cell carcinoma)
Chemical nature	AL (amyloid light chain)	AA (amyloid associated) due to misfolding of AA fibrils as a result of defective proteolysis
Organs involved	▪ Mesoderm derived structures such as peripheral nerves, heart, lung, skin, tongue, thyroid gland, joints and gastrointestinal tract. ▪ Parenchymal organs also involved (liver, spleen, kidney, heart)	Parenchymal organs (spleen, liver, kidney, adrenals, and lymph nodes)
Frequency	More common	Less common

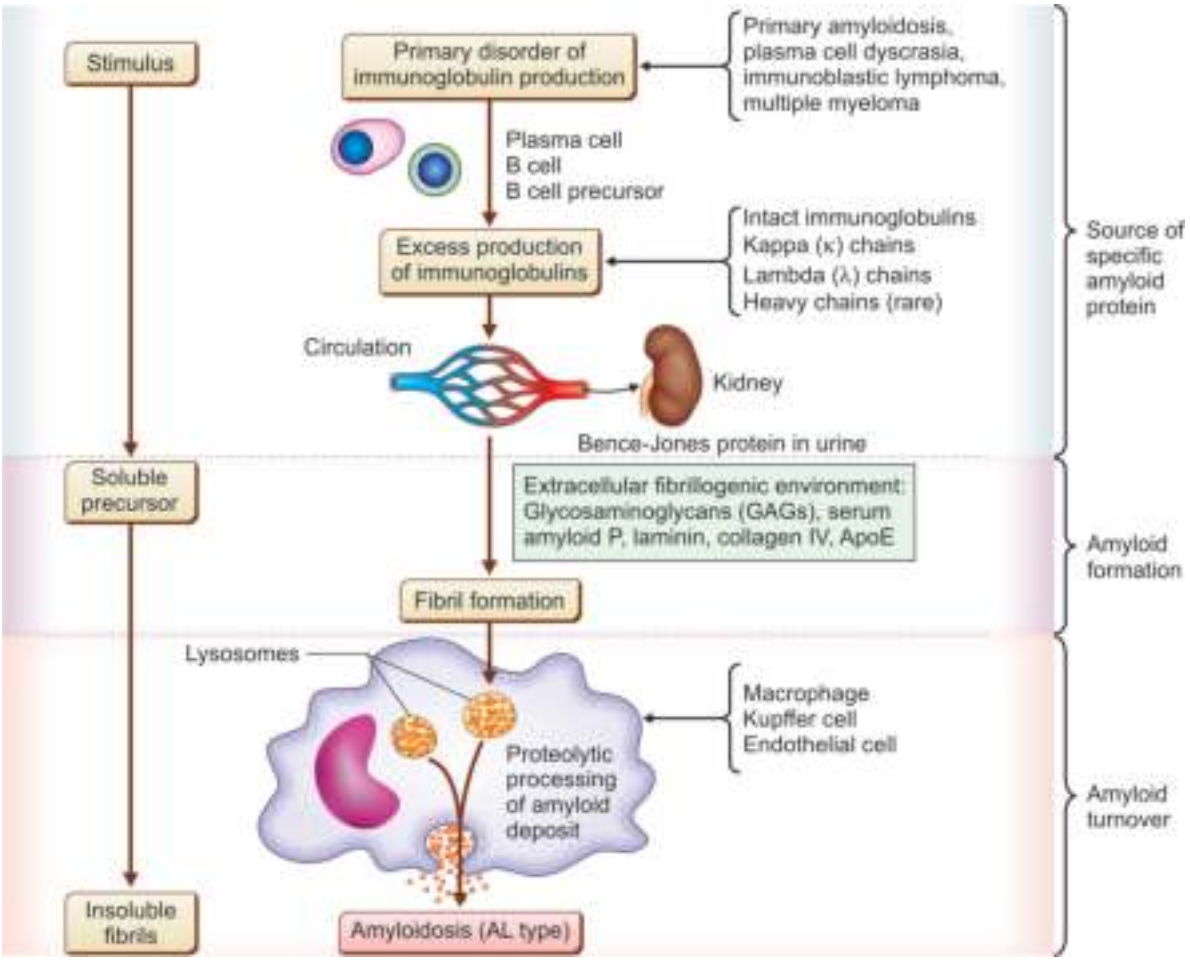


Fig. 4.83: Pathogenesis of amyloidosis (AL type). Primary disorders of B cells/plasma cells such as multiple myeloma leads to synthesis of excessive immunoglobulins. In the extracellular fibrillogenic environment, fibrils are formed. These fibrils are processed by macrophages resulting in amyloid deposition.

Table 4.80 Bronchopulmonary amyloidosis

Bronchopulmonary Amyloidosis	Amyloid Protein	Clinical Features
Diffuse parenchymal involvement as part of systemic amyloidosis	AL	▪ Cardiopulmonary failure ▪ Poor prognosis
Nodular parenchymal involvement limited to the lungs	AL	Often asymptomatic
Tracheobronchial involvement limited to the major airways	AL	▪ Dyspnea ▪ Wheeze ▪ Requires repeated resection

light chain (AL) amyloidosis occurs in 30% cases of multiple myeloma or B cell lymphomas. AL amyloidosis involves multiple organs, including heart (75%), kidneys (65%), liver (15%), soft tissues (175%), peripheral and/or autonomic nervous system (10%) and gastrointestinal tract (5%). Localized amyloid tumor may be found in the respiratory tract.

- Presenting symptoms of systemic AL amyloidosis are often consequence of advanced irreversible organ damage mimicking other common diseases of the older population, thus majority of patients are misdiagnosed. Affected patients have fatal outcome within a few months after diagnosis despite treatment. Hematopoietic stem cell (HSC) transplantation is preferred in 20% of cases. Chemotherapy is administered in patients, who have not undergone hematopoietic stem cell transplantation.

AMYLOID ASSOCIATED (AA) AMYLOIDOSIS

Amyloid associated (AA) amyloidosis is also known as reactive amyloidosis secondary to chronic inflammatory diseases, e.g. tuberculosis, bronchiectasis, chronic osteomyelitis, autoimmune diseases (rheumatoid arthritis, ankylosing spondylitis, dermatomyositis, scleroderma, regional enteritis, ulcerative colitis) and malignancies (e.g. Hodgkin's disease and renal cell carcinoma).

- **Amyloid associated (AA)** fibril, an acute phase reactant synthesized by liver (1,000-fold) under stimulation by **interleukin-1 (IL-1)** released from activated macrophages is derived from serum amyloid associated (SAA).
- **Denaturing of SAA** releases a subunit termed **apoSAA**, which renders it amyloidogenic. In contrast to AL protein derived from immunoglobulin light chain, the amino acid sequence of AA proteins is identical in all patients, regardless of the underlying disorder.

Pathophysiology

Normally SAA in chronic inflammation is degraded to soluble end products by the action of monocyte associated serine esterases. When degradation process is inhibited, insoluble end products form amyloid material. Defective proteolysis may produce misfolded, incompletely degraded SAA, leading to aggregation and deposition as AA fibrils in tissues/organs.

- Amyloid associated (AA) Amyloidosis involves liver, spleen, kidney, adrenal glands, and lymph nodes. However, no organ system is spared. Vascular involvement may be widespread. The liver and spleen are often enlarged, and become firm, and rubbery.
- The kidneys are usually enlarged. Sections of the spleen have large, translucent, waxy areas where the normal Malpighian bodies are replaced by pale amyloid, producing the sago spleen. Clinically significant involvement of the heart is rare. Pathogenesis of AA amyloidosis is shown in Fig. 4.84.

Pathology Pearls: Mediterranean Fever

- Mediterranean fever is autosomal recessive disorder characterized by attacks of fever accompanied by inflammation of pleural, peritoneal and synovial surfaces.
- Patient develops widespread tissue involvement such as kidneys, blood vessels, spleen, and respiratory tract. It is indistinguishable from reactive systemic amyloidosis.
- The amyloid fibril proteins are made up of amyloid associated (AA) proteins.
- Normal gene product '**pyrin**' inhibits neutrophils in acute inflammation.
- Mutation of gene product 'pyrin' leads to vigorous tissue damaging inflammatory response due to minor trauma.

AMYLOID-β PRECURSOR PROTEIN ASSOCIATED AMYLOIDOSIS

Amyloid-β precursor protein (APP) associated amyloidosis is derived from a much larger transmembrane glycoprotein called amyloid precursor protein, protein product of chromosome 21, which is deposited in cerebral blood vessels of Alzheimer's disease. Amyloid deposits contain fibrils of β-amyloid (Aβ-protein resides on chromosome 21) derived from larger Aβ-protein precursor (AβPP), which is a normal cell membrane constituent.

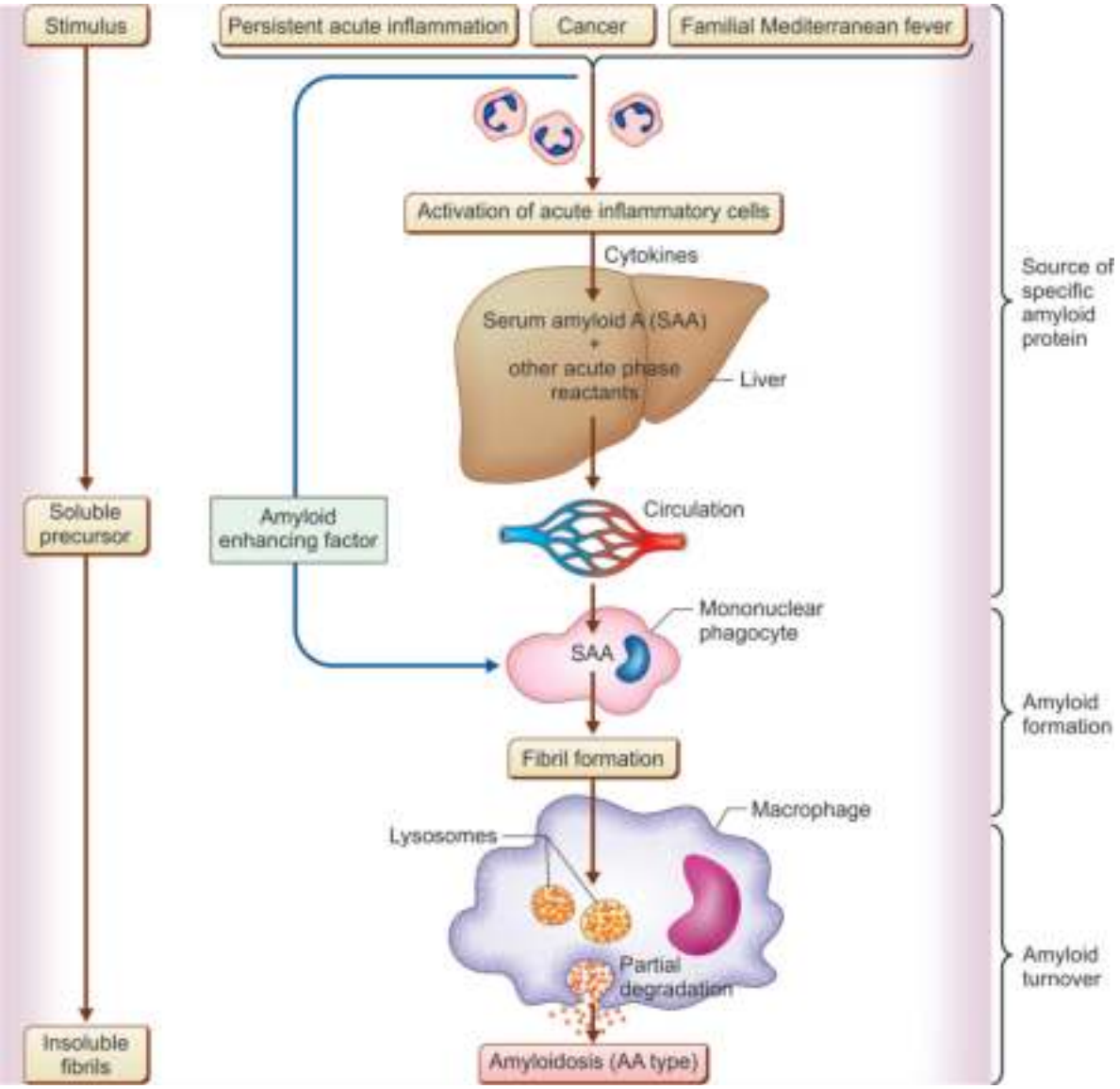


Fig. 4.84: Pathogenesis of amyloid associated (AA) amyloidosis. It is associated with the activation of polymorphonuclear leukocytes and macrophages, which in turn leads to the synthesis and release of acute phase reactants by the liver, including SAA. This SAA is released in association with amyloid-enhancing factor (AEF) by intact macrophages. This released product complexes with glycosaminoglycans and SAP as AA amyloid. The deposit is then processed by macrophages.

TRANSTHYRETIN PROTEIN ASSOCIATED AMYLOIDOSIS

Transthyretin (TTR) protein is a normal serum protein that transports thyroxine and retinol. Mutation in the genes encoding TTR protein is associated with amyloidosis affecting various tissues, including transthyretin amyloidosis and familial autosomal dominant polyneuropathy involving peripheral nerves and autonomic nervous system.

- **Amyloid of aging** is also known as senile systemic amyloidosis affecting 8th and 9th decades of elderly persons. Amyloid fibrils derived from **mutant form** of TTR protein are deposited in heart resulting in restrictive cardiomyopathy. In addition to heart, lungs, pancreas or spleen may be affected, suggesting that it is a systemic disorder.
- Hereditary amyloidosis is characterized by peripheral sensory and motor neuropathy, often autonomic neuropathy, and cardiovascular and renal amyloid. Carpal tunnel syndrome and vitreous abnormalities may occur. The fibrils in these familial polyneuropathies are made up of mutant ATTRs (transthyretin amyloidosis).
- Acquired transthyretin (TTR) protein is increased in insulinoma, glucagonoma and gastrointestinal carcinoids due to synthesis of transthyretin by these neoplasms.

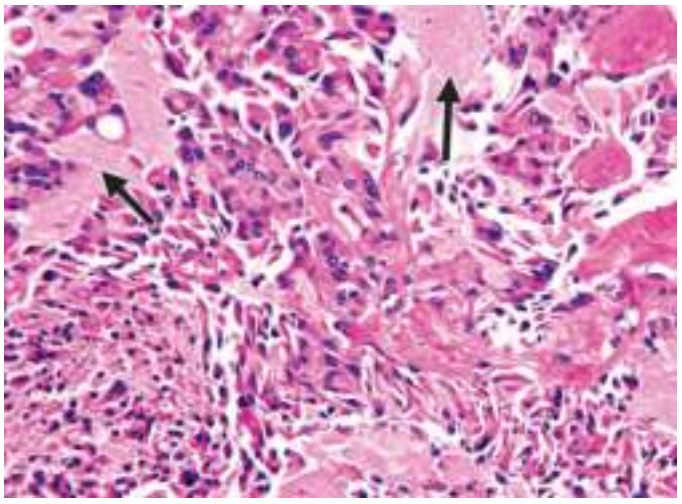


Fig. 4.85: Amyloid deposits in medullary carcinoma thyroid. The tumor is composed of round to polyhedral granular cells arranged in trabecular, glandular, carcinoid-like patterns. The tumor cells are separated by a distinctly fibrovascular stroma containing amyloid material representing deposition of procalcitonin (arrows) (400X).

β_2-MICROGLOBULIN PROTEIN HEMODIALYSIS ASSOCIATED AMYLOIDOSIS

The systemic amyloid deposition of β_2-microglobulin, a component of the MHC class molecules and a normal serum protein, occurs as a complication of long-term dialysis in patients with chronic renal failure due to increased concentration in serum. This protein does not pass-through conventional dialysis membranes. About 60–80% of patients on long-term dialysis develop amyloid deposition in the synovium, joints and tendon sheaths result in destructive arthropathy.

ENDOCRINE GLANDS–PROTEINS ASSOCIATED AMYLOIDOSIS

Microscopic deposits of localized amyloid deposit may be found in certain endocrine lesions, e.g. medullary thyroid carcinoma (amyloid-derived from procalcitonin), pheochromocytoma, undifferentiated carcinoma of stomach, islets of Langerhans in pancreas in type 2 diabetes mellitus (islet amyloid polypeptides or amylin) and islets tumors of pancreas (amyloid-derived from islet amyloid polypeptides). Amyloid deposits in medullary carcinoma of thyroid gland are shown in Fig. 4.85.

ORGANS INVOLVED IN AMYLOIDOSIS

Amyloid associated (AA) amyloidosis most commonly affects kidneys, liver and spleen. Amyloid light chain (AL) amyloidosis involves multiple organs including the heart (75%), kidneys (65%), soft tissues (15%), peripheral and/or autonomic nervous system (10%) and gastrointestinal tract (55%).

RENAL AMYLOIDOSIS

Kidneys are commonly involved in 80% cases of systemic amyloidosis. Patients present with proteinuria in nephritic/nephrotic range and ultimately go into chronic renal failure in a span of two years with fatal outcome. Renal failure is a common cause of death in amyloidosis.

- Renal amyloidosis occurs due to deposition of amyloid material in primary (AL in multiple myeloma) and secondary amyloidosis (AA in chronic disorders).
- Kappa (κ) or lambda (λ) chains of immunoglobulins synthesized by neoplastic plasma cells are deposited in the glomerular basement membranes and mesangial matrix, which immunoglobulins can be detected in serum or urine by electrophoresis.
- Amyloidosis is a well-known complication of chronic inflammatory disorders such as tuberculosis, bronchiectasis, rheumatoid arthritis, or osteomyelitis, which stimulate the production of amyloid from the serum amyloid A (SAA) protein, an acute-phase reactant secreted by the liver.

Surgical Pathology: Renal Amyloidosis

Gross Morphology

- Kidneys are enlarged and pale. Cut section of kidneys reveals firm and waxy surface.
- In advanced cases, the kidneys are contracted and shrunken due to vascular narrowing induced by deposition of amyloid.

Light Microscopy

- Renal amyloidosis shows amyloid deposits primarily affecting glomeruli and renal blood vessels, often causing marked vascular narrowing.
- Peritubular tissue is also involved. Amyloid nephropathy is shown in Fig. 4.86.
- Glomeruli show thickening of glomerular basement membrane. Amyloid material appears as amorphous, homogenous and eosinophilic in mesangial region, tubular basement membranes, renal vessels and interstitial tissue.
- Expansion of mesangial region obliterates the glomerular capillaries loops rendering the glomerular filter leaky to plasma proteins and impaired renal functions. There is no cellular response to the amyloid deposits.

Electron Microscopy

Electron microscopy reveals deposition of 7–10 mm thick amyloid fibrils in subendothelial region of glomerular basement membrane, mesangial matrix, tubular basement membrane and arterioles.

Histochemical Staining to Demonstrate Amyloid

- Amyloid material in glomerular mesangial matrix, glomerular and tubular basement membrane is demonstrated by Congo red staining under light microscopy, which reveals characteristic apple-green birefringence (color of a Granny Smith apple) under polarized microscopy.

- Amyloid material can also be demonstrated by various other stains, i.e. thioflavin T, thioflavin S, methyl violet, crystal violet.
- Pretreatment of tissue with potassium permanganate stained by Congo red demonstrates AL amyloid only but not AA amyloid. Congo red staining for amyloid deposition in the kidney in a case of diabetic glomerulosclerosis is shown in Fig. 4.87.

LIVER AMYLOIDOSIS

Amyloidosis is a well-known complication of chronic inflammatory disorders such as tuberculosis, bronchiectasis, rheumatoid arthritis, or osteomyelitis, which stimulate the production of amyloid from the serum amyloid A (SAA) protein, an acute-phase reactant secreted by the liver.

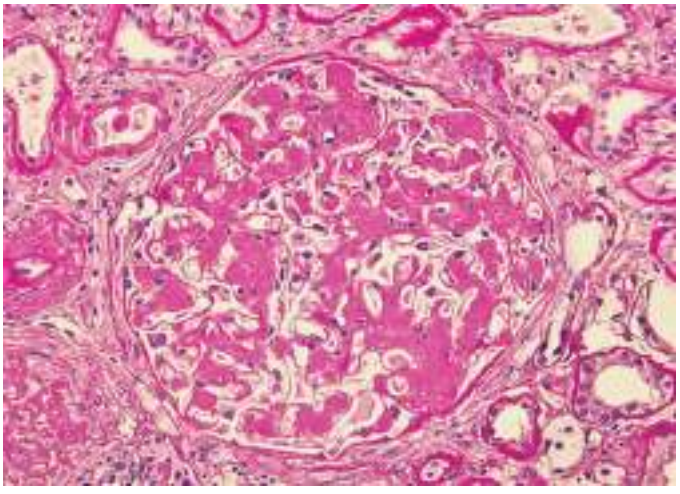


Fig. 4.86: Amyloid nephropathy: initially, amyloid material is deposited in the mesangium and then extending along the inner surface of GBM distorting glomerular lumina. Light microscopy shows amorphous acellular material extending to mesangial matrix and obstructing glomerular lumina (400X).

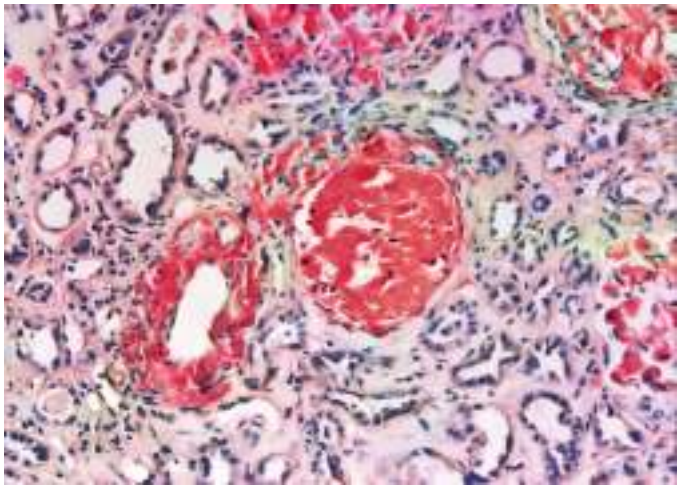


Fig. 4.87: Congo red staining for amyloid deposition in the kidney in a case of diabetic glomerulosclerosis. In histology and polarizing microscopy, Congo red is used to amyloid material in the tissues. Congo red stained preparations of tissue with amyloid material gives apple green birefringence indicative of the presence of amyloid fibrils (400X).

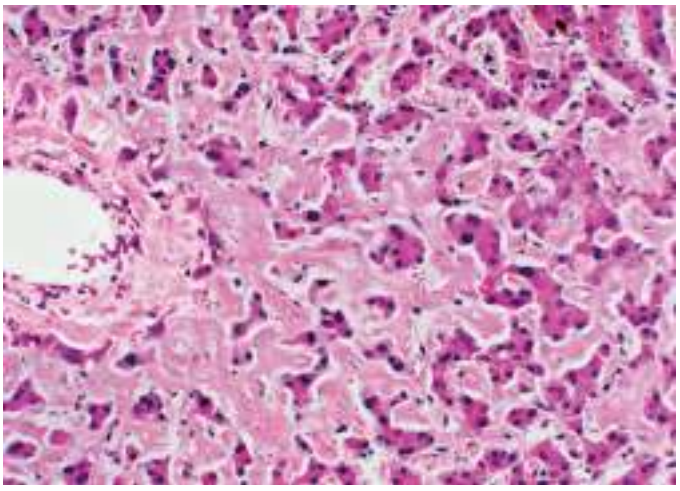


Fig. 4.88: Amyloid deposition in the liver. Histologic examination of liver shows amyloid deposition in the space of Disse between the hepatocytes and vascular sinusoids leading to compression of hepatic cords (400X).

- Liver is grossly enlarged (as much as 900 g), pale, and smooth surface, firm consistency. When sectioned, it has sharp rigid edges.
- On histologic examination, amyloid material is initially deposited in the '**space of Disse**' between the hepatocytes and vascular sinusoids. As more amyloid accumulates, it compresses the hepatic cords and sinusoids. The hepatic cords undergo nutritional and pressure atrophy and become displaced or replaced by bands and nodules of amyloid. Histologic features of amyloid liver are shown in Fig. 4.88.

SPLEEN AMYLOIDOSIS

Amyloidosis of the spleen often causes moderate or even marked enlargement (200–800 g). Amyloidosis of the spleen has two different anatomical patterns such as 'sago spleen' or 'lardaceous spleen'. In both the patterns, amyloid spleen is firm in consistency, and cut surfaces reveal pale gray, waxy deposits. Amyloid deposit in spleen is shown in Fig. 4.89.

- **Sago spleen:** Most commonly, the amyloid deposition is limited to the splenic follicles, resulting in the gross appearance of a moderately enlarged spleen dotted with tapioca-like gray nodules so-called sago spleen.
- **Lardaceous spleen:** Alternatively, the amyloid deposits may spare the follicles and mainly infiltrate the red pulp sinuses, producing a large, firm spleen mottled with waxy discolorations so-called lardaceous spleen.

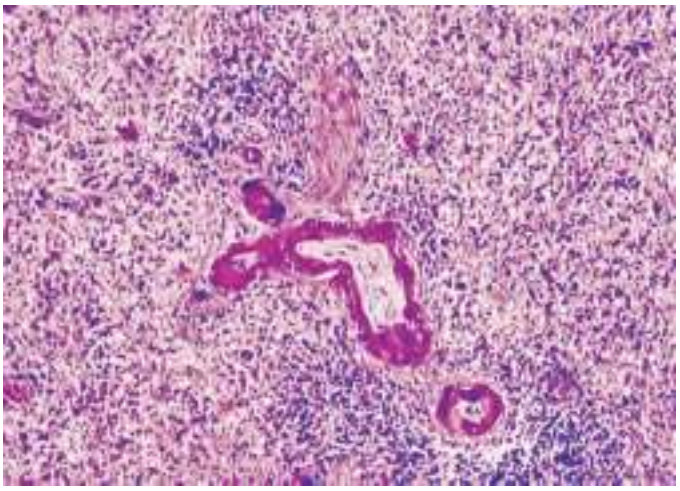


Fig. 4.89: Amyloid deposition in the spleen. Amyloid deposition in the spleen can occur in three major sites: red pulp, white pulp and blood vessels. Amyloid deposition in the red pulp of spleen demonstrates only AL protein in more than 50% of cases. Amyloid deposition in the white pulp of spleen can demonstrate either AL protein in 70% or AA protein in 35% of cases. In this case, amyloid deposition is seen in the blood vessels (100X).

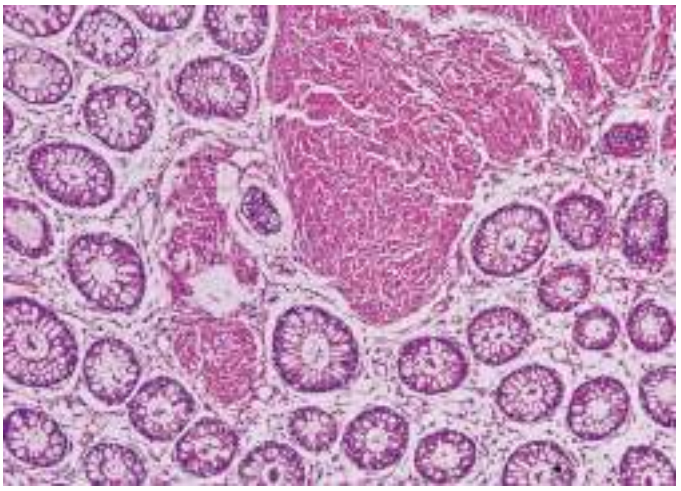


Fig. 4.90: Amyloid deposits in colorectal mucosa. In the colorectal mucosa, amyloid deposition is most often seen around blood vessels or diffusely in the lamina propria. Amyloid deposit is composed of AA protein. Subepithelial amyloid deposit may mimic collagenous colitis (400X).

CARDIAC AMYLOIDOSIS

Heart involvement may accompany systemic amyloid deposition usually associated with immunocyte dyscrasias or localized organ involvement (amyloidosis of aging). Amyloid deposits may cause minimal to moderately enlarged heart.

- Gross morphologic examination of cardiac amyloidosis reveals gray-pink dewdrop-like subendocardial elevation, particularly evident in the atrial chambers.
- Histologic examination reveals amyloid deposits located between the myocardial fibers eventually causing pressure atrophy or in the walls of the coronary arteries, which reduces the heart's ability to fill with blood in between heartbeats. When amyloidosis affects the electrical system of heart, heart's rhythm may be disturbed.

GASTROINTESTINAL TRACT AMYLOIDOSIS

The alimentary tract may be involved at any level, from the tongue to the rectum (submucosa). There may be direct involvement of gut or of autonomic nervous system. In amyloidosis of tongue (interstitial tissue) amyloid infiltrates the capillary walls and narrows the lumens of some of them; resulting in macroglossia. Lesions in the gut may cause obstruction, ulceration, protein loss, malabsorption, diarrhea and hemorrhage. Biopsy is taken from gingiva and rectum to demonstrate amyloid. Amyloid deposits in rectum are shown in Fig. 4.90.

SKIN AMYLOIDOSIS

Skin involvement occurs in primary amyloidosis. Patients present with slightly raised, waxy papules/plaques which are usually clustered in the axillae, anal or inguinal regions, which are seldom pruritic. Gentle rubbing of skin may induce purpuric bleeding into skin. Cutaneous may occur in secondary amyloidosis.

NERVOUS SYSTEM AMYLOIDOSIS

Cranial nerves are generally spared except for those involving pupillary reflexes. Neurologic manifestations include peripheral neuropathy, postural hypotension, inability to sweat; Adie pupil, hoarseness and sphincter incompetence. 25% of patients suffer from 'carpal tunnel syndrome' due to compression of nerves supplying fingers. This may cause severe pain especially during night, numbness and tingling of the fingers.

RESPIRATORY SYSTEM AMYLOIDOSIS

Amyloidosis can involve nasal sinuses, larynx, trachea, bronchi and alveolar septa. Pulmonary amyloidosis results from plasma cell production of monoclonal free immunoglobulin light chains (AL amyloid), which are either produced in the bone marrow and subsequently deposited in the lungs or pleura (systemic AL amyloidosis) or produced in the lungs.

- Diffuse parenchymal pulmonary amyloidosis usually presents with dyspnea, cough, hemoptysis, or a combination of these symptoms. The deposition of amyloid protein causes alveolar membrane and

capillary damage, which ultimately results in impaired gas exchange.

- Tissue biopsy is essential and green-yellow-orange birefringence under polarized light microscopy after Congo red staining is the gold standard for the diagnosis and typing of pulmonary amyloidosis, which is important and helpful for differentiated it from other diseases.

LABORATORY DIAGNOSIS OF AMYLOIDOSIS

No single set of clinical signs or symptoms points unequivocally to amyloidosis. When one suspects amyloidosis, the diagnosis ultimately rests on its histologic demonstration in biopsy specimens (heart, liver, skin, kidney, small intestine, sural nerve, rectum, gingiva, and tongue) and fine needle aspiration of abdominal fat. Biochemical tests, urine analysis, imaging techniques are also performed.

- Formalin fixed frozen sections are used to differentiate primary from secondary amyloid fibrils. Monoclonal antibodies are prepared to demonstrate different components of amyloid protein fibrils, amyloid P component and sulfated glycosaminoglycans (GAGs).
- Ultrastructurally, all forms of amyloid (AA, AL, ATTR types of amyloid) consist of interlacing bundles of parallel arrays of fibrils, which have a diameter of 7–13 nm. The protein in the amyloid fibrils contains a large proportion of crossed β-pleated sheet structure.
- Organs involved and clinical manifestations of amyloidosis are given in Table 4.81. Various stains used to demonstrate amyloid material are given in Table 4.82.

HISTOCHEMICAL STAINS

Iodine solution applied to cut surface of organ. Amyloid typically stains mahogany-brown. Application of dilute sulfuric acid leads to color reaction changes to blue ('starch-like' reaction).

Congo Red Stain

Congo red stain is the most common stain applied on formalin fixed biopsy specimens or aspirates to demonstrate amyloid. When the sections stained with Congo red are viewed under light microscopy, amyloid material imparts a pink or red colored hyaline-like material. Apple green birefringence is observed under polarized light. The fibrillary deposits organized in one plane have one color, and those organized perpendicular to that plane have the other color. This reaction is shared by all forms of amyloid and is caused by the crossed β-pleated configuration of amyloid fibrils.

Metachromatic Stains

Metachromasia refers to the coloring of a tissue constituent in a color different from that of the dye applied,

Table 4.81 Organs involved and clinical manifestations of amyloidosis

Organ Involvement	Clinical Manifestations
Tongue	Macroglossia
Esophagus	Megaesophagus and loss of peristalsis
Stomach	▪ Amyloid deposits in submucosa with loss of gastric rugae ▪ Amyloid deposit may be localized to antrum ▪ Stomach becomes rigid simulating linitis plastica leading to diminished or absence of stomach peristalsis
Small intestine	▪ Amyloid deposits in diffuse pattern (more common) causing broadened flat undulated mucosal folds (mucosal atrophy) leading to impaired motility ▪ Multiple small amyloid deposits causing small bowel dilatation and pseudo-obstruction
Colon	Pseudopolyps
Liver	Hepatomegaly
Spleen	Splenomegaly
Heart	Congestive heart failure with cardiomegaly
Kidney	Nephritic/nephrotic syndrome leading to chronic renal failure
Skin	Waxy papules
Brain	▪ Alzheimer's disease (dementia) caused by toxic Aβ deposits in neurons ▪ Amyloid precursor protein coded by chromosome 21 associated with Down's syndrome
Bone marrow	Plasmacytosis in multiple myeloma and osteolytic lesions in bones
Serum and urine	Monoclonal Ig and light chains

Table 4.82 Stains used to demonstrate amyloid material

Amyloid Stain	Color Imparted
Congo red stain examined under light microscope	Pink
Congo red stain examined under polarizing microscope	Apple green
Fluorescent stains (thioflavin T, thioflavin S)	Greenish fluorescence
Methyl violet (metachromatic stain)	Rose pink
Crystal violet (metachromatic stain)	Pink
PAS stain (metachromatic stain)	Magenta color
Von Gieson stain (metachromatic stain)	Khaki color

which takes place due to polarization, when certain negatively charged groups on the tissues react with cationic dyes. Metachromatic stains for amyloid protein include methyl violet, crystal violet, toluidine blues, periodic acid–Schiff and Van Gieson stains.

- **Methyl violet stain:** It is best used on frozen sections to demonstrate amyloid. Amyloid stains red purple/violet against a blue background. Addition of ammonium oxalate to methyl violet accentuates the metachromatic effect. Methyl green is used as counter stain in methyl violet method to demonstrate amyloid, which imparts purple red color.
- **Crystal violet stain:** Crystal violet stains amyloid a violet color against a blue background. Formic acid-crystal violet method accentuates the metachromatic effect.
- **Periodic acid-Schiff stain:** Amyloid appears magenta pink against background.
- **Von Gieson stain:** Amyloid stains khaki in a yellowish background.

Cotton Dye Sirius Red F3B

Cotton dye Sirius red F3B is a polyazo dye used principally in staining methods for amyloid and collagen. Cotton dye stains amyloid as red and exhibits apple green birefringence under polarized light.

Thioflavin T and Thioflavin Blue Stains

Fluorescein thiocyanate is used for immunofluorescence microscopy. Thioflavin T stain is not entirely specific to demonstrate amyloid. Thioflavin blue stain stains glycosaminoglycans of amyloid to appear blue, which allow amyloid to fluoresce green.

CHAPTER

5

Genetic Disorders

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LEARNING OBJECTIVES

HUMAN GENOME

- Human genome project
- Basic structure of DNA double helix molecule
  - Nuclear DNA and mitochondrial DNA
  - Nucleotide base pairs in DNA
  - Deoxyribonucleic acid (DNA) packaging
  - Ribonucleic acid

GENETICS AND MOLECULAR BIOLOGY

- DNA: chemical basis of hereditary
- Human DNA: genetic units
- Human chromosomes

THE NUCLEUS AND DNA REPLICATION

- Organization of the nucleus and its DNA
- DNA replication

GENE EXPRESSION, TRANSCRIPTION, TRANSLATION AND PROTEIN FOLDING

- Central dogma of biology
- Genetic code
- Transcription
- Translation
  - Protein folding
  - Denaturing of protein
  - Protein misfolding and aggregation

DNA DAMAGE AND REPAIR SYSTEMS

- DNA damage mechanisms
- DNA repair mechanisms
- Genetic disorders and faulty DNA repair systems

CHROMOSOMAL DISORDERS

- Changes in autosome chromosome number and structure
- Disorders of autosomal chromosome
  - Down syndrome (trisomy 21)
  - Edward syndrome (trisomy 18)
  - Patau syndrome (trisomy 13)
  - Cri-du-chat (5p minus, cat-cry) syndrome
  - DiGeorge syndrome
- Sex chromosome deviation
  - Barr bodies
  - X chromosome inactivation
- Disorders of sex chromosome
  - Klinefelter syndrome (trisomy 47, XXY)
  - Turner syndrome (monosomy 45, X/XO)
  - Jacob syndrome (trisomy XYY)
  - Triple X syndrome (47, XXX) and other multi-X chromosome anomalies

HUMAN GENE STRUCTURE AND MUTATIONS

- Gene structure
- Gene mutations
- Human genetic disorders

MENDELIAN (MONOGENIC) DISORDERS (SINGLE-GENE MUTATION)

- Modes of inheritance of Mendelian disorders
  - Autosomal dominant inheritance
  - Autosomal recessive inheritance
  - X-linked recessive inheritance
  - X-linked dominant inheritance
  - Mitochondrial inheritance
  - Codominant inheritance
  - Mendelian traits
- Autosomal dominant disorders
- Autosomal recessive disorders
- X-linked recessive disorders
- X-linked dominant disorders
- Mitochondrial DNA mutations associated disorders

BALANCED POLYMORPHISM

- Genetic disorders conferring resistance to infectious diseases
  - Hemoglobinopathies and resistance to malaria
  - Enzymopathies and resistance to malaria
  - Red blood cell-associated antigenic polymorphism and resistance to malaria
  - Cystic fibrosis and resistance to cholera
  - Tay-Sachs disease and resistance to tuberculosis
  - Phenylketonuria and lower risk for miscarriages
  - Niemann-Pick type C disease and resistance to flaviviruses such as Ebola and Marburg viruses
  - Myasthenia gravis and resistance to rabies virus
  - Congenital disorder of N-linked glycosylation (glycosidase 1 deficiency) and resistance to viral infections
  - Sickle cell anemia and susceptibility to pneumococcal infections
  - Hemosiderosis and susceptibility to typhoid fever

POLYGENIC AND MULTIFACTORIAL DISORDERS

- Diabetes mellitus type 2
- Essential hypertension
- Neural tube defects
- Developmental dysplasia of the hip

GENETIC POLYMORPHISM

- Single nucleotide polymorphism (SNP)
- Copy number variations
- Repetitive DNA sequence causing polymorphism

DISORDERS OF SEXUAL DIFFERENTIATION

- 46, XX karyotype testicular disorder of sexual development
- 46, XX karyotype female with normal ovaries and uterus but male or ambiguous genitalia
- 46, XY disorder of sexual differentiation
- Mixed genitalia and sex organs (46, XX ovotesticular disorder of sexual differentiation)
- Sex chromosome disorder of sexual differentiation [children may have either one X chromosome (XO) or an extra chromosome (XXY)]
- Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome
- Pseudohermaphrodite

GENOME IMPRINTING DISORDERS

- Prader-Willi syndrome
- Angelman syndrome

DIAGNOSTIC APPROACH OF GENETIC DISORDERS

- History and physical examination
- Pedigree construction
- Genetic testing
- Types of genetic testing
- Molecular genetic testing: methodology
- Biochemical genetic testing: methodology

HUMAN GENOME

Features of a person depend on genes present in DNA inherited from both the parents. Each gene synthesizes specific proteins. The genes are transmitted from the one generation to the next generation.

- Study of genes and the laws is known as '**genetics**'. The study of genetics consists of three major subdisciplines: transmission genetics, molecular genetics, and population genetics.
- Transmission genetics encompasses the basic principles of hereditary in which traits are transmitted from one generation to the next generation. Transmission genetics also addresses the relation between chromosomes and heredity, the arrangement of genes on chromosomes, and gene mapping.
- Applications of molecular diagnostics include: (a) diagnosis of genetic disorder, (b) prediction of disease progression, (c) paternity and forensic analysis, and (d) gene therapy and therapeutic design in management. Terminology used by geneticist is given in Table 5.1.

Table 5.1 Terminology used by geneticist

Term	Description
Allele	▪ Alternative of a gene present at a given locus ▪ Dominant allele is expressed even if it is paired with a recessive allele ▪ Recessive allele is only visible when paired with another recessive allele
Aneuploidy	Presence of extra copies of chromosome in addition to the normal pair (trisomy seen in Down syndrome and Klinefelter's syndrome) or deficient in number of chromosome (monosomy seen in Turner syndrome)
Anticodon	Consecutive sequence of three nucleotides on a tRNA molecule that is complementary to a specific codon on an mRNA molecule
Base substitution mutation	Base substitution mutation changes the base of a single DNA nucleotide
Cell nucleus	Carries genetic information
Central dogma of biology	The process by which the information in genes flows into the proteins: DNA→RNA→Proteins
Chromosome	Storage units of genes (condensed version of chromatin)
Chromatin	Substance consisting of DNA and associated protein
Chromosomal banding techniques	Demonstration of light and dark strips of chromosomes on staining with Giemsa stain (G-banding) and quinacrine fluorescence (Q-banding)
Codominance	Type of dominance in which two versions of a trait are expressed in the same individual
Codon	Three adjacent base nucleotide sequences in mRNA that code for specific amino acid production or start/stop signal during translation (consecutive sequence of three nucleotides on an mRNA molecule that corresponds to a specific amino acid)
Complete linkage	When two genes located on same chromosome are close to each other, so no crossing takes place and two are transmitted intact
Conservative gene mutation	Replacement of one amino acid with a biochemically similar amino acid
Deletion	▪ Small deletion of one or more nucleotide bases within a gene in DNA and large deletion removes an entire gene or several neighboring genes, hence alters function of the resulting protein(s) ▪ Insertion of intercalating agents into DNA molecule results in single nucleotide deletion ▪ Deletion can arise from strand slippage in DNA replication or from unequal crossing over
Diploid	Cells that carry two gametes (XX in females and XY in males)
DNA	Deoxyribonucleic acid contains the genetic instructions, which specify the biological development of all cellular forms of life
DNA polymerase	Enzyme that functions in adding new nucleotides to a growing strand of DNA during DNA replication
DNA replication	Process of duplicating a molecule of DNA
Duplication mutation	A piece of DNA is abnormally copied one or more times resulting in alteration in function of protein
Dominant	An allele that influences the appearance of the phenotype even in the presence of an alternative allele

Contd...

Table 5.1 Terminology used by geneticist (*Contd...*)

Term	Description
Epigenetics	Study of changes in the regulation of gene activity and expression that are not dependent on gene DNA sequence
Exon	Coding region of a gene (DNA), that codes for a particular translation of protein (one of the coding regions of an mRNA molecule that remain after splicing)
Expanding nucleotide repeats	Expanding nucleotide repeats are short DNA sequences that are repeated a number of times, in which the number of copies of the sequence increases
Frameshift mutation	▪ Insertion or deletion that alters the reading frame of a gene resulting in production of nonfunctional protein ▪ Reading frame consists of groups of three nucleotide bases, and each code for one amino acid ▪ Insertion, deletions and duplications can frameshift mutations
Forward mutation	Changes the wild-type phenotype to a mutant phenotype
Gain-of-function mutation	Gain-of-function mutation causes the appearance of a new trait or function or causes the appearance of a trait in inappropriate tissue or at an inappropriate time
Gene	Basic unit of genetic information determining the inherited characters (functional length of DNA that provides the genetic information necessary to build a protein)
Gene expression	Protein synthesis (gene product) is tightly controlled and regulated (active interpretation of the information coded in a gene to produce a functional gene product)
Genetic code	Combination of nucleotides that build different codons
Genome	Entire DNA content (set of chromosomes) in cell (entire complement of an organism's DNA; found within virtually every cell)
Genotype	The observed alleles for an individual's genetic constitution (DNA sequencing) at a genetic locus with respect to a single character or a set of characters
Haploid	Cells that contain a single copy of the genome (half the number of chromosomes) in replicating germ cells (gametes) such as ova and sperm during meiosis
Haplotype	Series of alleles on a single chromosome
Helicase	Enzyme that functions to separate the two DNA strands of a double helix during DNA replication
Heterozygous	Genotype with two different alleles at a locus of a gene for one trait
Histone	Family of proteins that associate with DNA in the nucleus to form chromatin
Homozygous	Genotype with two identical alleles of a gene for a single trait
In-frame deletion or insertion	Deletion or insertion of a multiple of three nucleotides that does not alter the reading frame
Incomplete linkage	Incomplete linkage occurs when crossover takes place in closely located genes (82% representing parental, while 18% recombinant)
Intron	Intron is noncoding region of a gene (DNA) present between two exons (pre-mRNA transcript) that may be removed during splicing
Insertion	▪ Addition of one or more nucleotide bases alters the number of DNA bases in a gene by adding a piece of DNA resulting in protein made by the gene, that may not function proper ▪ Insertion can arise from strand slippage in DNA replication or from unequal crossing over. Insertion of intercalating agents into DNA molecule causes single nucleotide insertion
Intragenic suppressor mutation	Intragenic suppressor mutation suppresses the effect of an earlier mutation within the same gene
Intergenic suppressor mutation	Intergenic suppressor mutation suppresses the effect of an earlier mutation in another gene
Karyotype	Orderly arrangement of photographs of chromosomes from a single cell; used in genetic counseling to identify chromosomal disorders
Lethal mutation	Lethal mutation causes premature death
Ligation	Process of joining two DNA molecule ends. It involves creating a phosphodiester bond between 3'-hydroxy of one nucleotide and 5'-phosphate of another nucleotide
Linkage disequilibrium	Allelic association when closely linked alleles are inherited together during many generations

Contd...

Table 5.1 Terminology used by geneticist (*Contd...*)

Term	Description
Locus	▪ Location of a gene on the chromosomes in genome ▪ Each locus contains two genes except for sex chromosomes
Loss-of-function mutation	Complete or partial loss of function
Non-conservative gene mutation	Replacement of one amino acid with a biochemically dissimilar amino acid
Missense mutation	A missense mutation alters the coding sequence (i.e. sense codon changes into different codon), so one amino acid is substituted for another amino acid in the protein made by a gene
Mitosis	Process by which a cell separates its duplicated genome into two identical halves
Meiosis	Process that transforms one diploid into four haploid cells
Messenger RNA (mRNA)	Nucleotide molecule that serves as an intermediate in the genetic code between DNA and protein
Molecular diagnostics	The use of DNA, RNA, mRNA to identify and /or characterize disease caused by infectious agents or gene abnormalities
Mutation	Permanent change in the genetic sequence (such as mispairing of base in DNA replication and spontaneous depurination and deamination)
Mutation rate	Mutation rate is the frequency of mutation influenced by genetic and environmental factors. Some mutations occur spontaneously
Mutagenic agents	▪ Intercalating agents insert into the DNA molecule and cause single nucleotide insertions and deletions. ▪ Oxidative reactions alter the chemical structures of bases ▪ Ionizing radiation alters nucleotide base structures and breaking phosphodiester bonds ▪ Ultraviolet light produces light pyrimidine dimers, which block DNA replication
Neutral mutation	A neutral mutation alters the amino acid sequence of a protein but does not change the function of the protein
Nonsense mutation	A nonsense mutation changes a sense codon into a nonsense codon, which altered DNA sequence causing premature termination of translation; but there is no substitution of one amino acid for another.
Nuclear envelope	Membrane that surrounds the nucleus; consisting of a double lipid-bilayer
Nuclear pore	One of the small, protein-lined openings found scattered throughout the nuclear envelope
Nucleolus	Small region of the nucleus that functions in ribosome synthesis
Nucleosome	Unit of chromatin consisting of a DNA strand wrapped around histone protein
Penetrance	Proportion of individuals of a particular genotype (allele of a gene) that express its phenotypic effect in a given environment
Phenotype	Observable characteristics of a specific gene or set of genes
Point mutation	Substitution of a single DNA base by another
Polymorphism	A variation in the base sequence of DNA
Polypeptide	Chain of amino acids linked by peptide bond
Polyribosome	Simultaneous translation of a single mRNA transcript by multiple ribosomes
Promoter of gene	Region of DNA that signals transcription to begin at that site within the gene
Proteins	Proteins are made up of amino acids
Protein expression	Different proteins are expressed in different cells according to the function of the cell
Proteome	Full complement of proteins produced by a cell (determined by the cell's specific gene expression)
RFLP (restriction fragment length polymorphism)	Variation in the size of DNA fragments generated by restriction enzymes
Recessive	An allele that influences the appearance of the phenotype only in the presence of another identical allele
Reverse mutation	Changes a mutant phenotype back to the wild-type phenotype
Ribonucleic acid (RNA)	Single-stranded nuclei acid that carries out the instructions coded in DNA

Contd...

Table 5.1 Terminology used by geneticist (*Contd...*)

Term	Description
Ribosomal polymerase	Enzyme that unwinds DNA and then adds new nucleotides to a growing strand of RNA for the transcription phase of protein synthesis
Ribosomal RNA (rRNA)	RNA that makes up the subunits of a ribosome
STR (short tandem repeats)	Short sequences of DNA, normally 2–5 nucleotide base pairs, that are repeated numerous pairs, that are repeated numerous times
Silent mutation	A silent mutation produces a sense codon into a synonymous codon, that encodes the same amino acid; there is no change in amino acid sequence of the protein
Spliceosome	Complex of enzymes that serves to splice out the introns of a pre-mRNA transcript
Splicing	Complex of enzymes that serves to splice out the introns of a pre-mRNA transcript
Suppressor mutation	Suppressor mutation suppresses the effect of an earlier mutation at a different site
Telomere	Region of repetitive DNA at the end of a chromosome
Transcription	Process during which a DNA sequence of a gene is copied to make RNA molecule
Transcriptome	Set of all RNA molecules transcribed in a cell
Transfer RNA (tRNA)	Molecules of RNA that serve to bring amino acids to a growing polypeptide strand and properly place them into the sequence
Transgene	A foreign gene that is introduced randomly somewhere in the genome
Transition	Base substitution in which a purine replaces a purine or a pyrimidine replaces a pyrimidine
Translation	Process during which a mRNA molecule is used to assemble amino acids into polypeptide chains
Transversion	Base substitution in which a purine replaces a pyrimidine or a pyrimidine replaces a purine
Triplet	Consecutive sequence of three nucleotides on a DNA molecule that, when transcribed into an mRNA codon, corresponds to a particular amino acid
VNTR (variable number of tandem repeats)	Variable number of tandem repeats of identical nucleotide sequences lined up one after another that vary in number from one individual to another

HUMAN GENOME PROJECT

Human Genome Project (HGP) was an International Scientific Research Project (1990–2003) with a goal to discover the complete set of 20,000–25,000 human genes and make them accessible for further biological study, and determine the complete sequence of three billion DNA bases in the human genome.

- **Genome sequencing:** Genome sequencing can provide nucleotide sequence of an individual's DNA. The primary purpose of genome sequencing is to determine the order of the four chemical building blocks called 'nucleotide base pairs' that make up DNA molecule; and to obtain information on medical value for future care, that can help in prevention of disease. Scientists can use DNA sequencing information to determine which stretches DNA contain genes and which stretches carry regulatory instructions, turning genes on or off.
- **Whole genome sequencing:** Whole genome sequencing can detect single nucleotide base variation, insertions/deletions, copy number changes and large structural variants. Human genome sequencing is performed by two methods: (a) the older, classical chain termination method is also known as 'Sanger method', (b) newer human genome DNA sequencing methods are high throughout sequencing (HTS) techniques or next-generation sequencing techniques, that can process a large number of DNA molecules quickly.

BASIC STRUCTURE OF DNA DOUBLE HELIX MOLECULE

Deoxyribonucleic acid (DNA) is a double helix molecule, while RNA is a single helix molecule. Both DNA as well as RNA have sets of nucleotides that contain genetic information. DNA molecule contains the instructions, an organism needs to develop, live and reproduce.

- The human genome is organized into chromosomes and wrapped in histone proteins, condensed into chromatin and arranged in nucleosomes. Histone modifications affect the chromatin state and gene expression.

- The nucleosome has 146 nucleotide base pairs surrounding the histone octamer. The chromatosome has 166 nucleotide base pairs surrounding histone octamer and binding the H1 histone. Nucleosome and chromatosome contain active genes.
- Solenoid defines the packing of DNA as a 30 nm fiber of chromatin and results from the helical winding of at least five nucleosome strands. Genes are less active in solenoid and 30 nm fiber of chromatin.
- Chromatin remains in interphase and chromosome in the metaphase. Structure of double-stranded DNA molecule is shown in Fig. 5.1. DNA packaging is shown in Fig. 5.2. Structure of histone is shown in Fig. 5.3.

NUCLEAR DNA AND MITOCHONDRIAL DNA

Nearly every cell in human body has similar DNA. Most DNA is located in the cell nucleus (where it is called nuclear DNA) having 3.2 billion nucleotide base pairs, but a small amount of circular DNA composed of 16,569 nucleotide base pairs is present in the mitochondria, where it is called mitochondrial DNA (**mtDNA**).

- Nuclear DNA has two copies of a gene per cell, while mitochondrial cell contains 100–1000 copies of a gene per cell. The mitochondrial DNA contains 2 ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs) and 37 genes, which code for 13 proteins.

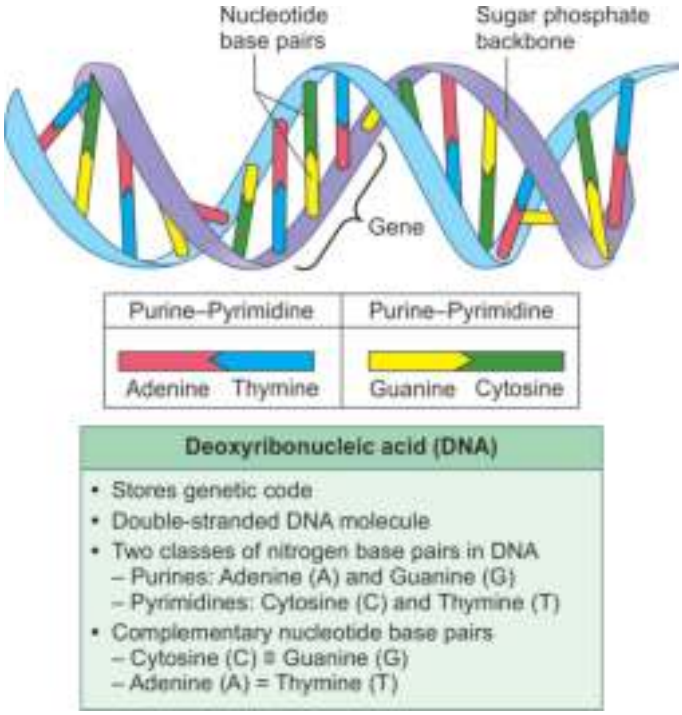


Fig. 5.1: Structure of double-stranded DNA molecule. Double-stranded DNA molecule is composed of two linear strands that run opposite to each other, or anti-parallel, and twist together. Each DNA strand within the double helix is a long, linear molecule composed of smaller units called nucleotides that form a chain.

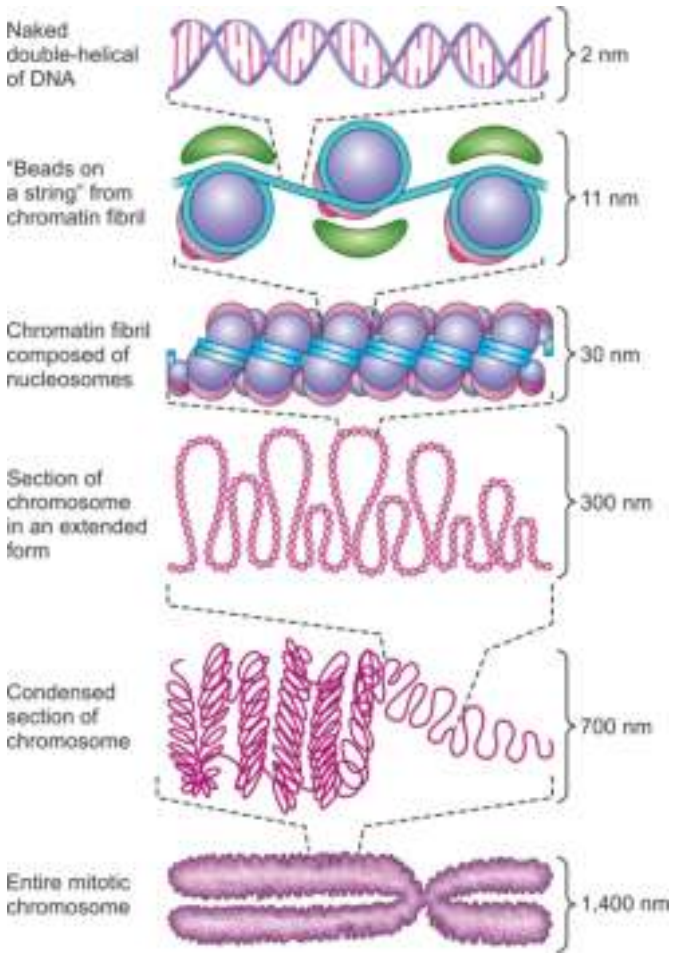


Fig. 5.2: DNA packaging. The process starts when DNA is wrapped around special positively charged protein molecules called histone octamer. The combined loop of DNA and protein is called a nucleosome (10 nm). Then nucleosomes are packaged into a thread, which sometimes described as 'beads on a string'. The end result is a fiber known as chromatin (30 nm) resulting in chromosome.

Each mitochondrion contains several copies of mitochondrial DNA.

- The codons are identical in nuclear DNA and mitochondrial DNA, except that mitochondrial DNA contains 60 codons for amino acids and 4 for stop codons. Transcription of mitochondrial DNA is continuous, as opposed to the discontinuous transcription of nuclear DNA. Comparison of nuclear and mitochondrial DNA is given in Table 5.2.

NUCLEOTIDE BASE PAIRS IN DNA

DNA molecule is composed of four chemical building blocks: adenine, guanine, cytosine and thymine. Each molecule is linked to other molecules, forming a chain.

- The DNA consists of two chains of nucleotides held together by hydrogen bonds. The purine nucleotides, adenine and guanine cross-link by hydrogen bonds to the pyrimidines, thymine and cytosine. Because

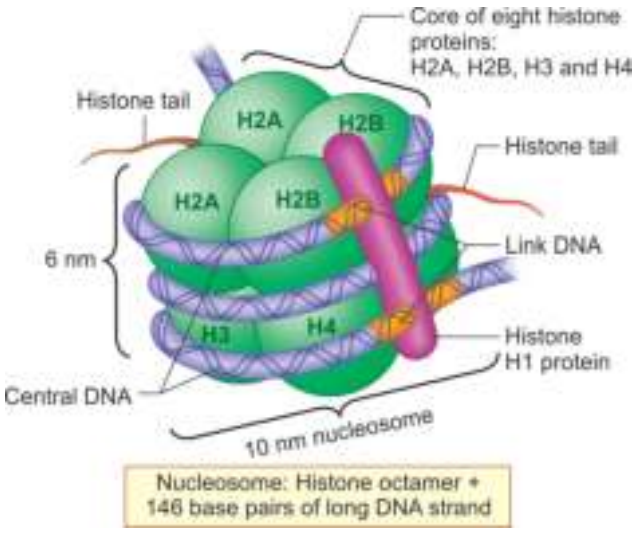


Fig. 5.3: Structure of histone. Histone is positively charged and highly basic proteins composed of amino acids, e.g. 'lysine' and 'arginine'; and found in nuclei that package and order the DNA into structural units called nucleosome. These are the chief protein components of chromatin, acting as spools around negatively charged DNA winds, and playing a role in gene regulation.

of this cross-linking, the nucleotide sequence of one strand of DNA sets the other strand's sequence.

- Separating the two DNA strands allow complementary nucleotides to bind to each DNA strand, which copies the DNA and replicates. Comparison of purine and pyrimidine is given in Table 5.3.

DEOXYRIBONUCLEIC ACID (DNA) PACKAGING

Negatively charged double-stranded DNA is coiled around a set of 8 highly basic histone proteins forming the nucleosome, which is the building block of chromatin packaging. **Histone proteins** contain '**lysine**' and '**arginine**' **amino acids**.

- Main function of the histone octamer is to compact DNA and regulates chromatin, therefore impacting gene regulation. DNA–protein complexes can be packaged by forming coils of nucleosomes, called chromatin fibers.
- The chromatin fibers are condensed into two strands of a replicated chromosome during mitosis (cell division) and visible under electron microscope.
- Chromatids connected by a centromere are called **sister chromatids**. Nonhistone proteins are additional set of proteins required for packaging of chromatin at higher level, i.e. chromatin fibers and chromosomes.
- Heterochromatin protein 1 and polycomb are common nonhistone proteins, which play a key role in regulation of gene expression.
- Both histone and nonhistone proteins provide structure to the DNA. The basic difference between histone and nonhistone proteins is in the structure they provide. Histone proteins are the spools around which DNA winds, whereas nonhistone proteins provide the scaffolding structure.

RIBONUCLEIC ACID

The structure of ribonucleic acid (RNA) is similar to DNA except that it has one strand instead of two. RNA also contains a series of four nucleotide base pairs except that one of these nucleotide base pairs is different from DNA. RNA uses a nucleotide base called uracil (**U**) (also called 5-methyl uracil) instead of thymine (**T**) in the DNA. It has ribose instead of deoxyribose. Comparison of structure of DNA and RNA is shown in Fig. 5.4 and Table 5.4.

- Messenger RNA (mRNA) records information from DNA and carries it to ribosomes for protein synthesis.
- Ribosomal RNA (rRNA) makes up about 60% of ribosomal structure. In RNA, every nucleotide

Table 5.2 Comparison of nuclear and mitochondrial DNA

Characteristics	Nuclear DNA	Mitochondrial DNA
Location of DNA	Nucleus	Mitochondria
Shape of DNA	Linear	Circular
Number of copies	2 copies per cell	100–1000 copies per cell
Number of nucleotide base pairs in DNA	3.2 billion	16,569
Number of genes	20,000 to 25,000	37
Genes code for	Majority of genome proteins	13 proteins, 2 ribosomal RNAs, and 22 transfer RNAs
Codons	Three letter sequence of mRNA nucleotides to specific amino acid, AUG is the codon for methionine; and start codons are UGA, UAA and UAG	60 codons for amino acids and 4 for stop codons
Transcription	Discontinuous	Continuous

Genetic code is universal.

Table 5.3 Comparison of purine and pyrimidine

	Purine	Pyrimidine
Main	A purine is a heterocyclic aromatic organic compound, consisting of a pyrimidine ring fused to an imidazole ring	Pyrimidine is a heterocyclic aromatic organic compound similar to benzene and pyridine, containing two nitrogen atoms at positions 1 and 3 of the six-member ring. It is isomeric with two other forms of diazine
Function	Production of RNA and DNA, proteins and starches, the regulation of enzymes and cell signaling	Production of RNA and DNA, proteins and starches, the regulation of enzymes and cell signaling
Nucleobases	Adenine and guanine	Cytosine, thymine, uracil
Structure	A pyrimidine ring fused to a imidazole ring. Contains two carbon-nitrogen rings and four nitrogen atoms	Contains one carbon-nitrogen ring and two nitrogen atoms
Melting point	214°C, 487 K, 417°F	20–22°C
Type of compound	Heterocyclic aromatic organic compound	Heterocyclic aromatic organic compound
Molecular formula	C5H4N4	C4H4N2

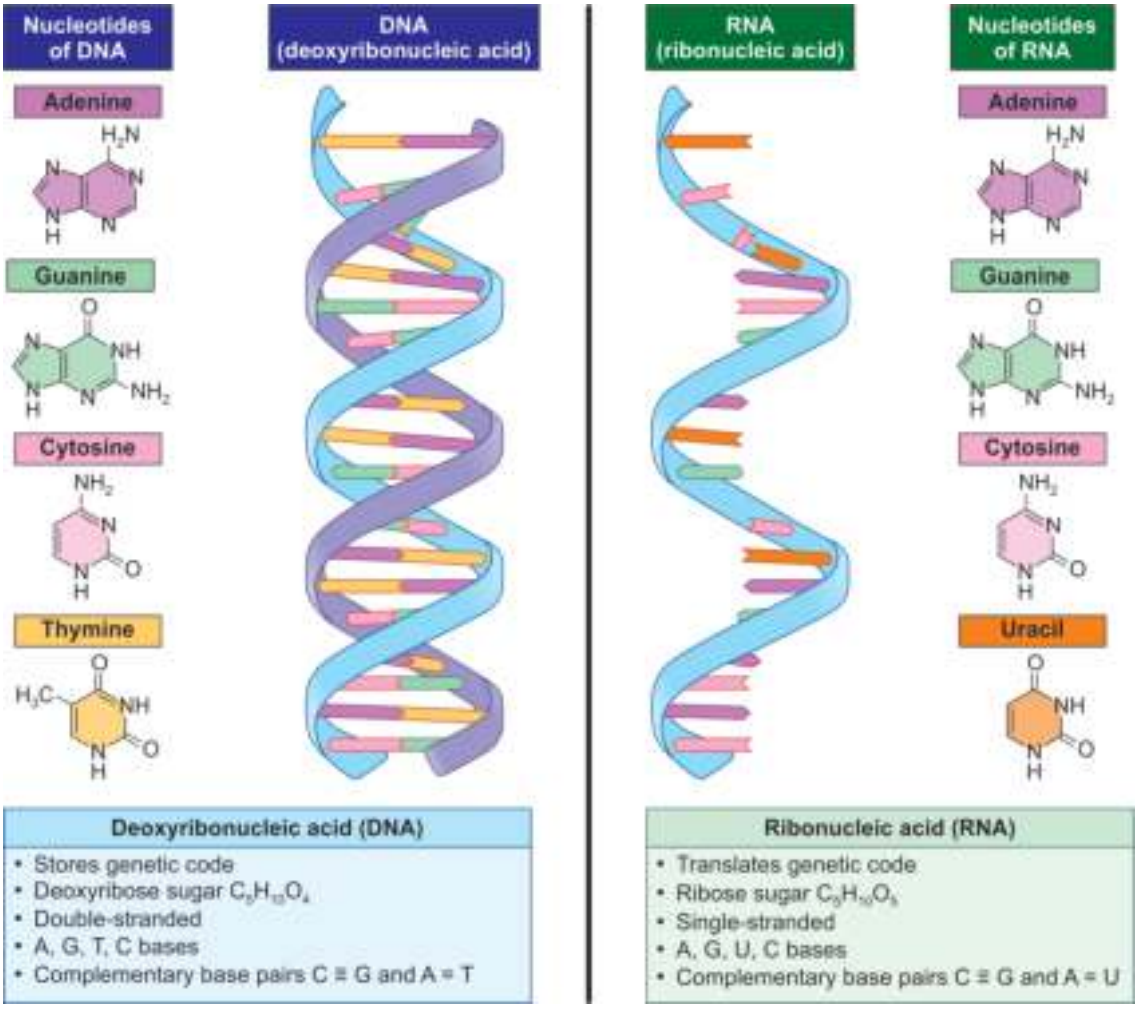


Fig. 5.4: Structure of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA is double-stranded, and stores genetic code and has adenine, guanine, thymine and cytosine base pairs. Ribonucleic acid is single-stranded, and translates genetic code and has adenine, guanine, uracil and cytosine base pairs.

residue has an additional OH group present at 2′-position in the ribose. This is a reactive group and makes RNA labile and easily degradable.

- **Transfer RNA (tRNA)** delivers amino acids building blocks to proteins at the ribosome to extend the chains.

Table 5.4 Comparison of structure of DNA and RNA

Characteristics	DNA (Deoxyribonucleic Acid)	RNA (Ribonucleic Acid)
Pentose sugar	Deoxyribose	Ribose
Nitrogen bases	▪ Adenine (A)–Thymine (T) ▪ Guanine (G)–Cytosine (C)	▪ Adenine (A)–Uracil (U) ▪ Guanine (G)–Cytosine (C)
Adenine pairing	Adenine (A) pairs with thymine	Adenine (A) pairs with uracil (U)
Complementary nucleotide base pairs	C≡G, (A) = (T)	C≡G, A = U
Purine and pyrimidine ratio	Purine is equal to pyrimidine (Chargaff's rule)	Purine is not equal to pyrimidine
Structure	DNA (double-stranded) antiparallel helix	RNA (single-stranded), hairpin and loops
Stability provided to DNA	DNA is more stable and less reactive (thymine provides stability to genetic material)	RNA is less stable and more reactive (due to the presence of 2-OH group)
Genetic material	Present in all living in all organisms	Present in viruses
Length of the molecule	Quite large consisting millions of nucleotides	Short consisting of thousands of nucleotides
Location	DNA present in nucleus, nucleolus and extra-chromosomal mitochondria	Messenger RNA (mRNA) in nucleus, transfer RNA (tRNA) and ribosomal RNA (rRNA) in cytoplasm
Function(s)	DNA stores genetic information; present only in one form	▪ **Ribosomal RNA (rRNA)** makes up about 60% of ribosomal structure ▪ **Messenger RNA (mRNA)** records information from DNA and carries it to ribosomes ▪ **Transfer RNA (tRNA)** delivers amino acids building blocks to proteins at the ribosome to extend the chains
Process	Transcription	Translation
Protein synthesis	DNA dependent on RNA for synthesizing protein	RNA can directly code for the synthesis of proteins RNA is better for transmission of information
Other functions	DNA being double-stranded and having complementary strand further resists changes by evolving a process of repair	RNA is essential for life processes such as metabolism, translation and splicing, which evolve around RNA
Lifetime	Long	Short
Mutations occurrence	Slower rate	Faster rate (viruses have shorter life span)

GENETICS AND MOLECULAR BIOLOGY

Nucleus is a spherical shape organelle in human cells, that contains the genetic material. The structure of the nucleus includes nuclear bilipid membrane, 3×10^9 nucleotide base pairs of the DNA distributed over 23 pair of chromosomes, gel-like nucleoplasm and nucleolus. The nuclear pore is a protein-lined channel in the nuclear envelope that regulates the transportation of molecules between the nucleus and the cytoplasm. This is collectively known as human genome, that contains around 30,000 genes, each of which codes for one specific protein.

DNA: CHEMICAL BASIS OF HEREDITARY

Genetics is a branch of biology concerned with the study of genes. Chemical basis of hereditary is the cellular DNA. The DNA is a biological print, that carries information for the cell to live, grow and replicate; it provides consistency and variability. It is important for inheritance; coding proteins and genetic instruction guide for life and processes.

- The DNA molecule is inherited from each parent. Gene is a specific sequence of nucleotides in DNA in RNA usually located on a chromosome. Gene is the 'functional unit of inheritance' controlling the transmission and expression one or more traits by encoding specific protein.
- Humans typically have 23 pairs of chromosomes, each of which contains DNA. One of each chromosome is received from each parent. During meiosis, when gametes are formed, the pair of chromosomes are split up often recombining in the process. During reproduction, the DNA in the gametes of each parent is combined. It is the crossing over and recombination of chromosomes, which are responsible for variation.

HUMAN DNA: GENETIC UNITS

Genetic units of human DNA include: (a) nuclear DNA with around 30,000 genes, (b) mitochondrial DNA (non-nuclear) with 37 genes, (c) nitrogen base pairs in DNA/RNA, and (d) DNA double helix.

- Nucleus stores the genetic information in the form of DNA, that holds the instructions, how the cell should work. The molecules of DNA are organized into special structures called chromosomes.
- Mitochondrial DNA is transmitted almost exclusively from the mother to offspring through the ovum. Mitochondrial DNA test talks about maternal ancestors of offspring.
- Nitrogen base pair is a molecule that contains nitrogen and has the chemical properties of a base. The nitrogen base pairs in DNA are adenine (A), guanine (G), thymine (T) and cytosine (C). RNA has same nitrogen bases except uracil (U) replaces thymine (T). Hence, nitrogen bases in RNA are adenine (A), guanine (G), uracil (U) and cytosine (C).
- A DNA molecule consists of two strands that wind around each other like a twisted ladder. Both strands of double-stranded DNA stabilized by hydrogen bonds between nucleotides, store the same biological information, which is replicated when the two strands of DNA separate. A large part of DNA (more than 98% for human beings) is noncoding, meaning that these sections do not serve as patterns for protein sequences. Genetic units of human DNA (deoxyribonucleic acid) are given in Table 5.5.

Table 5.5 Genetic units of human DNA (deoxyribonucleic acid)

Nuclear DNA

- Diploid genome (two sets of chromosomes)
- DNA packed in 23 pairs of chromosomes
- Homologous chromosomal pairs 22 (autosomes)
- Sex chromosomes (XX or XY)
- Nucleotide base pairs 3.2 billion
- Approximately 30,000 genes

Mitochondrial DNA (Non-nuclear DNA)

- Nucleotide base pairs (16,569)
- Approximately 37 gene
- Higher gene mutation rate
- Naturally occurring polymorphisms (128)
- Maternal inheritance

Nitrogen Bases of DNA and RNA

- Purines
  - Adenine (A) present in both DNA and RNA
  - Guanine (G) present in both DNA and RNA
- Pyrimidines
  - Cytosine (C) present in both DNA and RNA
  - Thymine (T) in DNA
  - Uracil (U) in RNA (thymine replaced by uracil)
- Hydrogen bonds can form between a purine and pyrimidine
- Watson-Crick pairing: A = T, G = C

DNA Double Helix Molecule

- Nucleotide base pairs
  - Adenine 'A' always binds to thymine 'T' in DNA
  - Adenine 'A' binds to uracil 'U' in RNA
  - Cytosine 'C' always binds to guanine 'G' in DNA and RNA
- DNA double helix molecules are oriented in opposite directions
- 5' End is beginning of DNA strand
- 3' End is the end of DNA strand

HUMAN CHROMOSOMES

Deoxyribose nucleic acid (DNA) molecule is tightly coiled around histones, packaged into thread-like structures called chromosomes in the cell nucleus.

- Human genome is made up of 23 chromosome pairs for a total of 46 chromosomes with a total about three billion DNA base pairs.
- Twenty-two of these chromosomal pairs are called **autosomes**, which look similar in both males and females. The 23rd chromosomal pair, the sex chromosomes X or Y, differ between males and females.
- Males have 22 chromosomal pairs of autosomes and one pair of sex chromosome (XY). Females have 22 chromosomal pairs of autosomes and one pair of sex chromosome (XX). Chromosomes are numbered roughly in order of decreasing in size.
- Each pair consists of two chromosomes that are alike in size and structure and carry information for the same characteristics. Sister chromatids are copies of a chromosome held together at the centromere. Each chromosome has a centromere and telomeres.
- Telomeres are the specific DNA sequences and associated proteins located at the tips (ends) of whole linear chromosomes. The centromere is a constricted region of the chromosome where the kinetochores form and the spindle microtubules attach.
- Haploid term refers to half the number of chromosomes in germ cells (23).
- Diploid term refers to two sets of chromosomes organized in homologous pairs found in non-germ cells (46). Structure of human chromosome is shown in Fig. 5.5. Terminology used for number of chromosomes in the cell is given in Table 5.6.

AUTOSOMES

Chromosomes other than the sex chromosomes are referred to autosomes. Human genome contains total of

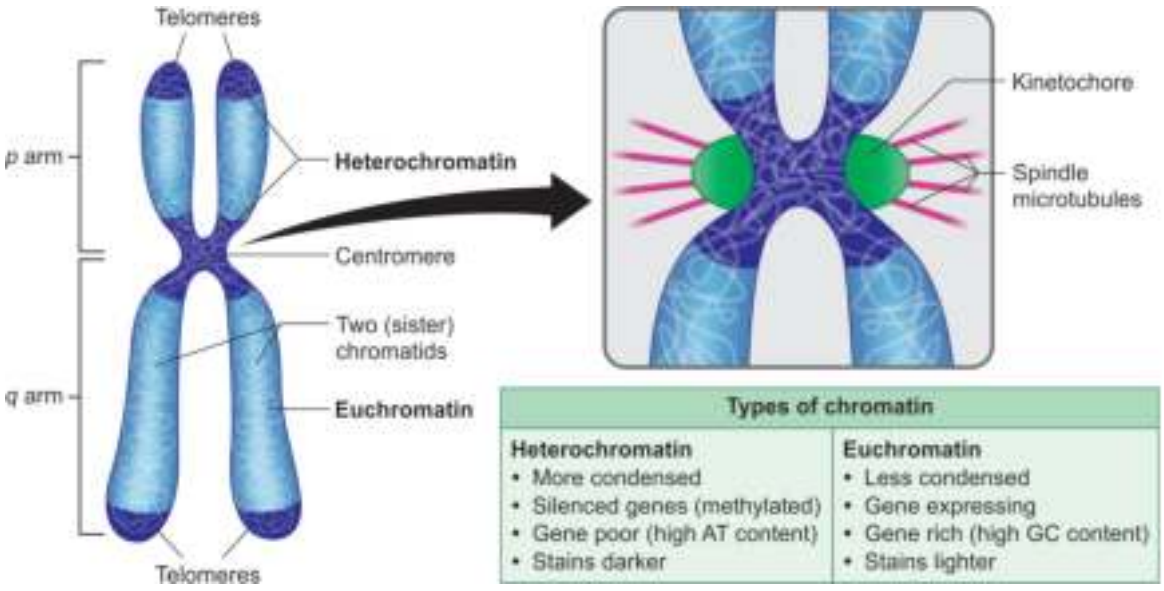


Fig. 5.5: Structure of human chromosome. Each cell normally contains 23 chromosomal pairs (total 46), i.e. 22 chromosomal pairs of autosomes and one chromosomal pair called sex chromosomes (XX, XY).

Table 5.6 Terminology used for number of chromosomes in the cell

Term	Characteristics
Haploid cell	Half the number of chromosomes in germ cells (23)
Diploid cell	Two sets of chromosomes organized in as homologous pairs found in non-germ cells (46)
Triploid cell	Number of chromosomes three times the haploid number (69)
Tetraploid cell	Number of chromosomes four times the haploid number (92)
Trisomy	Three copies of the same chromosome

46 chromosomes. Normal diploid cells have two copies of each autosome and two sex chromosomes either XX for females or XY for males. **Autosomes** are **homologous chromosomes**, which contain the same genes (regions of DNA) in the same order along chromosomal arms. Comparison of autosomes and sex chromosomes is given in Table 5.7.

Chromosome Morphology

The morphology of chromosomes can be best studied at the metaphase or anaphase of mitosis, when these are present as definite organelles, being most condensed or coiled.

- Chromosomes range, on an average from 0.5 to 30 μm in length and 0.2 to 3 μm in diameter. Variation in size of the chromosomes can be induced by a number of environmental factors. Cells dividing at low-temperature have shorter and more compact chromosomes than those dividing at higher temperature.

Table 5.7 Comparison of autosomes and sex chromosomes

Characteristics	Autosomes	Sex Chromosomes
Determines	Somatic traits	Gender
Copy of chromosomes	Males and females contain the same copy of autosomes	Males and females contain different copy of sex chromosomes, their size and behavior
Labelled with numbers from	1 to 22	XY, ZW, XO and ZO
Number of chromosomes in human genome	Majority of chromosomes	Few chromosomes
Chromosomes	22 pairs of autosomes are homologous in human beings	Female sex chromosomes (XX) are homologous; male sex chromosomes (XY) are nonhomologous
Position of the centromere	Identical	Not identical
Mendelian inheritance exhibits	Present	Absent
Number of genes	200 to 2000	X chromosome contains more than 300 genes; Y chromosome contains only a few chromosomes

- Colchicine, an alkaloid interferes with spindle formation and cell division resulting in shortening of the chromosomes. Rapid and repeated cell division results in smaller chromosomes, which appear because rate of cell division proceeds more rapidly than the formation of chromatic material as usual.
- Shape of the chromosome changes from phase to phase during cell division. In the resting phase of the cell, the chromosomes occur in the form of thin, coiled, elastic and contractile, thread-like structures, the chromatin threads.
- The position of centromere varies from chromosome to chromosome and it provides different shapes to the chromosomes. Eukaryotic chromosome can be divided into four major types based on the position of the centromere: metacentric, submetacentric, acrocentric, and telocentric.
  - Metacentric chromosomes are V-shaped and have centromeres in the center and forming two equal arms (1, 3, 16, 19, 20, X).
  - Submetacentric chromosomes are L-shaped and have centromere in the center and forming two equal arms (2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 17, 18).
  - Acrocentric chromosomes are J-like in shape, and have the centromere at one end thus giving a very short arm and exceptionally long arm (13, 14, 15, 21, 22, Y).
  - Telocentric chromosomes are rod-like in shape and have the centromere on the proximal end. Grouping and characteristics of chromosomes are given in Table 5.8.

Chromosome Structure

Functional chromosome contains the chromatids, centromere, secondary constrictions, nucleolar organizers, telomeres and satellites. The kinetophore is the multiprotein point of attachment for the spindle microtubules.

Chromatids

Chromosomes contain the genetic material of the cell. During metaphase after DNA replication, each chromosome is composed of two identical sister chromatids, which are held together by protein-mediated linkages. The chromatids are held together at a point along their length in the region of constriction of the chromosomes. The behavior of chromatids differs between mitotic and meiotic cells.

Centromere

Each chromosome contains one centromere, primary constriction, which is the specialized DNA sequence of a chromosomal locus that ensures delivery of one copy of each chromosome to each daughter cell during cell division. If there is no centromere, the cell cycle would not proceed.

- During mitosis or meiosis, spindle fibers attach to centromere via the kinetophore, the protein complex assembled at each centromere, which serves as the major point of contact between spindle microtubules and chromosomes.
- Kinetophores contain 50 different proteins, which take part in regulating and controlling their own interaction with spindle microtubules, verification of

Table 5.8 Grouping and characteristics of chromosomes

Group	Chromosomes	Characteristics	Location of Centromeres
Group A	1, 2, 3	Large metacentric chromosomes	Exactly in the middle of chromosome
Group B	4, 5	Large submetacentric chromosomes	Subterminal region dividing chromosome into short arm (p) and long arm (q)
Group C	6, 7, 8, 9, 10, 11,12 and X	Medium-sized submetacentric chromosomes	Subterminal region dividing chromosome into short arm (p) and long arm (q)
Group D	13,14,15	Large acrocentric chromosomes	Eccentrically located dividing chromosome in very short arm (p) and large long arm (q) capped by a telomere
Group E	16,17,18	Medium-sized acrocentric chromosomes	Eccentrically located dividing chromosome in very short arm (p) and large long arm (q) capped by a telomere
Group F	19, 20	Short metacentric chromosomes	Exactly in the middle of chromosome
Group G	21, 22 and Y	Short acrocentric chromosomes	Eccentrically located dividing chromosome in very short arm (p) and large long arm (q) capped by a telomere

1. French word petit meaning short is used to denote short arm of chromosome as 'p'.
2. Distal end of the chromosome is called telomere.
3. The centromere is a constriction at which the chromatids are joined.
4. Metacentric chromosomes (1, 3, 16, 19, 20, X), submetacentric chromosomes (2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 17, 18) and acrocentric chromosomes (13, 14, 15, 21, 22, Y).

anchoring, activation of the spindle checkpoint and generation of force to propel chromosome movement during cell division.

- Unattached kinetophores act as signal generator for mitotic checkpoint, which arrest mitosis until all kinetophores have correctly attached to spindle microtubules, thereby representing the cell cycle control mechanism protecting against loss of a chromosome (aneuploidy).
- In addition to the primary constriction or centromere, the arms of the chromosome may show one or more secondary constriction on the long arms of chromosomes 1, 10, 13, 16 and Y nucleolar organizers.

Telomeres

Telomeres are the stabilizing ends of a chromosome. Telomeres are tandem repeat DNA nucleotide sequences (TTAGGG) present at the tips of human chromosomes, that prevent their degradation or fusion with neighboring chromosomes, thus stabilize the genome and structural integrity during cell division.

- Telomeres progressively shorten throughout life. The greatest period of telomere shortening occurs during the first four years of life.
- Telomeres are crucial for the survival of cancer cells. Telomerase are maintained by an enzyme called telomerase in the vast majority of malignancies.

Euchromatin and Heterochromatin

Euchromatin and heterochromatin are two major categories of chromatin and heterochromatin.

- Euchromatin constitutes over 90% of human genome and has loose chromatin structure and active for transcription.
- Heterochromatin constitutes for 10% of human genome and has condensed chromatin structure and is inactive for transcription. There are main types of heterochromatin, which include constitutive and facultative heterochromatin.
  - Constitutive heterochromatin refers to the regions of DNA in the chromosome present throughout the cell cycle and does not code for proteins.
  - Facultative heterochromatin is the region of the DNA, in which the genes are silenced by modifications. Therefore, the genes are only activated under certain conditions and not present throughout the cell.
- Comparison of mitosis and meiosis is given in Table 5.9. Comparison of euchromatin and heterochromatin is given in Table 5.10.
- Comparison of constructive and facultative heterochromatin is given in Table 5.11.

Chromosome Banding Techniques

New banding techniques have been introduced for staining of chromosomes by which distinct patterns of stained bands and highly stained inter-bands become evident. These staining methods are important to identify the overall morphology of the chromosomes.

- **G-banding:** G-banding or Giemsa banding is a diagnostic technique used in cytogenetics to produce a visible karyotype by staining condensed chromosome. G-bands appear in the regions which are rich in sulfur-rich proteins. Giemsa-banding technique is employed in detection of genetic disorders through photographic representation of the entire chromosome complement. Giemsa-stained preparations are more permanent, and require ordinary microscopy optics and illumination.
- **Q-banding:** Introduction of quinacrine mustard has brought revolution in cytogenetics. Fluorochrome band technique using quinacrine produces a yellow fluorescence of different intensity (Q-bands) on exposure to the ultraviolet light.
  - The regions of chromosome rich in adenine and thymine get stained intensely.
  - The guanine-cytosine regions of chromosome remain unstained. These regions are called Q-bands.
  - Q-banding technique allows price identification of the different chromosome pairs and also the

Table 5.9 Comparison of mitosis and meiosis

Characteristics	Mitosis	Meiosis
Type of reproduction	Asexual reproduction (regeneration of the skin's epithelial cells)	Sexual reproduction (fertilization)
Number of cell divisions	Single cell division	Two-cell division
Chromosome pairing	Chromosome pairing absent	Chromosome pairing present
Resulting number of chromosomes	Diploid cells with the same number of chromosomes as the parent cell has	Haploid cells with half of the chromosomes that the parent cell has
Crossing over	No crossing over; homologous chromosomes remain	Crossing over occurs to provide genetic variation
Genetic information	Identical genetic information as the parent cells	Genetic differences when compared to the parent cells

Table 5.10 Comparison of euchromatin and heterochromatin

Characteristics	Euchromatin	Heterochromatin
Constitutes in human genome	Over 90% of human genome	10% of human genome
Location	Present near center of the nucleus	Present near periphery of nucleus
Packaging of DNA in the chromosomes	Loosely packed hence less condensed	Tightly packed hence more condensed
Staining pattern	Stains lighter due to the loose packaging (visible during cell division)	Stains darker due to the densely packed chromatin regions
DNA density	DNA density low	DNA density high
Genes	▪ Gene expression present ▪ Gene rich (higher in GC content)	▪ Gene expression absent (silenced genes-methylated) ▪ Gene poor (higher AT content)
Heteropycnosis (variable staining of chromosomes)	Variable staining of chromosomes absent	Variable staining of chromosomes present
Genetically	Active	Inactive
Tasks	Protecting of the integrity of gene to the handling or processes like regulation of gene	Transcription of the DNA of the DNA to the mRNA products
Transcription state (protein synthesis)	Transcriptionally inactive protein	Transcriptionally active protein

Table 5.11 Comparison of constructive and facultative heterochromatin

Characteristics	Constitutive Heterochromatin	Facultative Heterochromatin
Stability	Stable	Reversible
Constituents	Satellite DNA	Lines sequences
Polymorphism	Present	Absent
C bonds	Present	Absent

identification of structural chromosome rearrangements such as deletions, duplications, inversions (pericentric or paracentric), and aneuploidy (monosomy or trisomy).

- **C-banding:** C-banding is a diagnostic technique used in cytogenetics in which chromosomes are treated with strong alkaline sodium hydroxide followed by warm saline and then stained with Giemsa stain. C-bands are evident around the centromere and in other chromosomes that stain highly repetitive constitutive heterochromatin.
- **R-banding:** R-banding is a cytogenetic technique that produces the reverse of the G-band stain on chromosomes. Chromosomes on slides are incubated in phosphate buffer at high-temperature and stained with Giemsa stain. R-bands correspond to the regions on chromosomes having proteins lacking sulfur.

SEX CHROMOSOMES

A sex chromosome X or Y determines sex of person. Human beings have two sex chromosomes: X and Y chromosomes.

- Females have two XX chromosomes in their cells inherited from parents, while males have both X and Y chromosomes in their cells.
- Males inherit X chromosome from their mothers and Y chromosome from their fathers. The pseudoautosomal regions (**PAR1** and **PAR2**) of human X and Y chromosomes pair and recombine during meiosis. Therefore, genes in this region are not inherited in a strict sex-linked fashion.
- PAR1 region is located at the terminal region of short arms, and PAR2 region at the tips of the long arms of these chromosomes. Comparison of X and Y chromosomes in XX and YY genotypes is shown in Fig. 5.6 and Table 5.12.

X Chromosome

The X chromosome is five times bigger than Y chromosome, and contains 155 million nucleotide base pairs, 1000 genes, large amount of euchromatin and small amount of heterochromatin. SRY gene is absent in X chromosome.

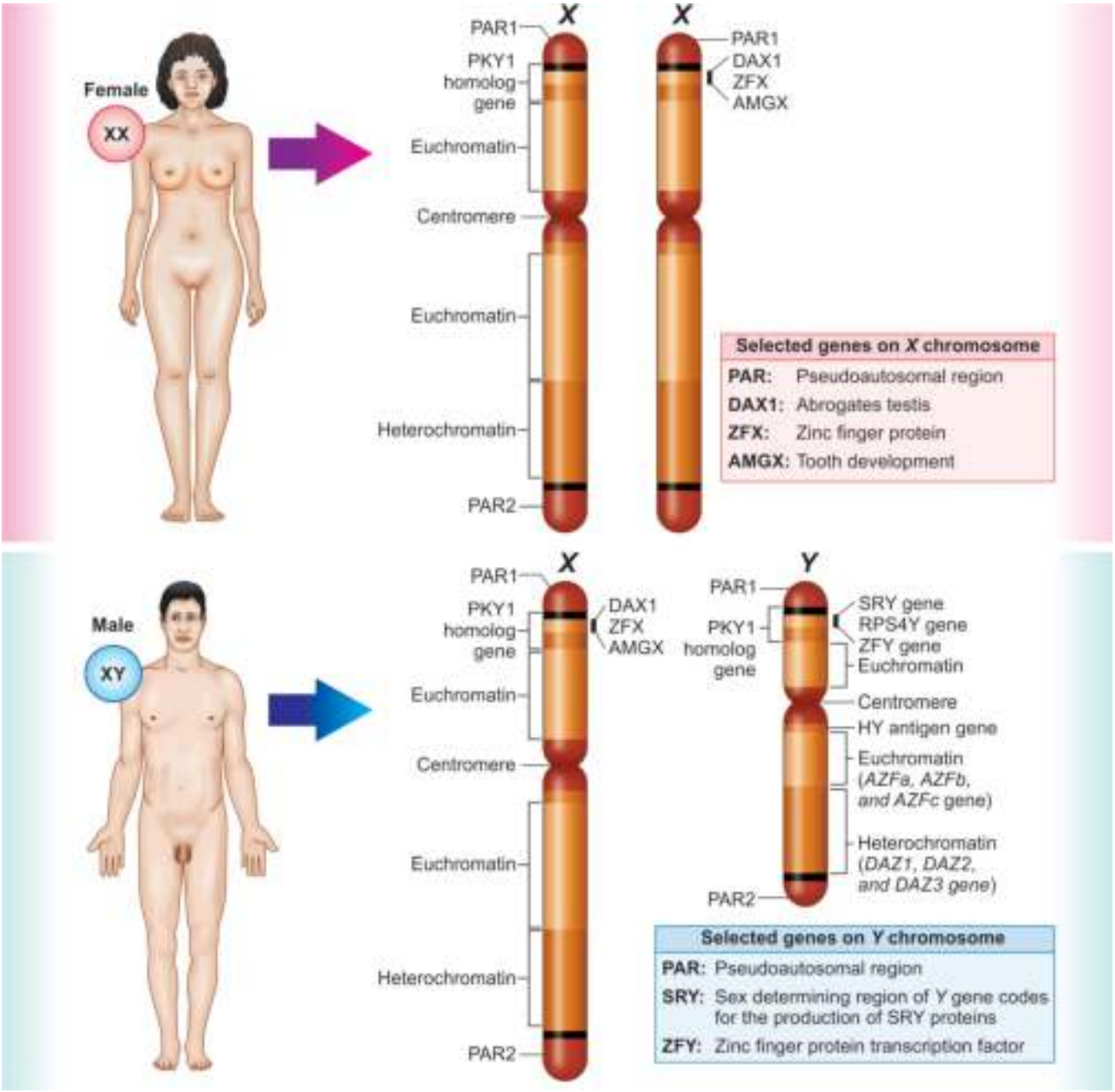


Fig. 5.6: Comparison of X and Y chromosomes in XX and YY genotypes.

- Genes on X chromosome give instructions to make protein. X chromosome represents 5% of the entire human genome. Each person normally has one pair of sex chromosomes in each cell.
- Early during embryonic development in females, one of the two X chromosomes is randomly and permanently inactivated in cells other than ovum. This phenomenon is called X-inactivation or lyonization.
- X chromosome ensures that females, like males, have one functional copy of the X chromosome in each cell. Some genes located at the ends of X chromosome escape inactivation, and such region on X chromosome is called pseudoautosomal region. Many genes located in pseudoautosomal region of X chromosome are essential for normal development.

Y Chromosome

Human Y chromosome has about 59 million nucleotide base pairs, i.e. the building blocks of DNA, which constitutes 2% of total DNA in male cell. Y chromosome contains 70 genes, which code for only

Table 5.12 Comparison of X and Y chromosomes in XX and YY genotypes

XX Genotype	XY Genotype
Female sex determination (XX genotype)	Male sex determination (XY genotype)
XX inherited from both parents	X inherited from mother
Large size chromosome	Small size chromosome
Genes (100) with 155 million nucleotide base pairs	Genes (70) with 59 million nucleotide base pairs
Constitutes 5% of human genome in females	Constitutes 1% of human genome in males
Pseudoautosomal regions (PAR1, PAR2) present	Pseudoautosomal regions (PAR1, PAR2) present
Sex determining region on Y (SRY) gene absent	Sex determining region on Y (SRY) gene present
ZFY (zinc finger protein-transcription factor) present	ZFY (zinc finger protein-transcription factor) present
PKX1 (protein kinase X-linked) gene present	PKX1 (protein kinase X-linked) gene present
Euchromatin large and heterochromatin small	Euchromatin small and heterochromatin large

1. X and Y chromosomes show several regions of homology in addition to pseudoautosomal regions (PAR1, PAR2). A crossover in PAR1 is essential in make meiosis to get proper segregation of chromosomes.
2.Relative few genes are functional on the Y chromosome, reflecting the limited somatic role of the Y chromosome, except for male-specific functions including spermatogenesis.
3. AZFa, AZFb and AZFc regions are deleted in azoospermia. The AZFc is located at the distal part of deletion interval 6 (subintervals 6C–6E) on the Y chromosome. While the AZFa and AZFb regions are essential in initiating spermatogenesis, the AZFc region is essential to complete the process of spermatogenesis.

Table 5.13 Genes on Y chromosomes involved in spermatogenesis

AMEY gene (codes for tooth enamel protein)
ANT3 gene (codes for adenine nucleotide translocase 3)
AZF1 gene (codes for azoospermia factor 1)
BRY2 (basic protein on the Y chromosome)
CSF2RA gene (codes for colony stimulating factor 2 receptor/ hematopoiesis)
DAZ1 gene (deleted in azoospermia resulting in male infertility)
DAZ2 gene (deleted in azoospermia resulting in male infertility)
IL-3RA gene (codes for interleukin 3 receptor)
MHC2 gene
PBDY gene spanning the pseudoautosomal boundary)
PRKY gene (codes for protein kinase, Y-linked)

Contd...

RBMY1A1 gene
SMCY putative gene for H-Y antigen
RPS4Y gene (codes for ribosomal protein subunit of low trans-criptional activity)
SRY gene (sex determining region)
STS gene (codes for steroid sulphatase)
TSPY gene (codes for testis-specific protein Y)
USP9Y gene
UTY (ubiquitously transcribed TPR gene on Y chromosome)
ZFY gene (codes for zinc finger protein-transcription factor)

Relative few genes are functional on the Y chromosome, reflecting the limited somatic role of the Y chromosome, except for male-specific functions including spermatogenesis.

23 proteins. Y chromosome is acrocentric and smaller than X chromosome.

- Y chromosome is unable to recombine with X chromosome, except for small pieces of pseudo-autosomal regions at the telomeres; which comprise about 5% of the chromosomes.
- The bulk of Y chromosome, which does not recombine is called 'NRY' or nonrecombining region of the Y chromosome. Y chromosome provides important roles in human males involving sex determination and spermatogenesis.
- Study of specific genes on Y chromosome important for spermatogenesis may allow the opportunity for new treatments for infertile men.
- Y chromosome provides important roles in human males involving sex determination and spermato-genesis. Study of specific genes on Y chromosome important for spermatogenesis may allow the opportunity for new treatments for infertile men. Genes on Y chromosomes involved in spermato-genesis are given in Table 5.13. Disorders of X and Y chromosomes are given in Table 5.14.

Table 5.14 Disorders of X and Y chromosomes

Disorders of X Chromosome
Paroxysmal nocturnal hemoglobinuria (PIG-A)
Menker's syndrome (MTP)
Alport's syndrome
Immunodeficiency with hyper-IgM
Adrenoleukodystrophy
Ocular albinism
Duchenne muscular dystrophy (DMD)
Retinitis pigmentosa
X-linked cleft palate
Fabry disease
Lesch-Nyhan syndrome
Hemophilia B
Hemophilia A (HEMA)
X-linked manic depressed illness
Color blindness
X-linked severe combined immunodeficiency (SCID)
Fragile X syndrome
Rett syndrome (MECP2)
Disorders of Y Chromosome
Azoospermia (AZFa, AZFb, AZFc gene deletion)
Microdeletion of Y-chromosome
Inborn errors of metabolism
Congenital adrenal hyperplasia
Alagille syndrome
α_1-Antitrypsin deficiency
Hereditary angioedema
Ataxia-telangiectasia

Contd...

THE NUCLEUS AND DNA REPLICATION

The nucleus is the small, round, largest and most prominent organelle in a cell, which consists of nuclear membrane, nucleoplasm, nucleolus and network of chromatin fibers packed in the form of chromosomes within DNA.

- Nucleus controls the cell, as it stores genetic instructors for manufacturing proteins. Muscle cells contain more than one nucleus.
- Mature red blood cells lack nuclei for making space for the large numbers of hemoglobin molecules. Without nuclei, the life span of red blood cells is short, and so the bone marrow must produce new ones constantly.
- Inside the nucleus lies the blueprint stored within DNA, that dictates cells to manufacture protein products. Every cell in the body except germ cells, contain the complete set of DNAs.
- When a cell undergoes division, the DNA must be duplicated so that each new cell receives a full complement of DNA.

ORGANIZATION OF THE NUCLEUS AND ITS DNA

Like most other cellular organelles, the nucleus is surrounded by a membrane called the nuclear membranous envelope made up of bilipid layer a thin fluid space in-between them. Spanning the nuclear membranes are nuclear pores.

- Nuclear pores allow small molecules (RNA, ribosomal proteins) and ions to freely pass, or diffuse, into or out of the nucleus; and also permit necessary proteins to enter the nucleus from the cytoplasm. If the proteins have special sequences that indicate that the proteins belong to the nucleoplasm. Inside the nuclear envelope, gel-like nucleoplasm is present, that contains solutes and building blocks of nucleic acids. A dark staining region in the center of nucleus is nucleolus most often visible under light microscope called a nucleolus.
- The nucleolus manufactures the components of ribosomes, which exit the cell's nucleus through the nuclear pores to the rest of the cell, where components of ribosome combine to form complete ribosome. Strands of DNA are wrapped around supporting histones. These proteins are increasingly bundled and condensed into chromatin, which is packed tightly into chromosomes when the cell is ready to divide. It is estimated that human beings have almost 22,000 genes distributed on 46 chromosomes. Structure of the nucleus is shown in Fig. 5.7.

DNA REPLICATION

When the cell divides, the two new daughter cells must contain the same genetic information (DNA), as the parent cell. The process of **duplication of DNA** is

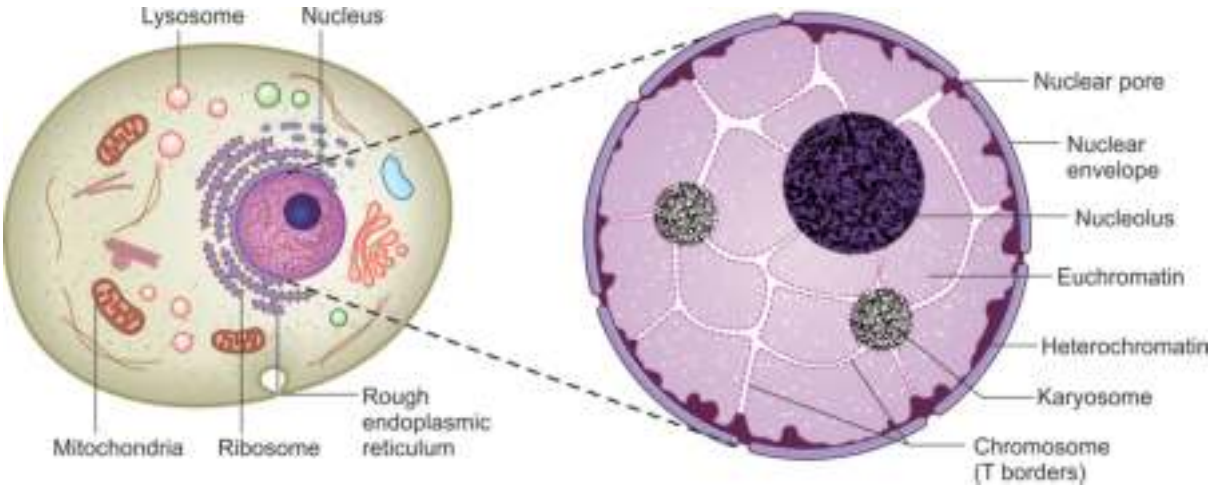


Fig. 5.7: Nuclear structure and organization. The nucleus is the control center of the cell. The nucleus of living cells contains the genetic material that determines the entire structure and function of that cell. The nuclear envelope surrounds the nucleus. Euchromatin is located near the center and heterochromatin is located near the periphery. Nuclear pores are specific portals of entry and exit to and from the nucleus. Transcription factors are regions where transcriptional complexes assemble with DNA and where transcription occurs. The nuclear matrix is a network of several proteins that add structure and support to the nucleus.

known as DNA replication, in which a double-stranded DNA molecule splits into its two single-strands during cell division. It is essential for cell division during growth, development and repair of damaged tissues.

- DNA replication is controlled by the Watson-Crick pairing of the nucleotide base pairs in the template strand with incoming deoxynucleoside triphosphates, and is directed by DNA polymerase enzymes. It is a complex process, particularly in eukaryotes, involving an array of enzymes such as helicase, gyrase, primase, DNA polymerase III, DNA polymerase I and ligase.
- Billions of new cells are produced in an adult human every day except permanent cells such as nerve cells, skeletal muscle and cardiac muscle. Cell division time of different cells varies. Three categories of cells are labile, stable and permanent cells.
  - Labile cells multiply continuously in various tissues such as epidermis, the mucosal layer of the internal hollow organs, bone marrow or seminiferous tubules of the testes.
  - Stable cells multiply only when needed. They spend most of the time in the quiescent G0 phase of the cell cycle and can be stimulated to enter the cell when required. Examples of stable cells include the liver, the proximal tubules of the kidney and endocrine cells.
  - Permanent cells are incapable of regeneration. Stable cells are considered to be terminally differentiated and hence do not proliferate in postnatal life such as neurons, cardiac muscle fibers, skeletal muscle fibers.
- Double-stranded DNA molecule take the shape of a long-twisted ladder. Each molecule of DNA is a double helix formed from two complementary strands of four types of nucleotide subunits help together by hydrogen bonds between adenine–thymine (A–T) and guanine–cytosine (G–C) base pairs with a 'sugar-phosphate backbone'. Adenine always binds with thymine, and cytosine always binds with guanine. The particular sequence of the nucleotide base pairs along DNA molecule

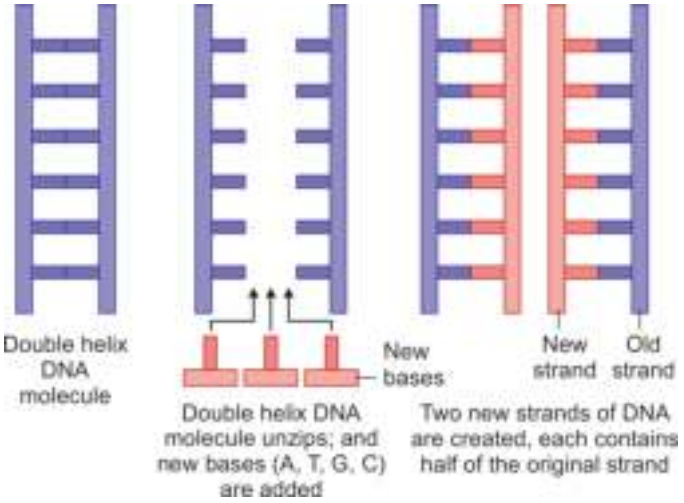
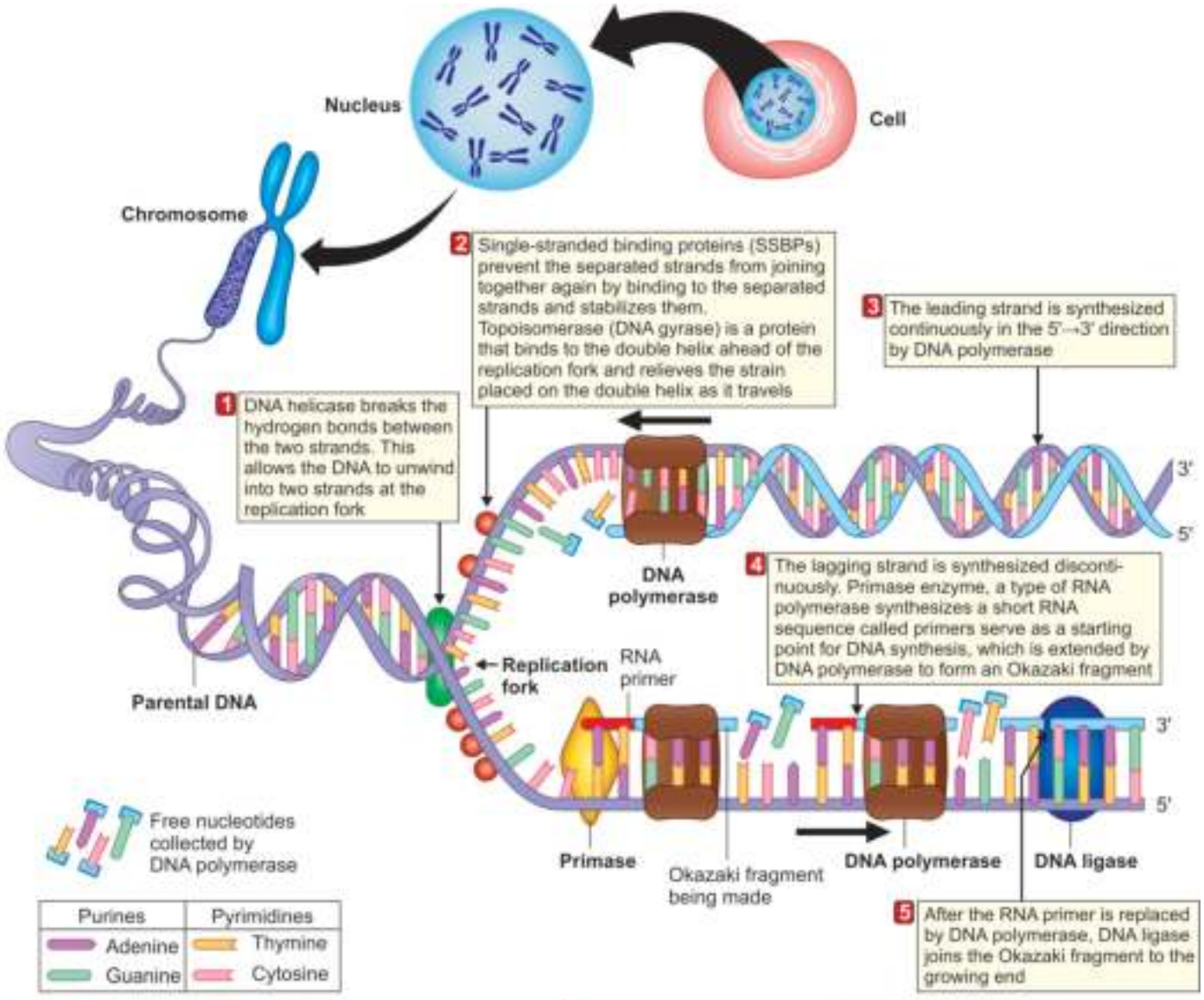


Fig. 5.8: DNA replication process creating of two new strands. DNA replication is semiconservative process, in which each strand in the DNA double helix acts as a template for the synthesis of a new, complementary DNA strand. In this process, newly formed double helix contains one new and one old strand. The sequence of the bases encodes genetic information. The three steps in this process of DNA replication are initiation, elongation and termination.



DNA replication

- Each of the nitrogenous bases can only pair with its partner (A—T and G—C). A purine (adenine and guanine) must base pair with a pyrimidine (cytosine and thymine).
- Each new strand contains one original and one new strand; therefore, DNA replication is said to be semiconservative and depends on complementary base pairing.
- Two new strands formed will be identical.

Free nucleotides

- Free nucleotides (deoxynucleotide triphosphates) are formed in the ribosomes of a cell, that make up the DNA and RNA.
- Four different types of DNA nucleotides include: adenine (A), thymine (T), guanine (G) and cytosine (C).
- Free nucleotides are collected by DNA polymerase and attached to the new strand by complementary base pairing.
- The extra phosphate groups carry energy, which is used for the formation of covalent bonds.

DNA polymerase

1. DNA polymerase 3 is essential for DNA replication of the leading and the lagging strands. DNA polymerase 1 is essential for removing of the RNA primers from the fragments and replacing it with the required nucleotides. These enzymes cannot replace each other as both have different functions to be performed.
2. DNA polymerase moves in 5'→3' direction.
3. DNA polymerase moves in opposite directions on each strand.
4. DNA polymerase links nucleotides together to form a new strand, using the pre-existing strand as a template.
5. DNA polymerase catalyzes the covalent phosphodiester bonds between sugars and phosphate groups.
6. DNA polymerase proof reads the complementary base pairing.
7. DNA polymerase uses 3'→5' exonuclease activity to remove the incorrectly inserted nucleotides from the 3' end of the new strand during polymerization.

Fig. 5.9: DNA replication process creating of two new strands. DNA replication duplicates the entire genome of the cell by number of DNA enzymes.

determines the DNA's instructions or genetic code. Duplication of the genetic information occurs by the use of one DNA strand as a template for the formation of a complementary strand. If one strand of DNA molecule has a region with the sequence AGTGCCT, then the sequence of the complementary strand would be TCACGGA.

- DNA is packed into tightly coiled structure called chromatin, which loosens prior to DNA replication, allowing the cell replication machinery to access the DNA strands.
- DNA replication process creating of two new strands is shown in Figs 5.8 and 5.9. Enzymes involved in DNA replication are given in Table 5.15.

Table 5.15 Enzymes involved in DNA replication

Enzyme in DNA Replication	Function
DNA helicases	▪ DNA helicase proteins bind to the double-stranded DNA and unwinds its strands by breaking hydrogen bonds down the center of strands ▪ DNA single-stranded binding proteins bind to the DNA as a tetramer and stabilize the single-stranded structure that is generated by the action of the helicases ▪ DNA replication is 100 times faster when these proteins are attached to the single-stranded DNA
DNA gyrase (also called topoisomerase)	▪ DNA gyrase relieves the buildup of torque (tension due to supercoiling of DNA) during unwinding and prevents DNA breakage ▪ DNA gyrase enzyme catalyzes the formation of negative supercoils and is thought to aid with the unwinding process ▪ There are two types of DNA strands: leading and lagging strands. • Leading strand or template strand (sense strand or also called coding strand): the strand which supports the continuous DNA synthesis is called as leading strand • Lagging strand or noncoding strand (antisense strand) is replicated in a discontinuous manner in short stretches (also known as 'Okazaki fragments')
RNA primase	▪ A free 3'-OH group is required for DNA replication ▪ RNA primase helps in synthesis of a free 3'-hydroxyl group at the initiation sites by laying down RNA primers
DNA polymerase III	DNA polymerase III is main enzymes involved in DNA synthesis with 3'→5' exonuclease activity (proofreading activity), that participates in proofreading and allows the incorrect base pair to be excised
DNA polymerase I	DNA polymerase I replaces RNA primers with DNA and repairs and damage with DNA; and also serves to connect Okazaki fragments by deleting RNA primers and replacing the strand with DNA
DNA ligase	▪ DNA ligase facilitates the sealing (joining) of DNA strands together by catalyzing the formation of a covalent phosphodiester linkage between 3'-hydroxyl and 5'-phosphate groups. and fills in the gap ▪ RNA editing may also take place before translation begins ▪ Genetic message is carried by mRNA moves out of nucleus into cytoplasm for translation and converts into polypeptide sequence of amino acids
Telomerase	Lengthens telomeric DNA by adding repetitive nucleotide sequences to the ends of chromosomes. This allows germ cells and stem cells to avoid Hayflick limit on cell division

1. Single-stranded binding protein (SSBP) stabilizes the separated DNA strands and prevent their re-association, so that each strand can serve as a template for new DNA synthesis.

2. Purified DNA ligase is used in gene cloning to join DNA molecules together to form recombinant DNA.

3. The Hayflick limit concept states that a normal human cell can only replicate and divide 40–60 times before it cannot divide anymore and will break down by apoptosis.

Pathology Pearls: DNA Replication Process

- DNA replication is the process by which DNA makes a copy of itself during cell division. During DNA replication, double helix structure of the DNA is unzipped by helicase enzyme, which breaks the hydrogen bonds holding the complementary bases of DNA together (adenine with thymine, cytosine with guanine). The separation of the two strands of DNA creates a 'Y' shape called a DNA replication fork.
- One of the strands of DNA molecule is oriented in the 3'→ 5' direction (towards the replication fork) called 'leading strand'. The other strand is oriented in the 5'→ 3' direction (away from the replication fork) called 'lagging strand'. As a result of their different orientations, the two strands (i.e. leading and lagging strands) are replicated differently.
- DNA polymerase binds to leading strand and then walks along it, adding new complementary nucleotide bases (adenine, cytosine, guanine and thymine) to the strand of DNA in 5'→ 3' direction. This sort of DNA replication is known as continuous.
- Primase enzyme also synthesize numerous RNA primers at various points along the lagging strand. The primer acts as the starting point for DNA synthesis. Chunks of DNA, called Okazaki fragments, are then added to the lagging strand also in 5'→ 3' direction. Once all of the nucleotide base pairs are matched (A with T, C with G), exonuclease enzyme strips away the primer(s). These gaps created by stripping away of primer(s) are then filled by more complementary nucleotides. Proofreading of new strand ensures no mistake in the new DNA sequence.
- Finally, DNA ligase enzyme seals up the sequence of DNA into tow continuous double-strands. The result of DNA replication is two DNA molecules consisting of one new and one old chain of nucleotides.
- That is the reason that DNA is described as semiconservative, half of the chain is part of this original DNA molecule and half is a brand new. Following DNA replication, the new DNA automatically winds up into double helix.

DNA REPLICATION: PHASES

In order to fit within a cell's nucleus. DNA is packed into tightly coiled structures called chromatin, which loosens prior to DNA replication machinery to access the DNA strands.

- DNA replication faithfully duplicates the entire genome of the cell. During DNA replication, a number of **different enzymes** work together to pull apart the two DNA strands, so each DNA strand can be used as a template to synthesize new complementary strands.
- The two new daughter DNA strands are formed, each contains one pre-existing strand and one newly synthesized strand. Thus, DNA replication is said to be "**semiconservative**". Three main steps in DNA replication include initiation, elongation and termination.

DNA Replication: Initiation Phase

The two complementary strands of DNA molecule are separated, much like unzipping a zipper.

- DNA helicase uses ATP to break hydrogen bonds between complementary nucleotide base pairs resulting in separation of two polynucleotide strands of DNA. The separation of the two single-strands of DNA creates a 'Y' shape called a replication fork.
- Single-stranded binding protein (**SSBP**) stabilizes the separated DNA strands and prevent their re-association, so that each DNA strand can serve as a template for new DNA synthesis. SSBP also fasten DNA replication 100 times.
- The stress produced by supercoiling of double helix DNA is released by DNA gyrase (also called topoisomerases) resulting in prevention of DNA breakage. During DNA replication, the leading strand is synthesized continuously in the 5′→ 3′ direction by DNA polymerase.
- DNA polymerase catalyzes the covalent phosphodiester bonds between sugars and phosphate groups. DNA polymerase links free nucleotides (i.e. deoxynucleoside triphosphates) and proofreading of complementary base pairing to new DNA strand, consequently mistakes are very infrequent occurring approximately once in every billion pairs. The lagging DNA strand is synthesized discontinuously by forming short segments known as Okazaki fragments.

DNA Replication: Elongation Phase

During elongation of DNA replication, the leading DNA strand is made continuously in the 5′→ 3′ direction, while the lagging DNA strand is made in pieces called Okazaki fragments.

- Okazaki fragments prevent elongation in the 3′→5′ direction. Each DNA strand becomes a template along which a new complementary strand is built. DNA polymerase catalyzes the elongation of DNA strand in the 5′→ 3′ and brings in the correct nucleotide base pairs to complement the template DNA strand, synthesizing a new strand base by base.
- DNA polymerase is an enzyme that adds free nucleotides to the end of a chain of DNA, making a new double-strand. This growing DNA strand continues to be built until it has fully complemented the template strand.

DNA Replication: Termination Phase

Termination of DNA replication occurs when replication forks converge on the same stretch of DNA, after DNA polymerase completes transcription of sequences

in DNA and gaps of backbone of double-stranded DNA are filled in and ligated and catenanes (**interlocked compounds**) are removed.

- DNA ligase repairs irregularities or breaks in the double-stranded DNA molecules, seals recombination fragments and connects Okazaki fragments (small DNA fragments formed during replication of double-stranded DNA).
- DNA replication is stopped with the creation of two new identical DNA molecules. Each new DNA molecule contains one strand from the original molecule and one newly synthesized strand. Thus, **DNA replication** is said to be 'semiconservative', because half of the original DNA molecule is conserved in each new DNA molecule. DNA replication takes place precisely, so that new cells in the body contain exact same material as their parent cells.
- Errors made during DNA replication, such as accidental addition of inappropriate nucleotide base pairs and formation of mismatch nucleotide base pairs resulting in formation of dysfunctional gene and genetic disease.

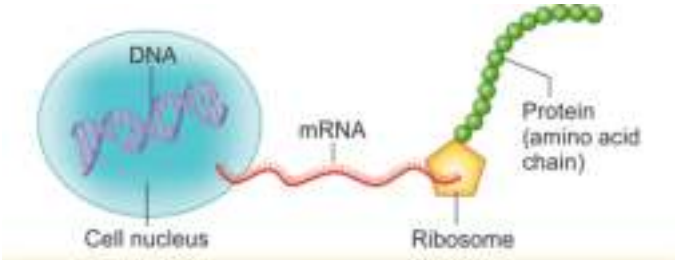
GENE EXPRESSION, TRANSCRIPTION, TRANSLATION AND PROTEIN FOLDING

DNA contains the information to synthesize proteins essential for the cell structure and function. Protein is the basic component of living cells, that is made up of carbon, hydrogen, oxygen, nitrogen and one or more chains of amino acids.

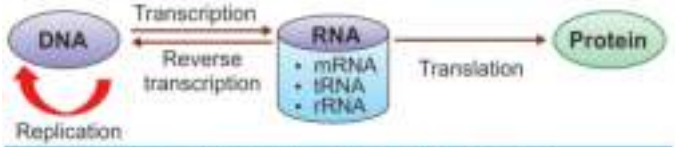
- DNA replication is the process by which a double-stranded DNA molecule is copied to produce two identical DNA molecules during cell division. Two new daughter cells must contain the same genetic information (DNA) as the parent cell. A gene is expressed through the processes if transcription and translation.
- During transcription, the enzyme RNA polymerase uses DNA as a template to produce a pre-messenger RNA (pre-mRNA). The pre-messenger RNA is processed to form a mature messenger RNA (mRNA) molecule, which leaves the nucleus and attaches to ribosome and translates to produce polypeptide chains of amino acids and then protein molecule by the original gene.
  - Transcription is the process of making an RNA copy of a gene sequence called primary RNA transcript that gets a cap added to the 5′ end and undergoes polyadenylation and splicing to produce messenger RNA (mRNA).
  - The mature messenger RNA leaves the cell nucleus and enters the cytoplasm, where it directs the synthesis of the protein molecules, which it encodes.
  - Transcription involves ribosomal RNA (rRNA), transfer RNA (tRNA), messenger RNA (mRNA), and various enzymes.
  - Ribosomal RNA (rRNA) constitutes 60% of ribosome, that is used to manufacture ribosomes.
  - Messenger RNA (**mRNA**) carries DNA sequence information to ribosomes in the cytoplasm. Transfer RNA (**tRNA**), the smallest structure decodes information and carries amino acids to the ribosomes; and hence provides linkage between mRNA and amino acids' transfer amino acids to ribosomes.
- Proteins provide structure and support to the cells and transport hormones. One of the most important classes of proteins is enzymes, which accelerate necessary biochemical reaction that take place inside the cell. Most genes contain the information required to construct functional molecules are called proteins. A few genes make other molecules that help the cell to assemble proteins. The journey from gene to protein synthesis is tightly controlled within each cell.
- Together, transcription and translation are known as gene expression. Protein synthesis takes place in cellular structures is called ribosome, found outside the nucleus. DNA alone cannot account for the expression of genes. RNA is needed to help out the instructions in DNA. The process by which genetic information is transferred from the nucleus to the ribosomes is called transcription.
  - DNA alone cannot account for expression of genes. RNA is needed to help in carrying out of the instructions in DNA. Like DNA, RNA is made up of nucleotide consisting of a 5′-carbon ribose, a phosphate group and a nitrogen base. Three main differences between DNA and RNA include: (a) RNA is generally single-stranded instead of double-stranded, (b) RNA uses the sugar ribose instead of deoxyribose, and (c) RNA contains uracil in place of thymine.

- The sequence of four nucleotide base pairs in a gene (purines: adenine–guanine and pyrimidines: cytosine–thymine) covalently linked to a phosphodiester backbone, translates to an amino acid sequence.
- There are 1200 nucleotide base pairs present in the double-stranded DNA. Three nucleotides are called a triplet or codon that codes for one particular amino acid in the protein. The nucleotide sequence in the DNA transcribed into a molecule of messenger RNA (ribonucleic acid). The mechanism by which cells turn the DNA to code for a protein product, which is a two-step process, with an RNA molecule and the intermediate product.

- Protein synthesis in human involving DNA replication, transcription and translation is shown in Fig. 5.10. Transcription and translation are shown in Figs 5.11 and 5.12. Reverse transcription of DNA from RNA is shown in Fig. 5.13. Comparison of DNA replication and gene transcription is given in Table 5.16. Comparison of transcription and translation in human cell is given in Table 5.17.



Gene expression: DNA replication, transcription and translation



Gene expression (DNA→RNA→Protein)	
• DNA replication	DNA→DNA copy occurs in nucleus.
• Transcription	DNA→mRNA occurs in nucleus, then mRNA moves on to ribosomes into the cytoplasm for translation.
• Translation	Protein synthesis occurs in cytoplasm (mRNA transcript is decoded into protein with the help of a ribosome and tRNA).
Human genome contains around 30,000 genes, each gene codes for one protein.	

Fig. 5.10: Protein synthesis in human involving DNA replication, transcription and translation. Human genome contains around 30,000 genes, each gene codes for one protein. The process by which DNA is copied to RNA is called transcription and that by which RNA is used to produce proteins is called translation.

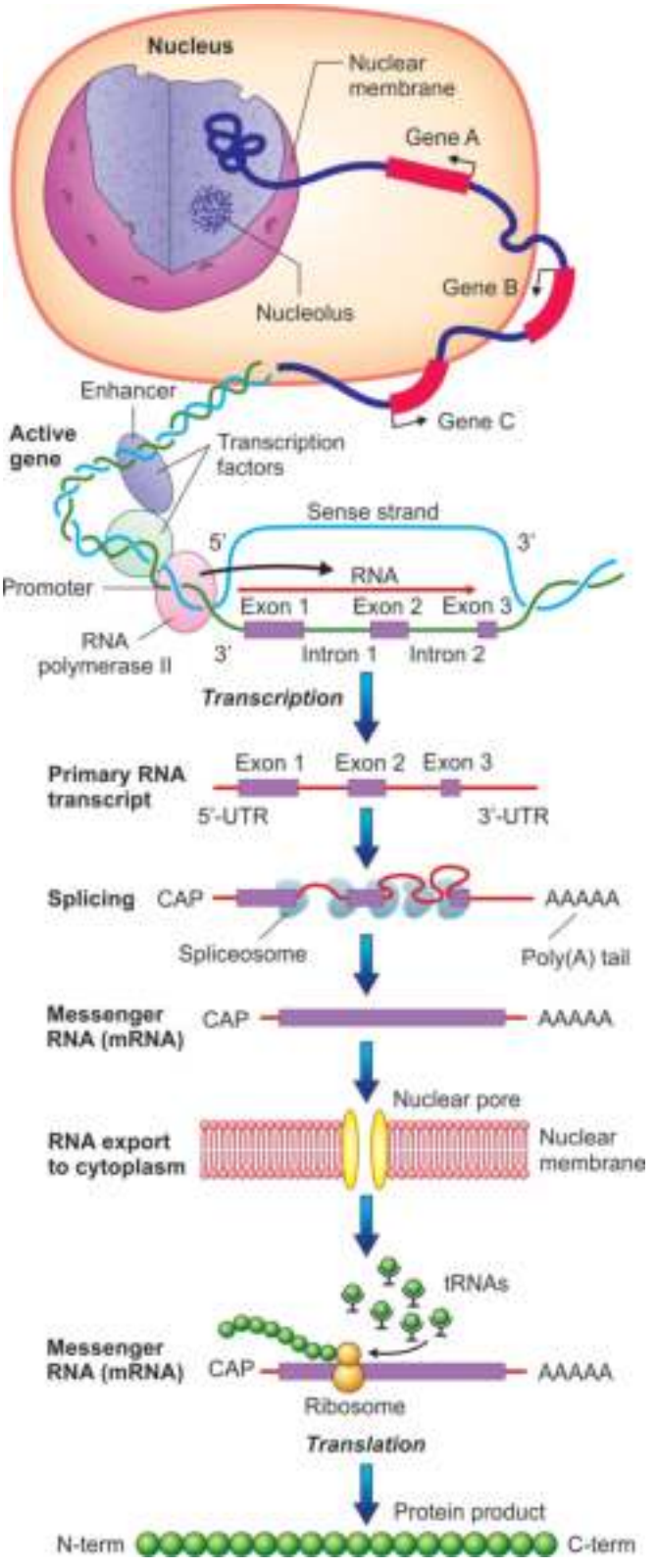


Fig. 5.11: Transcription and translation (RNA synthesis and its translation into protein in the cytosol). Transcription process yields a primary RNA transcript that gets a cap added to the 5' end and undergoes polyadenylation and splicing to produce messenger RNA (mRNA). Following transport of mRNA to the cytoplasm, mRNA is translated in the cytosol to protein molecules.

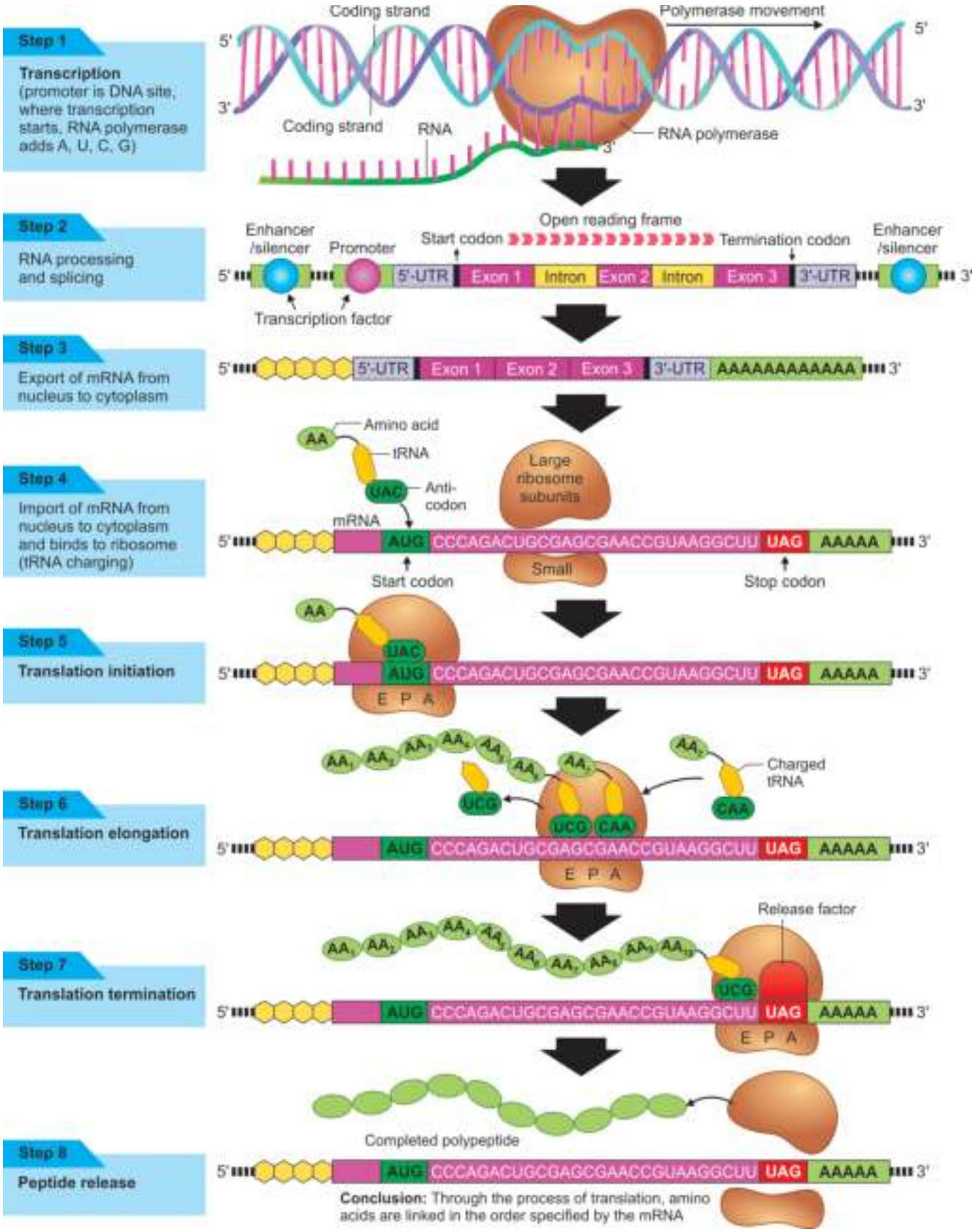


Fig. 5.12: Transcription and translation. Schematic representation demonstrates the process by which DNA is copied to RNA is called transcription, and that by which RNA is used to produce proteins is called translation.

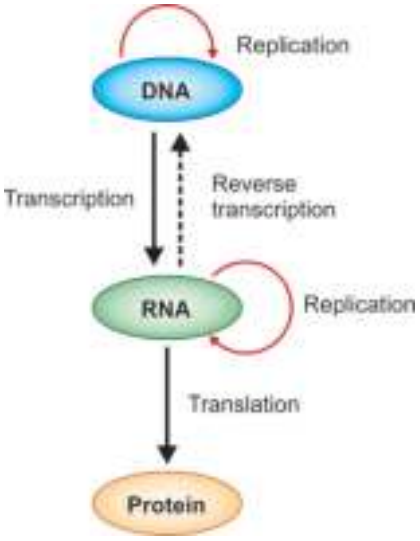


Fig. 5.13: Reverse transcription of DNA from RNA. A reverse transcriptase is an enzyme present in RNA virus, used to generate complementary DNA (cDNA) from an RNA template, a process is known as reverse transcription. DNA transcript of the RNA is incorporated into the genome of the cell, possibly altering permanently its behavior and potentially leading to tumor formation. Retroviral reverse transcriptase has three sequential biochemical activities: RNA-dependent DNA polymerase activity, ribonuclease H (RNase-H), and DNA-dependent RNA polymerase activity.

Pathology Pearls: Reverse Transcription Polymerase Chain Reaction (RT-PCR)

- Reverse transcription polymerase chain reaction (RT-PCR) is a technique to generate a complementary strand of DNA (cDNA) from single-stranded ribonucleic acid (RNA) template by transcriptase enzyme. Process involves three steps: denaturation, annealing and elongation.
- Denaturation is done by heating the DNA sample to separate it into two strands.
- The process permits exponential amplification of a desired sequence by using DNA primers that anneal to the sequence of interest. A heat-tolerant DNA polymerase (Taq) copies the strands results in elongation. Real-time or quantity, polymerase chain reaction detects the PCR amplification during their synthesis, and is more sensitive and quantitative than conventional PCR.
- The combination of reverse transcription polymerase chain reaction (RT-PCR) allows the detection of low abundance RNAs in a sample blood, semen, tissue, hair on crime scene and production of the corresponding complementary DNA (cDNA), thereby facilitating the cloning of low copy genes. One cycle of PCR yields two identical copies of the DNA sequence. A standard reaction of 30 cycles would yield 1,073,741,826 copies of DNA.

Table 5.16 Comparison of DNA replication and gene transcription

Characteristics	DNA Replication	Transcription
Function	▪ Making of DNA copy for a 'daughter cell' ▪ Synthesis of an identical DNA copy	▪ Making of RNA copy of a gene, copy of gene is called messenger RNA (mRNA) ▪ Synthesis of RNA using DNA as a template
Enzymes	DNA helicase, DNA polymerase, DNA ligase	DNA helicase, DNA polymerase
Nucleotides	dUMP, dCMP, dAMP, dGMP	UMP, CMP, AMP, GMP
Occurrence (happens in cell cycle phase)	▪ S phase of cell cycle ▪ Along both leading and lagging DNA strands ▪ Preparation for cell division	▪ G1 and G2 phase of cell cycle ▪ Along one template DNA strand ▪ Preparation for gene expression
Bonding	Copied DNA bonded to template	Copied RNA dissociates from DNA template
Requiring primer or not	RNA primer required	RNA primer not required
Products	▪ Deoxyribonucleic acid (DNA) molecules ▪ Double-strands bonded to original strands ▪ Stays within nucleus ▪ Product not degraded	▪ Ribonucleic acid (RNA) molecules ▪ mRNA exported to the cytoplasm ▪ mRNA exported to the cytoplasm ▪ Product degraded after performing work
Copying	Entire genome (all chromosomes)	Individual gene only
Unwinds or unzips	Entire DNA molecule	Only DNA with gene to be transcribed
Post-copy processing	DNA ligase joins Okazaki fragments	RNA 'editing/processing' splicing of noncoding 'introns' and joining of coding sequence 'exons'

Table 5.17 Comparison of transcription and translation in human cell

Characteristics	Transcription Process	Translation Process
Step of gene expression process	First step of gene expression process involving synthesis of RNA from DNA	Second step of gene expression process involving synthesis of proteins from RNA, which are copied from genes
Components	DNA, RNA polymerase core enzyme	mRNA, small and large ribosomal subunits, initiation factors, elongation factors, tRNA
Template	DNA (genes in human gene)	mRNA (messenger RNA)
Enzymes	RNA polymerase	Ribosome enzyme that contains ribosomal RNA (rRNA) and proteins
End product	RNA (production of several functional forms of RNA)	Protein
Location	Template is the genes in the nucleus	Template is the messenger RNA on rough endoplasmic reticulum in cytoplasm
Controlling factor	RNA polymerase	Ribosomes and ribosome complex
Production	Several functional forms of RNAs	Production of proteins
Main events in each stage of transcription and translation	▪ **Initiation:** Initially, RNA polymerase binds to the promoter region of DNA, DNA is denatured. Complementary ribonucleosides are inserted, joined by phosphodiester bonds ▪ **Elongation:** The RNA strand begins to separate from the DNA template strand ▪ **Termination:** A termination sequence signals the RNA to form a hairpin secondary structure, before dissociating from the DNA	▪ **Initiation:** Small and large ribosomal subunits and tRNA attach to mRNA ▪ **Elongation:** Two additional charged tRNA molecules attach to the mRNA; their amino acids bind and the next tRNA attaches ▪ **Termination:** GTP-dependent release factors lease the last amino acids in the polypeptide chain from the tRNA, before the tRNA is released

Salient Features: Transcription, Translation and Protein Folding

Transcription Process

- DNA in nucleus serves as a template for transcription. Gene transcription involves binding of RNA polymerase II to the promoter of genes being transcribed with other proteins (transcriptional factors) that regulate the transcriptional rate. Messenger RNA (mRNA) is processed before leaving the nucleus.
- The pre-messenger RNA (primary RNA transcript) is a copy of the whole gene and includes introns and exons. In the nucleus, a structure is called a spliceosome cuts out introns (noncoding regions) with in pre-mRNA transcript and reconnects the exons to form the mature messenger RNA (mRNA).
- Prior to its exit from the nucleus, a methylated guanosome nucleotide is added to the 5' end of the RNA (cap) and a string of adenine nucleotides are added to the 3' (poly(A) tail). This process protects the mRNA from degradation and facilitates transport into the cytoplasm.

Translation Process

- During translation, the mRNA transcript is "read" by a functional complex consisting of the ribosome and tRNA molecules. tRNAs bring the appropriate amino acids in sequence to the growing polypeptide chain by matching their anti-codons with codons on the mRNA strand.
- Messenger RNA (**mRNA**) moves into cytoplasm and becomes associated with ribosomes and the ribosome moves down the mRNA reading the sequence of nitrogen bases.
- Messenger RNA (mRNA) carries message from DNA to ribosomes in cytoplasm. Triplets (3) of nucleotides on the mRNA constitute a **codon sequence** that codes for a protein.
- Transfer RNA (**tRNA**) is involved in the next stage of protein synthesis, i.e. translation.
- AUG codon on messenger RNA (mRNA) is the **initiator codon**. AUG binds to the **P site** of the ribosome. AUG helps align the tRNA, the mRNA, and the ribosome and then signals the beginning of the gene to be expressed.
- Transfer RNA (**tRNA**) carry amino acids to mRNA. Each tRNA has an **anticodon** that binds to a complementary **codon** on the mRNA. Anticodons codon-codon complementary base pairing occurs.
- The 2nd transfer RNA (**tRNA**) molecule binds to the 2nd codon of the mRNA and a di-peptide is formed. The 1st transfer RNA (**tRNA**) is released from the E site of the ribosome.
- Translation process continues until a nonsense 'stop codon' (UAA, UGA, UAG) tells the ribosome that protein synthesis is complete.
- The **codons** from mRNA have now been translated into polypeptide, i.e. one amino acid at a time.
- The mRNA, polypeptide, tRNA, and ribosome disassemble. Polypeptide enters its lumen, where the polypeptide folds and is modified further.

Protein Folding in Endoplasmic Reticulum

- After translation, next step is folding of polypeptide in the endoplasmic reticulum.
- Signal recognition particle binds to signal peptide on the endoplasmic reticulum.
- Signal recognition particle (SRP) attaches to receptor; translocation channel opens; polypeptide enters into lumen of endoplasmic reticulum.
- A signal peptidase removes the signal peptide. Ribosome units and mRNA break away. Protein folds into final shape.

CENTRAL DOGMA OF BIOLOGY

A gene that encodes a polypeptide is expressed in two steps. In this process, information flows from DNA → RNA → protein, a directional relationship is known as the **central dogma of molecular biology**.

Concept: Central Dogma of Molecular Biology

- Central dogma describes the flow of genetic information from DNA → RNA → proteins
- One gene: one polypeptide
- Transcription: DNA → RNA
- Translation: RNA → protein

GENETIC CODE

Genetic code is the term used to the four nucleotide bases of DNA—adenine (A), cytosine (C), guanine (G), and uracil (U) are strung together in the cell and the ribosome can read them and turn them to potein. In the genetic code, three nucleotides present in a row are called triplets or codons that encodes single amino acid. Schematic representation of genetic code is shown in Fig. 5.14.

- The first step in decoding genetic information is transcription, during which a nucleotide sequence is copied from DNA to RNA. Second step is to join amino acids together to form a protein. The order in which amino acids are joined determine the properties, shape and function of a protein.
- Most of the amino acids are encoded by more than one codon. The code is based on sets of DNA bases and universal from bacteria to human. There are 64 possible base triplets and only 20 amino acids.
- Out of 64 codons, 61 codons code for amino acids, while 3 codons do not code for any amino acids;

Genetic Code

First base of codon	Second base of codon: U	C	A	G	Third base of codon
U	UUU, UUC: Phenylalanine	UCU, UCC, UCA, UCG: Serine	UAU, UAC: Tyrosine	UGU, UGC: Cysteine	U, C, A, G
	UUA, UUG: Leucine		UAA, UAG: Stop codon	UGA: Stop codon; UGG: Tryptophan	
C	CUU, CUC, CUA, CUG: Leucine	CCU, CCC, CCA, CCG: Proline	CAU, CAC: Histidine; CAA, CAG: Glutamine	CGU, CGC, CGA, CGG: Arginine	U, C, A, G
A	AUU, AUC, AUA: Isoleucine	ACU, ACC, ACA, ACG: Threonine	AAU, AAC: Asparagine; AAA, AAG: Lysine	AGU, AGC: Serine; AGA, AGG: Arginine	U, C, A, G
	AUG: Methionine; start codon				
G	GUU, GUC, GUA, GUG: Valine	GCU, GCC, GCA, GCG: Alanine	GAU, GAC: Aspartic acid; GAA, GAG: Glutamic acid	GGU, GGC, GGA, GGG: Glycine	U, C, A, G

Amino acids

- *Alanine*
- *Arginine*
- *Asparagine*
- *Aspartate*
- *Cysteine*
- *Glutamine*
- *Glutamate*
- *Glycine*
- *Histidine*
- *Isoleucine*
- *Leucine*
- *Lysine*
- *Methionine*
- *Phenylalanine*
- *Proline*
- *Serine*
- *Threonine*
- *Tryptophan*
- *Tyrosine*
- *Valine*

Genetic code feature

- Genetic code codes for 20 amino acids found in proteins.
- Amino acids are organic compounds composed of nitrogen, carbon, hydrogen and oxygen, along with other elements in side chain.
- Essential amino acids cannot be made by the body. As a result, they must be derived from food. The 9 essential amino acids are: *histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine.*
- Codon has 3 nucleotide bases; and 43 combinations of 64 codons.
- Codon properties: Codon contains signals, **start codon** (AUG) and **stop codon** (UAA, UGA, UAG).

Fig. 5.14: Schematic representation of genetic code. DNA holds all of the genetic information necessary to build a cell's proteins. The nucleotide sequence of a gene is ultimately translated into an amino acid sequence of the gene's corresponding protein.

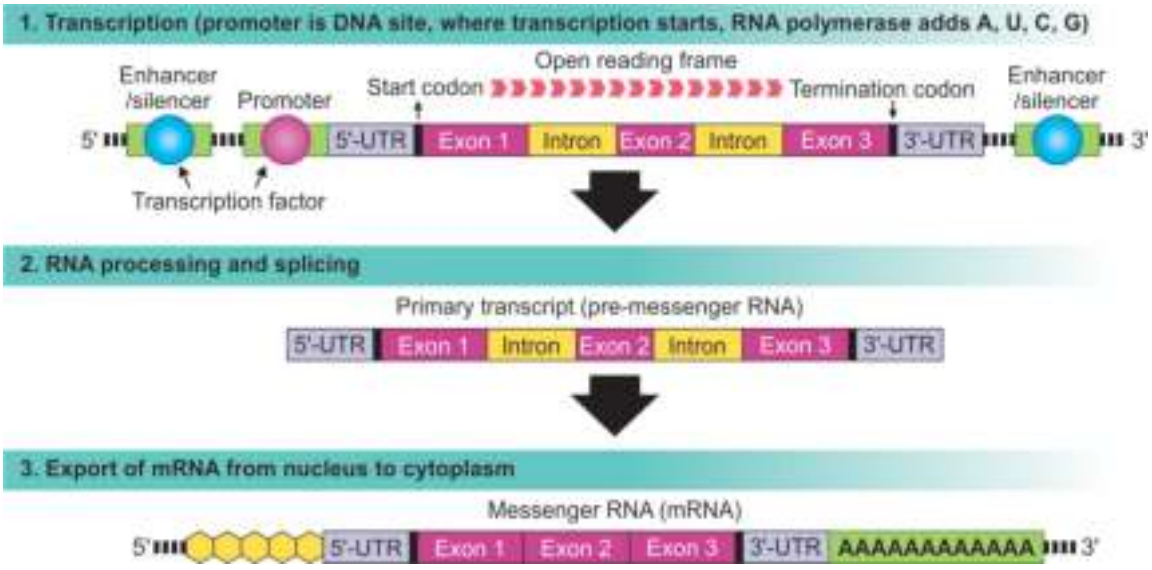


Fig. 5.15: Schematic representation of transcription. Transcription is the process by which the information in DNA strand is copied into a new molecule called messenger RNA, that leaves the cell nucleus and enters the cytoplasm and then serve as blueprints for protein synthesis during the process of translation (*see* Fig. 15.17).

and they function as '**stop codons**' and designate the termination of translation. Each codon codes for one of the 20 amino acids used in the synthesis of proteins, hence, it is specific.

TRANSCRIPTION

Transcription is the first step in gene expression. During this process, the DNA sequence of a gene is copied (transcribed) to make an RNA molecule in the nucleus. RNA polymerase is the main transcription enzyme. Schematic representation of transcription is shown in Fig. 5.15.

- DNA transcription unit is composed from its 3′→5′ end, of an RNA-coding region flanked by a promoter region and a terminator region. Regions to the left, or moving towards the 5′ end, of the transcription start site are considered. In RNA, adenine forms base pair with uracil instead of thymine.
- DNA transcription takes place in broad two steps. First, precursor messenger RNA (pre-mRNA or primary RNA) is synthesized, with the help of RNA polymerase enzymes in the nucleus.
- Then primary RNA transcript (**pre-mRNA**) gets a cap added to the 5′ end and undergoes polyadenylation and splicing to produce messenger RNA (mRNA). Following transport of mRNA to the cytoplasm, mRNA is translated in the cytosol to protein molecules. Transcription occurs in three steps: initiation, elongation (addition of nucleotides to the mRNA strand) and termination.

PROCESSING OF MESSENGER RNA (PRE-MESSENGER RNA→MATURE MESSENGER RNA)

Genetic information is stored in the genes in human genome, arranged in the strands of the DNA double helix in the nucleus. The genetic information is transcribed from DNA into a messenger RNA template by a process of transcription. Before the messenger RNA (mRNA) can be translated into the proteins, noncoding regions (**introns**) must be removed and protein-coding regions (**exons**) joined by RNA splicing to produce mature messenger RNA (mature mRNA). Schematic representation of process of primary RNA transcript to messenger RNA is shown in Fig. 5.16.

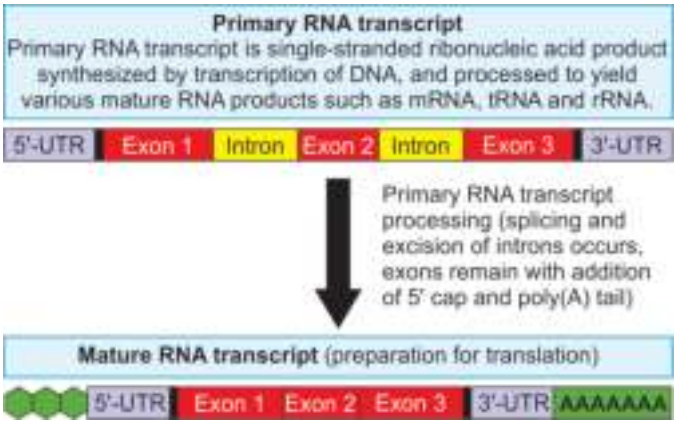


Fig. 5.16: Schematic representation of process of primary RNA transcript to messenger RNA. The genetic information is transcribed from DNA into primary RNA transcript (pre-messenger RNA) that becomes a messenger RNA (mRNA) after processing, that can be translated into the proteins, noncoding regions (introns) must be removed and protein-coding regions (exons) joined by RNA splicing to produce mature messenger RNA (mature mRNA).

Pre-messenger RNA (Pre-mRNA)/Precursor mRNA/Primary RNA Transcript

Precursor mRNA (pre-mRNA)/primary RNA transcript is synthesized from a DNA template in the cell nucleus by transcription. It is the primary transcript that becomes a messenger RNA (mRNA) after processing.

- Pre-mRNA comprises the bulk of heterogenous nuclear RNA (hnRNA). Pre-mRNA contains both coding sequences (**exons**) and noncoding sequences (**introns**).
- During RNA processing, intervening noncoding sequences (**introns**) are cut away by **spliceosome** and rejoining of exons to produce the final mature mRNA molecule containing only exons. Without RNA splicing of introns, primary transcript cannot leave the nucleus.
- Prior to exit of primary transcript from the nucleus, 7-methylguanosine cap is added to the 5′ end of the pre-mRNA of the growing transcript by a phosphate linkage, while elongation is still in progress.
- The 5′ cap protects the nascent mRNA from degradation and assists in ribosome binding during translation into the cytoplasm. In addition, factors involved in protein synthesis recognizes the 5′ cap to initiate translation by ribosomes.
- A poly(A) tail is added to the 3′ end of the pre-mRNA once elongation is complete. The resulting mature mRNA then exits the nucleus, and binds on the ribosomes in the cytoplasm.

Mature Messenger RNA (mRNA)

A fully processed mRNA includes a 5′ cap, 5′-UTR coding region, 3′-UTR, and poly(A) tail. This is known as a poly-A and is used for stability and guidance so that the mRNA can exit the nucleus find the ribosome in the cytoplasm.

- Mature mRNA binds to ribosomes and forms a template for protein production.
- Ribosomal RNA (**rRNA**) constitutes 60% of ribosome and consists of large and small ribosomal subunits.
- Mature messenger RNA (**mRNA**) carries the genetic information copied from DNA in the form of a series of three-base codes, each of which specifies a particular amino acid. Transfer RNA (**tRNA**) decodes information and provides linkage between mRNA and amino acids transfers amino acids to ribosomes in the cytoplasm.

TRANSCRIPTION PHASES

Transcription involves three steps: initiation, elongation and termination. In the initiation phase, the DNA molecule unwinds and separates to form a small open complex. During elongation phase, RNA polymerase moves along the template DNA strand that synthesizes a messenger RNA (mRNA), RNA polymerase will keep transcribing until it gets signals to halt. The process of ending transcription is called **termination**, and it happens once RNA polymerase transcribes a sequence of DNA is known as terminator. Transcription process involving initiation, elongation and termination phases is given in Table 5.18. Schematic representation of transcription phases is shown in Fig. 5.17.

Transcription Initiation Phase

Initiation of transcription occurs when the RNA polymerase enzyme binds to a region of a gene is called promoter region—a particular sequence of nucleotides. This signals the DNA to unwind so the enzyme RNA polymerase can 'read' in one of the DNA strands.

- RNA polymerase enzyme uses one of the DNA strands (template strand) as a template to make a new complementary RNA molecule.
- RNA polymerase enzyme then aligns the correct nucleic acid (A, C, G, or U) with its complementary base on the coding strand of DNA.

Transcription Elongation Phase

Once the RNA polymerase is in position at the promoter region, transcription elongation occurs by the addition of new nucleotides to the growing mRNA strand. This process builds a strand of mRNA.

- During transcription elongation phase, RNA polymerase moves along template strand of DNA in the 3′→5′ direction. For each nucleotide in the template, RNA polymerase adds a matching (complementary) RNA nucleotide to the 3′ end of the RNA strand.
- The RNA transcript is nearly identical to the non-template, or coding strand of DNA. However, RNA manuscript has the nucleotide base uracil (U) in place of thymine (T), as well as slightly different sugar in the nucleotide base.

Table 5.18 Transcription involving initiation, elongation and termination phases

Phases	Characteristics
Initiation phase	Initiation occurs when the enzyme RNA polymerase binds to a region of a gene called the promoter
Elongation phase	Process of addition of nucleotides to the mRNA strand
Termination phase	Transcription ends, when RNA polymerase receives polyadenylation signal in the RNA transcript to stop. Cutting results in release of RNA transcript from RNA polymerase enzyme

Transcription within the cell nucleus produces an mRNA molecule, which is modified and sent into cytoplasm for translation. The transcript is decoded into a protein with the help of a ribosome and tRNA molecules.

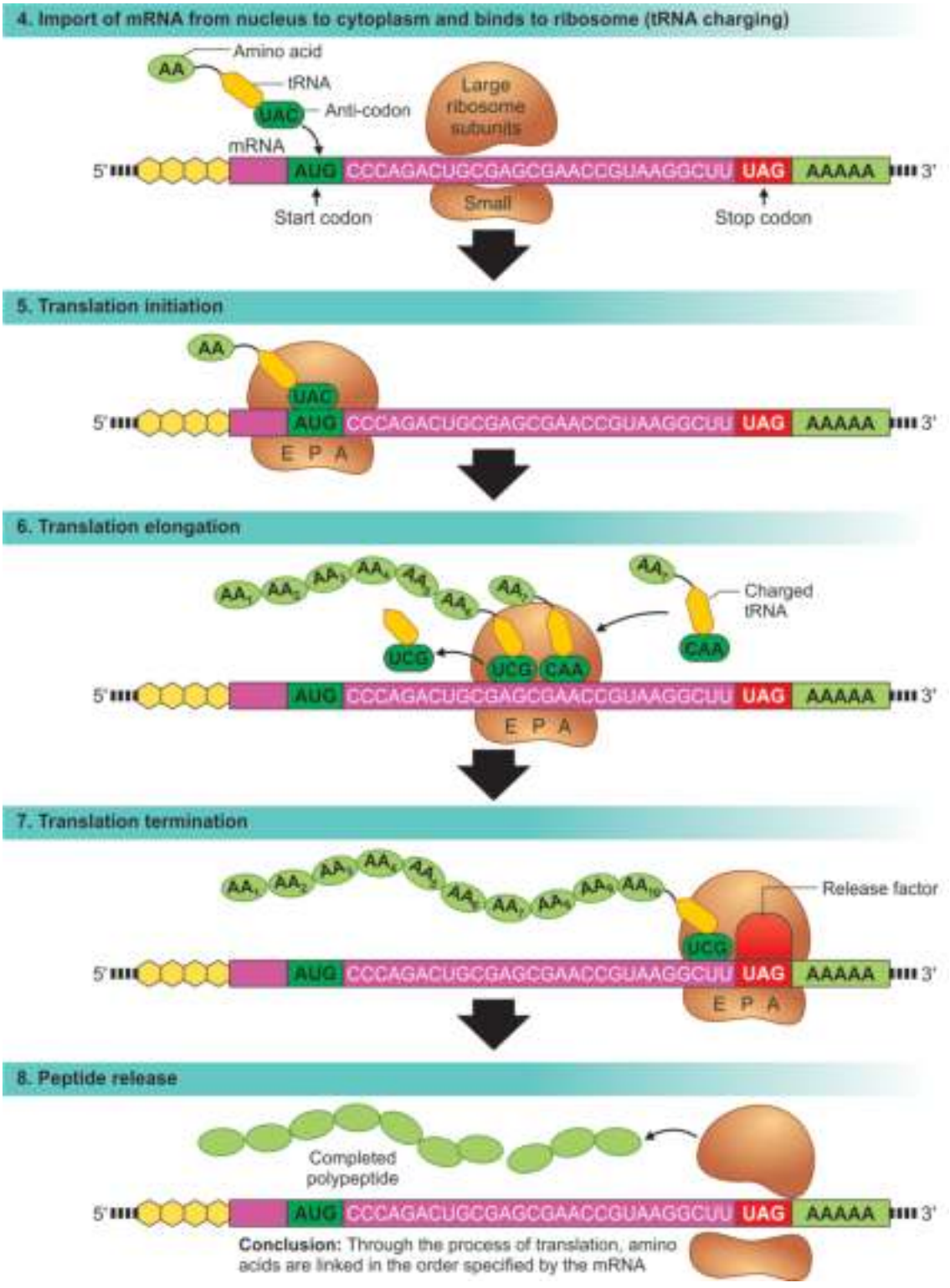


Fig. 5.17: Schematic representation of transcription phases. Transcription involves three steps: initiation, elongation and termination (*see* **Fig. 15.15**).

Transcription Termination Phase

RNA polymerase keeps on transcribing until it receives **polyadenylation signal** in the RNA transcript to stop. The process of ending transcription is called termination. The **polyadenylation signal** is recognized by an enzyme, that cuts the RNA transcript nearby releasing it from RNA polymerase enzyme.

TRANSLATION

Translation is the process of translating the sequence of a messenger RNA (mRNA) molecule to a sequence of amino acids is called polypeptide chains during protein synthesis in human cell. The substrate on which translation takes place is the ribosome found in cytoplasm.

- Translation is the process in which ribosomes in the cytoplasm or endoplasmic reticulum (ER) synthesize proteins after the process of transcription of DNA to RNA. Based on the size of sedimentation and genetic information, three major types of cellular RNAs include messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA). Features of major classes of cellular RNAs involved in translation are given in Table 5.19.
- Messenger RNA carries the genetic information out of the nucleus for protein synthesis.
- Transfer RNA is the smallest and decodes the information.
- Ribosomal RNA is the largest and constitutes 50% of a ribosome, which is a molecular assembly involved in protein synthesis.
- Catalytic RNAs are involved in many reactions in cytoplasm of the cell.

TRANSLATION PHASES

In translation, messenger RNA (mRNA) is decoded in the ribosome decoding center to produce a specific amino acid chain, or polypeptide. Translation proceeds in three phases: initiation, elongation and termination. Translation phases are given in Table 5.20.

Translation Initiation Phases

Initiation of translation occurs when mRNA, **tRNA** and an amino acid meet up inside the ribosome.

- Ribosome binds to mRNA at start codon (AUG), that initiates translation of mRNA into amino acids. Each **tRNA** contains a set of **three nucleotides** is known as **anticodon**. The anticodon of a given tRNA binds to specific mRNA codon.
- The **tRNA** molecule also carries the appropriate amino acid encoded by codon to the ribosome to be added to the growing polypeptide chain. One amino acid synthesis occurs at a time.

Translation Elongation Phase

Elongation of nascent polypeptide (i.e. beginning but not completed) chains by covalent addition of successive adding of amino acid residues, which are carried to the ribosome and correctly positioned by its tRNA. Elongation of nascent polypeptide chain requires elongation factors, which are cytosolic proteins.

Translation Termination Phases

Ribosomes are attached to the membrane of the endoplasmic reticulum. When a stop codon is encountered, polypeptide chain formation stops.

- The transfer RNA (**tRNA**) stops carrying the amino acids to the ribosome. It leads to dissociation of polypeptide chain from the ribosome by a releasing factor.
- **Protein folding** occurs in a cellular compartment called endoplasmic reticulum (ER). This is a vital

Table 5.19 Features of major classes of cellular RNAs involved in translation

Classes of RNAs	Comments
Messenger RNA (mRNA)	mRNA migrates from nucleus to cytoplasm and binds to ribosomes and carries DNA sequence information to ribosomes
Transfer RNA (tRNA), smallest	Decodes information and provides linkage between mRNA and amino acids' transfers amino acids to ribosomes in the cytoplasm
Ribosomal RNA (rRNA), constitutes 60% of ribosome	Structural component of ribosomes involved in reading the order of amino acids and linking amino acids together

Catalytic RNAs are involved in many reactions in cytoplasm of the cell.

Table 5.20 Translation phases (translation, elongation and termination)

Process	Characteristics
Translation initiation	Ribosome binds mRNA at start codon (AUG), that initiates translation of mRNA into amino acids
Translation elongation	Polypeptide chains extends (elongates) by successive adding of amino acids transferred from transfer RNA (tRNA)
Translation termination	When a stop codon is encountered, polypeptide formation stops resulting in dissociation of ribosome from the polypeptide and then its (polypeptide) release

cellular process because proteins must be correctly folded into specific three-dimensional shapes in order to function correctly.

- Two types of proteins are synthesized: (a) structural proteins such as collagen and keratin, and (b) regulatory proteins such as enzymes, antibodies and hormones.

Pathology Pearls: General Features of RNA (Ribonucleic Acid)

- RNA is composed of nucleotides. Each nucleotide within the RNA structure has a nitrogen base, a ribose sugar and a phosphate. RNA is involved in many biological processes.
- RNA has a single-stranded nucleotide chain structure. RNA comprises ribose sugar in its nucleotides instead of deoxyribose sugar present in the DNA.
- RNA nucleotide base pairs comprise adenine, guanine, cytosine and uracil instead of thymine in DNA.
- RNA can catalyze important biological reactions similar to proteins but DNA cannot do so. RNA molecules functioning such as protein enzymes are called ribozyme.

Pathology Pearls: Classification of RNA (Ribonucleic Acid)

RNAs are divided into two groups: (a) **messenger RNAs (mRNAs)** are protein-coding RNAs, which mediate translation of genetic information in DNA into protein, and (b) **functional RNAs** are noncoding RNAs, which generate functional molecules instead of encoding proteins. Functional RNAs are described as below:

- **Transfer RNA (tRNA):** It brings amino acid toward the mRNA in the process of translation. All kinds of tRNAs are connected to a single kind of amino acid and carry it to the ribosome.
- **Ribosomal RNAs (rRNAs):** These are essential components of ribosomes, which guide assembly of amino acid chain. Ribosomal RNA which is composed of mRNA and tRNA with pretending to be large macromolecular machinery.
- **Small nuclear RNAs (snRNAs):** These are part of RNA transcription system in cells. Some small nuclear RNAs provide guidance to ribosomal RNAs (rRNAs) modifications. Others are combined with various protein subunits to form ribonucleoprotein processing complex.
- **Small nucleolar RNAs (snoRNAs):** More than 20, small nucleolar RNAs (snoRNAs) have been described, which are responsible for making various modifications after transcription for rRNA, tRNA, or small nuclear RNAs.
- **Micro-RNA (miRNA):** Micro-RNAs are conserved in a high level consisting of 18–26 nucleotides, encoded from the DNA regions, which are translated to protein. Micro-RNAs direct post-transcriptional regulation of gene expression. Micro-RNAs regulate a wide range of biological processes including development, cell proliferation, cell metabolism and apoptosis.

PROTEIN FOLDING

After translation, next step is folding of polypeptide in the endoplasmic reticulum. Protein folding is the process by which a protein structure assumes its functional conformation or shape. All protein molecules are heterogenous unbranched chains of amino acids. Proteins have no biological activity in an unfolded state.

- All proteins are made up of amino acids linked together by peptide bonds.
- Proteins have four levels of organization: primary, secondary, tertiary and quaternary.

Primary Structure of Protein

The primary structure of protein consists of linear structure of amino acids that are linked by peptide bonds and form linear chains of polypeptides.

- Primary structure of protein determines how the protein folds and interacts at the level of interactions. Dipeptide consists of two amino acids, oligopeptide 3–9 amino acids and polypeptide 8 or more amino acids linked together.
- Protein consists of several polypeptides. Hemoglobin contains four polypeptides. Human DNA polymerase contains at least 10 polypeptides.
- All amino acids have a carbon atom that form four bonds: amino group, carboxyl group, a hydrogen bond and one of 20 side chains.

Secondary Structure of Protein

Secondary structure of protein is two-dimensional structure and has secondary structures: α-helix polypeptide chain (rod-shaped and coiled) and β-pleated sheets.

- The α-helix of secondary structure of protein, a polypeptide chain is held by hydrogen bonds between the amino hydrogen and carboxyl oxygen atoms in the peptide backbone, that extend in one direction.
- β-Pleated sheets are made of β-strands (chains) connected laterally by two or more hydrogen bonds forming a backbone.
- In a β-pleated sheet, the β-chains are folded so that they lie alongside each other.
- Each β-chain is made of 3–10 amino acid residues. Hydrogen bonding of the peptide backbone causes the amino acids to fold into repeating pattern.

Tertiary Structure of Protein

Tertiary structure of protein is a three-dimensional arrangement of polypeptide chain. It is stabilized by four interactions of: (a) ionic bonding, (b) hydrogen bonding, (c) disulfide linkages, and (d) dispersion forces.

- Three-dimensional folding pattern of a protein occurs due to interactions between the side chains—the 'R' groups, which include ionic, hydrogen bonds, hydrophobic interactions, and disulfide bonds.
- Hydrophilic amino acids with polar side chains are present on outside to make the protein soluble.
- Hydrophobic amino acids tend to cluster together in the center of the protein. Hydrophobic amino acid side chains do not interact with water or other hydrophilic molecules.
- Sulfhydryl groups tend to form disulfide bonds with other sulfhydryl group. **Cysteine** is commonly involved in sulfhydryl bonds, which limits structure of the protein.
- Three-dimensional shape of a polypeptide chain is the result between all these tendencies as discussed.
- Fibrous proteins are structural proteins (e.g. cytoskeleton proteins, elastin, collagen). These have α-helices and β-pleated sheets, which tend to make the polypeptide as rigid structure. Globular chains are compact and flexible, which contain α-helices and β-pleated sheets interspersed with randomly coiled structure. The globular proteins usually have enzymatic activity.

Quaternary Structure of Protein

Quaternary structure of protein involves the association of two or more amino acid polypeptide chains into functional proteins held together by hydrophobic interactions of the amino acids and disulfide bridges.

DENATURING OF PROTEIN

Each protein has its own unique shape. If a protein loses its shape, it ceases to perform its specific function. The process of losing shape of the protein is called denaturation.

- Denaturation occurs in secondary, tertiary and/or quaternary structures; with intact primary structure and is usually caused by external stress on protein, such as chemical solvents, inorganic salts, exposure to acids or bases and high temperature. Disulfide bonds can be broken with reducing agents, which destroy hydrogen bonds, ionic bonds, or hydrophobic interactions of the protein resulting in denaturation.
- Denatured protein is usually nonfunctional. Since primary structure of the protein remains intact, denaturation of some proteins can be reversed. One example of irreversible denaturation of protein is fried egg. Many proteins do not fold by themselves, but instead get assistance from 'chaperone proteins' (chaperonins) found in endoplasmic reticulum and mitochondria.

Pathology Pearls: Chaperone Proteins

- **Chaperone proteins (chaperonins)** have ability to prevent non-specific aggregation by binding to non-native proteins. Examples of chaperon proteins are the '**heat shock proteins**'.
- Chaperonins may assist in protein folding when the nascent polypeptide is being synthesized by the ribosome.
- Chaperonins operate by binding to stabilize an otherwise unstable structure of a protein in its folding pathway, which have the ability to prevent nonspecific aggregation by binding to non-native proteins.

PROTEIN MISFOLDING AND AGGREGATION

Protein misfolding is a common cellular event, that can occur throughout the life span of a cell. In young and healthy cells, the misfolded protein load is disposed of by protein quality control (PQC) systems.

- In cells in aged persons and certain persons with genetic disease, misfolded proteins are not disposed of by protein quality control system.
- Genetic disorders can be caused by genetic mutations, translational errors, abnormal modification of protein, thermal or oxidative stress and incomplete formation of complex. Depending on the properties of the protein and the efficiency of the protein quality systems, the accumulated protein may be assembled or degraded into aggregates and toxic oligomers.
- Different protein misfolding disorders include phenylketonuria, α_1-antitrypsin deficiency, Parkinson's disease, familial neurophyseal diabetes insipidus, and certain acetyl-CoA dehydrogenase deficiency.

DNA DAMAGE AND REPAIR SYSTEMS

Deoxyribonucleic acid (DNA) in the living cell is subjected to many chemical alterations. The genetic information encoded in the DNA has to be uncorrupted. Any chemical alterations in DNA must be corrected. DNA damage is structural abnormality in the DNA, that can be recognized by DNA repair enzymes and correctly repaired. Major types of DNA damage are given in Table 5.21.

Table 5.21 Major types of DNA damage

Type of DNA Damage	Characteristics
Single-base alterations in DNA sequence (not affecting DNA replication, transcription and translation)	▪ Deamination: cytosine→uracil; adenine→hypoxanthine ▪ Depurination (caused by UV radiation, ionizing radiation, alkylating agents, reactive oxygen species) ▪ Insertion of adenine in place of cytosine ▪ Alkylation of base ▪ Insertion or deletion of base ▪ Base analog incorporation
Double nucleotide base pair alteration	▪ Ultraviolet light induced thymine-thymine (pyrimidine dimer) ▪ Bifunctional alkylating agent cross-linkage
Helical chain breaks	▪ Ionizing radiation ▪ Radioactive disintegration of backbone element ▪ Oxygen-derived free radical formation
Cross-linkage	▪ Between nucleotide base pairs in same DNA strand or opposite DNA strand ▪ Between DNA and protein molecules (histones)

DNA DAMAGE MECHANISMS

DNA damage refers to alterations in the chemical structure of DNA such as a break in a DNA strand, a nucleotide base missing from the sugar–phosphate backbone of DNA, and a chemical alteration in the base.

- Damage to DNA that occurs naturally can result from metabolic or hydrolytic processes. DNA damage can be caused by errors during DNA replication, alkylating agents, reactive oxygen species (ROS) during normal cellular respiration and other biochemical pathways, ultraviolet rays, X-rays and γ-radiation. DNA damage leads to genome instability, increased cancer risk, accelerated aging process and neurodegenerative disorders.
- DNA damage mechanisms include: (a) hydrolysis (deamination and depurination), (b) alkylation, (c) oxidation, (d) radiation reactions, (e) nucleotide base analogue and intercalating agents.
- The comet assay, or single cell gel electrophoresis assay (**SCGE**) is a common technique performed to analyze all types of DNA damage. It is a convenient diagnostic tool for measuring universal DNA damage in individual's cells.
- Mechanisms of DNA damage, DNA repair systems and outcome of DNA repair systems failure are shown in Fig. 5.18. Human DNA repair genes are given in Table 5.22.

HYDROLYSIS (DEAMINATION AND DEPURINATION)

Hydrolytic DNA damage may result from the biochemical reactions, various metabolites as well as overabundance of reactive oxygen species. A single nucleotide base pair transformation within a DNA molecule may be sufficient to cause a mutation or inactivate the DNA. Hydrolytic DNA damage involves deamination or total removal of individual nucleotide base pairs.

Deamination

Deamination is a reaction, where cytosine (C) is lost from DNA to produce uracil (U) resulting in mutagenic U:G mispairs. Deamination is the removal of an amino group from a molecule. The enzymes that catalyze this reaction are called deaminases.

- The deamination of cytosine to uracil takes place at significant rate in liver and kidney. Spontaneous deamination of cytosine forms uracil, which is recognized and removed by DNA repair enzymes.
- Deamination converts adenine (A) to hypoxanthine, that selectively affects nucleotide base pairs with cytosine (C) instead of thymine (T). This results in a post-replicative transition mutation, where the original adenine (A) to thymine (T) nucleotide base pair transforms to guanine (G) to cytosine (C) nucleotide base pair.
- Deamination of 5-methylcytosine forms thymine. This conversion of a DNA nucleotide base pair from cytosine (C) to thymine (T) can result in transition mutation.

Depurination

Depurination of DNA occurs by spontaneous hydrolysis of the β-N-glycosyl-linkage between deoxyribose and purine nucleotide base; leading to subsequent loss of purine bases, adenine (A) or guanine (G) from the DNA; leaving the DNA's sugar–phosphate chain intact, producing an abasic site (i.e. deoxyribose lacking a nucleotide base) in the DNA. Loss of DNA nucleotide base pairs can be mutagenic and if left unrepaired, they

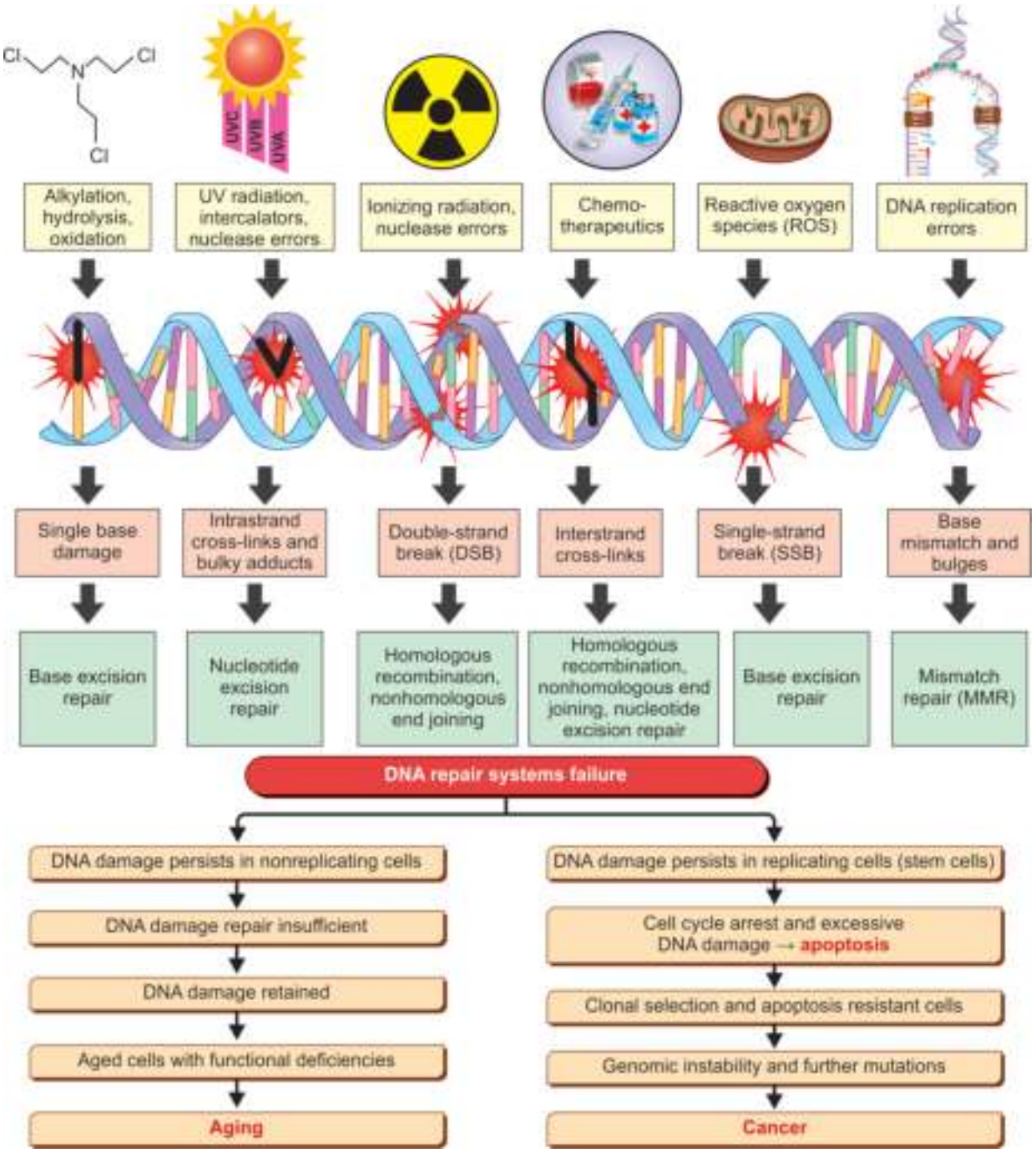


Fig. 5.18: Mechanism of DNA damage, DNA repair systems and outcome of DNA repair systems failure.

can inhibit transcription. Pyrimidine dimers can disrupt polymerases and prevent proper DNA replication.

ALKYLATION AND DNA DAMAGE

Methyl and ethyl group added to DNA nucleotide base pairs alters the structure of DNA. DNA alkylation refers to the addition (transfer) of alkyl (methyl or ethyl) groups to the reactive sites of specific nucleotide bases and to the phosphates in DNA backbone, resulting in alkylation products such as O^2-alkylamine, O^4-alkylamine, O^6-alkylamine, and O^8-alkylamine, which cause DNA mutations.

- Alkylation does not immediately result in mispairing. However, alkylation makes the bond between sugar and nucleotide base pair more labile, that may break and result in an apurinic site, a sugar without its purine.
- Hence, DNA molecule cannot be replicated properly, unless it is first repaired. If such cells sometimes attempt to replicate apurinic DNA, these frequently

Table 5.22 Genes involved in human DNA repair

DNA Repair Mechanism	Genes Proteins
Base-excision repair (BER)	UNG gene (12q24.11), SMUG1 gene (12q13.13), MBD4 gene (3p21.3), TDG gene (12q23.3)
Direct reversal of DNA damage	MGMT gene (10q26.3), ALKBH2 (ABH2) gene (12q24.11), ALKBH3 (DEPC1) gene (11p11.2), APEX1 gene (14.q11.2), APEX2 gene (Xp11.21), LIG3 gene (17q12), PARP1 gene (1q42.12), PARP2 gene (14q11.2)
Repair of DNA-protein cross-links	TDP1 gene (14q32.11), TDP2 (TTRAP) gene (6p22.3), SPRTN (Spartan) gene (1q42.12-q43)
Nucleotide-excision repair (NER)	XPA gene (9p22.33), XPB gene (13q233.1), XPC gene (3p25.1), XPD gene (19q13.32), XPE gene (11p11.2), XPF gene (16p13.12), XPG gene (2q13.3), XPV gene (6p21.1), RAD23B gene (9q31.2), ERCC1 gene (19q13.32)
Mismatch excision repair (MMR)	MLH1 gene (3p21), MSH2 gene (2p15), MSH3 gene (5q11.12), MSH4 gene (1p31.1), MSH5 gene (6p21.33), MSH6 gene (2p16), MLH3 gene (14q24.3), PMS1 gene (2p32.2), PMS2 gene (7p22.1), HFM1 gene (1p22.2)
Double DNA break repair	BRCA1 (17q21), BRCA2 (13q12), ATM gene (11q22.3)
Homologous recombination	RAD51 gene (15q15.1), RAD51B gene (14q24.1), RAD51D gene (17q12), HELQ gene (4q21.23), SW15 gene (9q34.11), SRCC2 gene (7q36.1), SRCC3 gene (14q32.33)
Nonhomologous end joining	XRCC6 gene (22q13.2), XRCC5 gene (2q35), PRKDC gene (8q11.21), LIG4 gene (13q33.3), XRCC4 gene (5q14.2), DCLRE1C gene (10p13), NHEJ1 gene (2q35)
Fanconi anemia gene encoding protein	FANCA gene (16q24.3), FANCB gene (Xp22.31), FANCC gene (9q22.32), FANCE gene (6p21.31), FANCF gene (11p14.3), FANCG gene (9p 13.3)
DNA replication fork repair	MLM gene (9p21), WRN gene (8p), RecQ4 gene (8q24.3)
Telomere maintenance	TERT gene (5p15.33)

ATM gene (11q22.3) is important checkpoint. TP53 gene (17p1.2) enhances transcription of PTEN. PTEN gene (10q23.3) protects p53 from MDM2-mediated degradation.

insert the wrong nucleotide base pairs across from an apurinic site, and this results in mutation.

- Alkylation changes nucleotide base-pairing properties resulting in mutation. Moreover, all the nitrogen and oxygen atoms involved in nucleotide base-pairing are also subject to allylation, which can directly disrupt nucleotide base-pairing leading to mutation. Many environmental carcinogens are electrophilic molecules that can attack DNA and alkylate it.
- Alkylating agents such as nitrogen mustard, cyclophosphamide, chlorambucil, melphalan and busulfan are administered to treat cancer patients by causing mutated DNA in clinical practice. These alkylating agents are nonspecific for cell cycle phase, but remain active during most parts of the cell cycle.
- Alkylating agent is a volatile organic solvent ethyl methanesulfonate (EMS, CH_8O_3S), which transfers ethyl (CH_3–CH_2) groups to DNA. The product of this methylation, O^6-methylguanine produces random point mutations in DNA by nucleotide substitution, particularly by guanine alkylation. The product also produces single-stranded break in DNA and chromosomal aberrations.

OXIDATIVE DNA DAMAGE

Oxidative DNA damage occurs from reactive oxygen species (ROS) derived from normal cellular metabolic processes and generated by ionizing radiation and chemical agents.

- However, cells also produce antioxidants that neutralize oxygen-derived free radicals. DNA damage occurs at guanine residues due to high oxidation potential of the nucleotide base pair relative to cytosine (C), thymine, and adenine.
- An important oxidation product is 8-hydroxyguanine, which mispairs with adenine, resulting in guanine (G): cytosine (C) to thymine (T): adenine (A) transversions.

RADIATION REACTIONS AND DNA DAMAGE

Both **ultraviolet rays** and **ionizing radiation** cause damage to DNA. Ionizing radiation is a high-energy radiation that releases electrons from atoms and molecules generating ions inducing DNA breaks, particularly, double-stranded DNA breaks.

- Secondary effects of radiation are the generation of reactive oxygen species (ROS) that oxidize proteins and lipids and also induce severe damage to DNA,

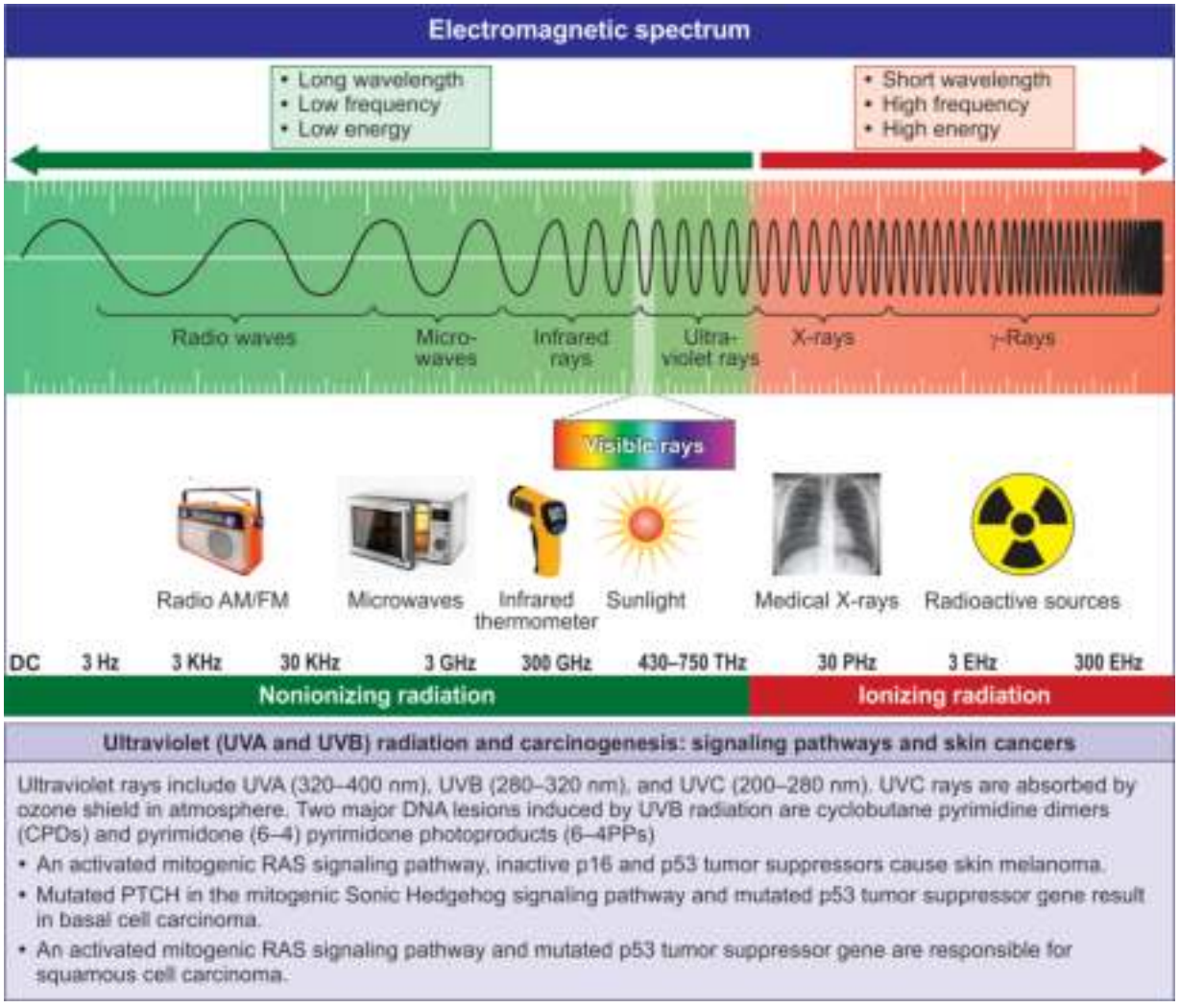


Fig. 5.19: Ultraviolet radiation-induced DNA damage and associated cancers.

like generation of abasic sites and single-strand DNA breaks.

- Collectively, all these cellular changes induce cell death and mitotic failure. Ultraviolet radiation-induced DNA damage and associated cancers are shown in Fig. 5.19.

Ultraviolet Radiation-induced DNA Damage

Ultraviolet radiation (UVB radiation: 290–320 nm) cross-links adjacent pyrimidines (thymine and cytosine) on the same DNA strand forming pyrimidine dimers, usually thymine dimers.

- Pyrimidine dimers disrupt DNA polymerases and prevent proper replication of DNA, because DNA replication machinery fails to convey which nucleotide base pairs to insert opposite the dimer. If DNA replication proceeds, wrong nucleotide base pairs are inserted at random resulting in mutation.
- Ultraviolet (UV) radiation from the sun is most important carcinogen in human beings. Exposure of the skin to UV radiation has multiple cellular and clinical effects including development of skin cancers (cutaneous squamous cell carcinoma, basal cell carcinoma and malignant melanoma). UVB radiation-induced DNA damage adversely affects signaling pathways resulting in impairment of cell cycle arrest, DNA repair and apoptosis.
- UV radiation-induced mutations in signature genes (UVA, UVB, ROS), tumor suppressor genes (TP53, PTCH, p16) and TNF-α gene lead to production of thymine dimers, dipyrimidine dimers, 'reactive oxygen species' and TNF-α, which cause damage to cellular biomolecules and DNA.

Ionizing radiation-induced DNA Damage

Ionizing radiation (gamma rays and X-rays) directly affects structure by inducing DNA breaks, particularly double-stranded breaks.

- The radiation causes formation of reactive oxygen species (ROS) which are indirectly involved in DNA damage. Reactive oxygen species, especially those

containing oxygen, are most reactive, which attack neighboring molecules.

- When oxygen-derived free radical attacks a DNA molecule, then it can change a nucleotide base pair, and frequently causes a single-strand or double-strand DNA break(s). Single-stranded DNA breaks are usually repaired by using template strand just by rejoining the ends of the damaged single-strand. But double-stranded DNA breaks are difficult to repair resulting in mutation.
- Ionizing radiation can also cause break in chromosomes. Exposure to ionizing radiation is known to increase the risk for development of cancers especially leukemia.
- Radiotherapy is high energy ionizing radiation commonly employed treatment in the management of more than 50% of human malignancies. Ionizing radiation destroys the cancer cells in the treated region by damaging DNA of cancer stem cells as well as normal cells in the surrounding region. The side effects of radiotherapy usually improve within a few weeks after radiotherapy.

BASE ANALOGUE AND INTERCALATING AGENTS: DNA DAMAGE

Mutations can be caused by compounds that substitute for normal nucleotide base pairs (base analogs) or slip between the nucleotide base pairs (intercalating agents) to cause error in replication.

Base Analogues

Base analogues are molecules which have similar structure to one of four nitrogen nucleotide base pairs present in DNA (adenine, guanine, cytosine and thymine).

- The base analogs form a structure identical to one of the DNA nucleotide base pairs and then can be used to form the new strand in semiconservative replication of DNA. Usually, substitution of a nucleotide base analogue results in altered nucleotide base pairings and structural changes that can affect DNA replication and transcription of genes. Since 5-bromouracil can pair with either adenine (A) or guanine (G).
- Base analogue 5-bromouracil causes frequent mistakes in forming correct base pair during DNA replication, which results in mutation. Nucleic acid analogues are structurally similar to natural occurring DNA and RNA, which are used in medicine and molecular biology research.

Intercalating Agents

Intercalating agents are **hydrophobic molecules** containing several polycyclic rings that resemble the ring structure of purine or pyrimidine base pair of the DNA. Intercalating compounds such as ethidium bromide, acridine orange, actinomycin D and proflavine bind to the major or minor grooves of DNA double helix and cause addition or deletion of bases during replication.

- Intercalating compounds may result in a frameshift mutation, which can alter the codon reading frame and result in interference in DNA replication and transcription.
- Proflavine, an intercalating compound, is used as an antimicrobial agent as a topical agent, that denatures bacterial DNA resulting in lysis of bacteria. Due to its intercalating property, it adversely affects host DNA, which has potential chances to induce skin cancer and other cancers.

POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds containing carbon and hydrogen, and composed of multiple aromatic rings. PAHs occur naturally in coal, crude oil, and gasoline.

- Incomplete combustion of organic matter such as coal, wood, tobacco or garbage results in emission of PAHs. Examples of PAHs include benzene, toluene, naphthalene and anthracite.
- Polycyclic aromatic hydrocarbons (PAHs) induce carcinogenesis by causing DNA damage through the formation of adducts. A common marker for DNA damage due to PAHs is benzo(a) pyrene diol epoxide (BPDE). BPDE compound is found to be very reactive, and known to bind covalently to proteins, lipids, and guanine residues of DNA to produce BPDE adducts. If left unrepaired, BPDE-DNA adducts may lead to permanent mutations resulting in cell transformation and ultimately tumor development.

DNA REPAIR MECHANISMS

DNA damage is minimized by DNA repair systems. DNA repair systems recognize the correct damage of DNA molecules and repair the defects in DNA.

- DNA damage is repaired by various mechanisms include: (a) direct repair (reversal), (b) excision repair (free base-excision repair, nucleotide-excision repair), (c) mismatch repair, and (d) recombination repair of double-strand DNA break.
- Most of the DNA repair mechanisms depend on the presence of two strands of DNA because nucleotides in the damaged region are removed and replaced. Nucleotide base pairs are also replaced in mismatch repair, free base-excision repair and nucleotide-excision repair mechanisms.

Table 5.23 Type of DNA damage and repair mechanism

Type of DNA Damage	DNA Repair Mechanism
Methylated (O6 or N7) guanine	Direct repair (pyrimidine dimers; other specific types of alterations)
Hydrolytic DNA damage (deamination/depurination)	Base-excision repair (BER)
Bulky DNA lesions, DNA-protein adducts (DNA damage due to abnormal bases, modified bases, and pyrimidine dimers)	Nucleotide-excision repair (NER)
Mismatched nucleotide base pairs (replication errors, including mispaired bases and strand slippage)	Base-excision repair (BER)
Single-stranded DNA breaks (reactive oxygen species, ionizing radiation, spontaneous loss of sugar-phosphate)	Base-excision repair (BER), nucleotide-excision repair (NER)
Double-stranded DNA breaks	Homologous recombination (HR), nonhomologous end joining (NHEJ)

DNA damage is an alteration in the chemical structure of DNA, such as a break in a strand of DNA, a base missing from the backbone of DNA.

- Poly-ADP-ribosyl-polymerase (PARP) plays a central role in nucleotide-excision repair (NER) and base-excision repair (BER) and enables repair of damaged DNA caused by alkylating agents and chemotherapeutic agents. PARP is also involved in many other cellular processes including transcription and modulation of chromatin structure.
- However, nucleotide base pairs are not replaced by direct-repair mechanism. DNA repair mechanisms occur by using a common four-step pathway are given below. Type of DNA damage and repair mechanism are given in Table 5.23.

Pathology Pearls: Steps of DNA Repair Mechanisms

1. **Detection:** Damaged region of the DNA is recognized.
2. **Excision:** Endonuclease gives a small cut to the sugar-phosphate backbone on one or both sides of the DNA damage to remove one or more nucleotides.
3. **Polymerization:** DNA polymerase enzyme adds nucleotides to the newly exposed 3'-OH group by using the other strand of DNA as a template and replacing damaged nucleotides as well as some undamaged nucleotides in all three base-excision, mismatch and nucleotide repair mechanisms.
4. **Ligation:** DNA ligase seals and fills in the small gaps produced by the excision and removal of the damaged nucleotides in the sugar-phosphate backbone in all three: base-excision, mismatch and nucleotide repair mechanisms.

DIRECT DNA DAMAGE REVERSAL MECHANISM

Direct DNA damage reversal system acts directly on damaged single nucleotide chain, that restores the DNA genome to its normal original state in a single-reaction step. But only a few damaged nucleotides can be repaired directly. Pyrimidine dimers are repaired by a light-dependent direct system called photoreactivation.

EXCISION REPAIR MECHANISM

Excision repair mechanism involves excision of a segment of the polynucleotide containing a DNA damage site followed by synthesis of the correct nucleotide sequence by DNA polymerase enzyme and then ligation. Excision repair occurs by two mechanisms: base-excision repair and nucleotide-excision repair.

Base-excision Repair Mechanism

Base-excision repair mechanism refers to repair of damage to a single base caused by oxidation, alkylation, hydrolysis or deamination. The damaged nucleotide base pair is removed by DNA glycosylase enzyme, synthesis by DNA polymerase enzymes, and sealing by DNA ligase enzyme.

Nucleotide-excision Repair Mechanism

Damage to the DNA distorts the configuration of the molecule. An enzyme complex recognizes the distortion in DNA. DNA strand becomes separate. Single-stranded DNA-binding proteins stabilize the single-stranded regions of DNA. DNA glycosylase enzyme cleaves the damaged strand on both sides of the damage. Part of the damaged DNA strand is removed, and the gap is filled in by **DNA polymerase** and sealed by **gyrase**.

MISMATCH REPAIR MECHANISM

DNA replication is extremely accurate. Each new copy of DNA has less than one error per billion nucleotide base pairs. However, in the process of DNA replication, mismatched nucleotide base pairs are incorporated into the new DNA. Most of the errors that initially arise during DNA replication are corrected in MMR machinery human at genes and never becomes permanent mutations. Some of these errors in nucleotide base pairs are corrected during proofreading by polymerases.

- During proofreading, incorrect nucleotide base pairs are inserted to the newly synthesized strand of DNA. Mismatched nucleotide base pairs and other DNA errors are corrected by mismatch repair.
- **Exonucleases** remove nucleotide base pairs on the newly synthesized strand of DNA between the GATC sequence and the mismatch. DNA polymerase then replaces the nucleotide base pairs, correcting the mismatch, and DNA ligase seals the nick in the sugar-phosphate backbone.
- Human beings, who possess mutations in mismatch-repair genes often exhibit elevated **somatic mutation rates** and are frequently susceptible to **colon cancer**.

RECOMBINATION REPAIR OF SINGLE-STRAND AND DOUBLE-STRAND BREAK MECHANISM

Ionization, certain chemical agents and reactive oxygen species (ROS) can produce both single-strand breaks (SSBs) and double-strand breaks (DSBs) in the DNA backbone.

- Breaks in single-strand of DNA molecule are repaired using the same enzyme systems that are used in base-excision repair. The damaged nucleotide base pair is removed by DNA glycosylase enzyme, synthesis by DNA polymerase enzymes, and sealing by DNA ligase enzyme.
- Double-strand break in DNA inhibits DNA replication that may lead to chromosomal rearrangements such as deletions, inversions, duplication, and translocations.
- Two major pathways for repairing double-strand breaks in DNA include homologous recombination and nonhomologous end joining.

Homologous Recombination Repair Mechanism

Homologous recombination repair mechanism is a broken DNA molecule by using the identical or nearly identical genetic information contained in another DNA molecule, usually a sister chromatid or homologous chromosome.

- The process of homologous recombination is responsible for crossing over of chromosomes that results in creation of new combinations of DNA sequences in each chromosome.
- The breast cancer suppressor BRCA1 and BRCA2 genes are essential for the maintenance of genome integrity in human cells through their role in DNA repair by homologous recombination. Inherited mutations of BRCA1 and BRCA2 genes predispose women to breast and ovarian cancers.

Table 5.24 Disorders associated with defects in DNA repair systems

Disorder	Genetic Defect	Symptoms
Xeroderma pigmentosum	Defects in nucleotide-excision repair	Freckle-like spots on skin, sensitivity to sunlight, predisposition to skin cancer
Cockayne's syndrome	Defects in nucleotide-excision repair	Dwarfism, sensitivity to sunlight, premature aging, deafness, intellectual disability
Trichothiodystrophy	Defects in nucleotide-excision repair	Brittle hair, skin abnormalities, short stature, immature sexual development, characteristic facial features
Hereditary nonpolyposis colon cancer (HNPCC) also known as Lynch syndrome	Defects in mismatch repair	Predisposition to colon carcinoma
Fanconi anemia	Possibly defects in the repair of inter-strand predisposition to leukemia	Increased skin pigmentation, abnormalities of skeleton, heart, and kidneys, predisposition to leukemia
Li-Fraumeni syndrome	Defects in DNA damage response	Predisposition to cancer in many different tissues
Werner syndrome	Defect in homologous recombination	Premature aging, predisposition to cancer
Ataxia-telangiectasia	Defective repair of broken DNA	Impairment of certain areas of brain and cerebellum causing difficulty with movement and coordination, impaired immune system and predisposition to lymphoid malignancies
Bloom syndrome	Genome instability	Autosomal recessive disorder characterized by short stature and predisposition to cancer
Progeria (Hutchinson-Gilford progeria syndrome)	Genome instability due to lamin A (LMNA) gene mutation	Genetic disorder causing premature aging (two years child looking older)
Rothmund-Thompson syndrome	Mutation in DNA helicase RECOL4 gene causing defect in DNA replication	Presents with sparse hair, eyebrows and eyelashes, slow growth, small stature, chronic diarrhea, vomiting and abnormalities in teeth and nails; and predisposition to osteosarcoma, squamous cell carcinoma of skin and basal cell carcinoma of skin

Nonhomologous End Joining Repair Mechanism

Nonhomologous end joining repair mechanism occurs in double-strand breaks without using a homologous template. This pathway is most often used when the cell is in G1 phase of cell cycle and a sister chromatid is not available for repair through homologous recombination.

- Nonhomologous end joining repair mechanism uses proteins that recognize the broken ends of DNA molecule, bind to the ends and then join them together.
- Nonhomologous end joining repair mechanism is more prone to errors such as deletions, insertions and translocations than homologous recombination.
- Errors in nonhomologous end joining repair mechanism may be associated in translocation in various cancers such as Burkitt lymphoma, Philadelphia chromosome in chronic myelogenous leukemia, and B cell lymphoma.

GENETIC DISORDERS AND FAULTY DNA REPAIR SYSTEMS

Several human diseases are associated with defective DNA repair system and increased rate of mutation. Defects in DNA repair are the underlying cause of several genetic diseases. Many of these diseases are characterized by a predisposition to cancer. Disorders associated with defective DNA repair system include xeroderma pigmentosum, Cockayne's syndrome, trichothiodystrophy, hereditary nonpolyposis colon cancer, Fanconi anemia, Li-Fraumeni syndrome, Werner syndrome, ataxia-telangiectasia, Bloom syndrome, progeria (Hutchinson-Gilford progeria syndrome) and Rothmund-Thompson syndrome. Genetic disorders associated with defects in DNA-repair systems are given in Table 5.24.

CHROMOSOMAL DISORDERS

Normal cells are diploid, containing 46 chromosomes (23 pairs). Out of these, there are 22 pairs of autosomes and 1 pair of sex chromosomes (XX in females or XY in males). Chromosomes are rod-shaped filamentous bodies present in the nucleus, which become visible during cell division in the mitotic metaphase.

- Chromosomes are composed of thin chromatin threads called chromatin fibers, which undergo folding, coiling and supercoiling during prophase so that the chromosomes become progressively thicker and smaller in length. Therefore, chromosomes become readily observable under light microscope.
- Study of human chromosomal abnormalities is called cytogenetics.
- Chromosomal disorders may result in congenital malformations, mental retardation, miscarriage, stillbirths, fertility problems and/or malignancy. Chromosomal disorders can occur due to numerical abnormality (aneuploidy or polyploidy) or structural abnormality (structural rearrangement).
- Duplication of chromosomes in somatic cell lines involves mitosis.
- Mitosis is a type of cell division that results in two identical daughter cells, each having the same number and kind of chromosomes as the parent nucleus. The major purpose of mitosis is for the growth and replacement of worn-out cells. Mitosis consists of four phases: prophase, metaphase, anaphase, and telophase. Meiosis is limited to replicating germ cells (ova or sperms).
- Meiosis is a process where a single germ cell (sperm in male or ovum in female) divides to produce four cells containing half the original amount of genetic information.
  - In meiosis, the chromosome/chromosomes duplicate during interphase, and the homologous chromosomes exchange genetic information (chromosomal crossover) during the first division, called meiosis I (formation of two daughter cells).
  - The daughter cells undergo meiotic division again in meiosis II (formation of four daughter cells), splitting up sister chromatids to form haploid gametes. Two gametes (sperm and ovum) fuse during fertilization, creating a diploid cell with a complete set of paired chromosomes.
- Sex determination of an individual is shown in Fig. 5.20. Terminology used for chromosomal appearances and abnormalities are given in Table 5.25.

CHANGES IN AUTOSOME CHROMOSOME NUMBER AND STRUCTURE

Normally, all the individuals have same number of chromosomes. Presence of a whole set of chromosomes is called **euploidy**. Gametes normally contain only one set of chromosomes; this number is called **haploid (1n)**.

- Somatic cells usually contain two sets of chromosomes, i.e. diploid (2n), three sets of chromosomes, i.e. triploid (3n), and four sets of chromosomes, i.e. tetraploid (4n). The condition, in which the set of chromosomes in multiples of 'n' is called polyploidy.

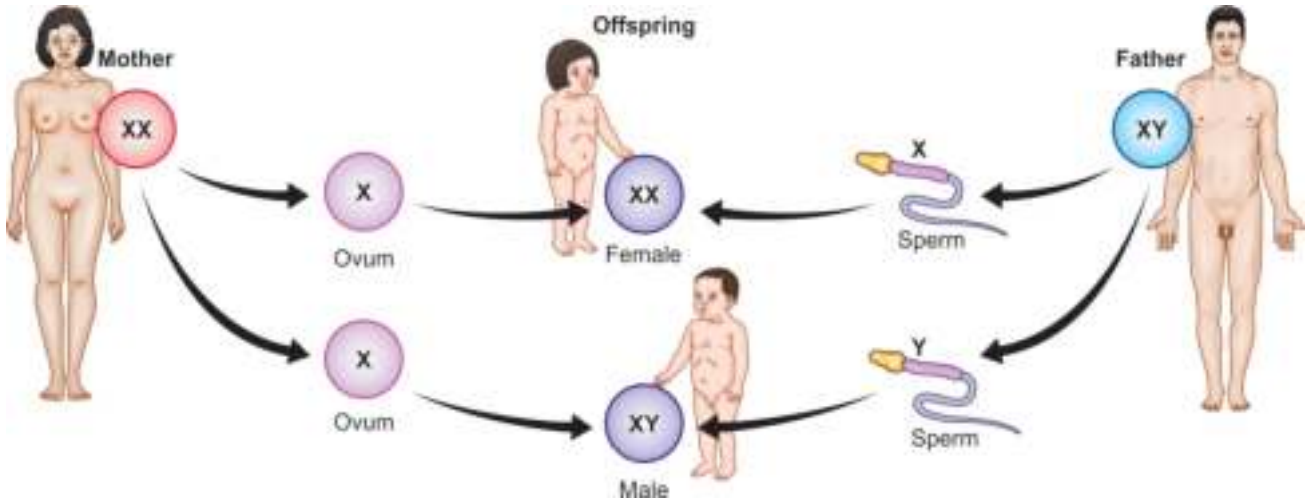


Fig. 5.20: Sex determination of an individual. In this system, the sex of an individual is determined by a pair of sex chromosomes. Normally, cells from female contain two X chromosomes (XX), and cells from male contain X and Y chromosomes (XY).

Table 5.25 Terminology used for chromosomal appearances and abnormalities

Term	Description
Centromere	The region that connects the two arms of the chromosome
Chromosomal marker	▪ A small extra piece of chromosomal material that travels with the other chromosomes ▪ Example of chromosomal marker is 46, XY, +mar
Deletion	▪ Loss of all or part of one chromosomal arm, usually a part toward the end of an arm ▪ An acentric chromosome (without a centromere) is typically lost completely after a few cell divisions. ▪ Example of deletion is 46, XX, 4p– or 46, XX, del (13) (q12q14)
Duplication	▪ A segment of chromatin that is repeated on the same or another chromosome. Many are incompatible with life ▪ Example of duplication is 46, XY, dup (1) (q11q22)
Fragile site	▪ There is a site on one arm that has a demonstrable fixed break point ▪ Example of fragile site is 46, fra(X) (q27.3), Y
Insertion	▪ There must be two break points in one chromosome that allow a segment to insert between a break point in another chromosome ▪ Example of insertion is 46, XX, ins (11) (p14q23q24)
Isochromosome	▪ The arms of a chromosome are mirror images of each other. Monocentric isochromosomes have two long arms or two short arms ▪ Dicentric isochromosomes have mirrored material between centromeres ▪ Example of isochromosome is 46, X, i(Xq)
Inversion	▪ Two break points in a chromosome allow a 180° turn of a segment with fusion ▪ The inversion is "paracentric" if it only involves one arm, while "pericentric" inversions involve the centromere and parts of p and q arms ▪ Example of inversion is 46, XX, inv (8) (p23q22)
Monosomy	▪ Only one of a chromosome pair is present in monosomy ▪ Autosomal monosomies yield nonviable embryos ▪ Only monosomy X is rarely liveborn ▪ Example of monosomy is 45, X or 45, XY, –14
Mosaic pattern	▪ Cells with an abnormal karyotype are present along with normal cells ▪ Example of mosaic pattern is 45, X/46, XX
p arm	The short arm of the chromosome
q arm	The long arm of the chromosome
Ring chromosome	Breakage of a chromosome at two points is followed by repair with fusion to a circular chromosome. Example of ring chromosome is 46, XX, r(1) (p36q44)

Contd...

Table 5.25 Terminology used for chromosomal appearances and abnormalities (*Contd...*)

Term	Description
Translocation	▪ There is an exchange of material between arms on different chromosomes. The translocation is "reciprocal" if there is an exchange of two chromosome segments on different (nonhomologous) chromosomes ▪ Chromosomal translocation is 'balanced' if no genetic material is lost ▪ Unbalanced chromosomal translocation involves more breakpoints that lead to loss of genetic material in the exchange ▪ Example of translocation is 46, XY, t(9;22)
Triploidy	Three haploid sets of chromosomes are present in a cell (e.g. 69, XXY)
Trisomy	▪ An extrachromosome is present, making three copies ▪ Extragenetic material reduces viability ▪ Example of trisomy is 47, XX, +18 e

- When a change in the chromosome number does not involve entire sets of chromosomes, but only a few of the chromosome, it is called aneuploidy.
- Examples of changes in autosome chromosome number and structure are monosomy (2n–1), trisomies (2n+1), nullisomy (2n–2) and tetrasomy (2n+2).
- Mosaicism is defined as the mitotic error in the early development, that give rise to two population of cells with different chromosomal complement in some individuals, e.g. 45X/47XXX mosaicism.

CHANGES IN AUTOSOME CHROMOSOME NUMBER

Normal human somatic cell is diploid (46 chromosomes). Germ cell is haploid (23 chromosomes).

- Polyploidy refers to increase in number (a whole set) of chromosomes more than as in diploid, which occurs due to failure of cytokinesis after telophase stage of cell division. It can also bring about visible effects on the phenotype. Polyploidy is rarely compatible with life and usually results in spontaneous abortion.
- Triploidy (3X) is three times the haploid number; tetraploidy (4X) is four times the haploid number and so on. Chromosomal disorders caused by aneuploidy (numerical abnormalities) are given in Table 5.26.

Aneuploidy

In normal persons, somatic cells divide through mitosis to produce two identical diploid (2n) daughter cells. Meiosis occurs in germ cells, which produce four haploid (n) gametes (sperms or ova), that are generally unique from each other and the original parent (germ) cell.

Table 5.26 Chromosomal disorders caused by aneuploidy (numerical abnormalities)

Chromosomal Disorder	Trisomy or Monosomy	Incidence	Clinical Manifestations
Autosomal disorders			
Down syndrome	Trisomy 21	1 in 1000 live births	▪ Mental retardation ▪ Facial features (epicanthic folds, macroglossia, flat occiput) ▪ Simian crease ▪ Cardiac defects ▪ Increased risk of leukemia
Edward syndrome	Trisomy 18	1 in 5000 live births	▪ Multiple malformations (small head, small jaw, clenched fists with overlapping fingers and severe intellectual disability, cardiac defects) ▪ Most patients die during infancy
Patau syndrome	Trisomy 13	1 in 5000 live births	▪ Multiple malformations (severe mental disability, holoprosencephaly, polydactyly overlapping of fingers, i.e. clinodactyly, flexion of fingers, cleft lip and palate, neural tube defects, cardiac defects such as VSD, ASD, PDA) ▪ Most patients die during infancy
Sex chromosomal disorders			
Klinefelter syndrome	Trisomy XXY (47, XXY)	1 in 500–600 live male births	▪ Testicular hypoplasia ▪ Irreversible infertility (azoospermia) ▪ Eunuchoid body habitus ▪ Long extremities ▪ High FSH levels ▪ Low testosterone level ▪ Absence of secondary sexual characters

Contd...

Table 5.26 Chromosomal disorders caused by aneuploidy (numerical abnormalities) (*Contd...*)

Chromosomal Disorder	Trisomy or Monosomy	Incidence	Clinical Manifestations	
Turner syndrome	Monosomy X (45, X)	1 in 2000–3000 live female births	▪ Streak ovaries ▪ Primary amenorrhea and infertility ▪ Webbed neck ▪ Short stature	▪ Broad chest ▪ Coarctation of aorta ▪ Absence of secondary sexual characters
XYY syndrome (Jacob syndrome)	Trisomy XYY (47, XYY)	1 in 1000 live male births	▪ Taller than average height ▪ Low muscular tone (hypotonia) ▪ Curved pinky finger (clinodactyly)	▪ Widely spaced eyes (hypertelorism) ▪ Cystic acne during adolescence
XXX syndrome	Trisomy XXX (47, XXX)	1 in 1000 live births	▪ Epicanthic folds ▪ Low muscular tone (hypotonia) ▪ Curved pinky finger (clinodactyly) ▪ Flat feet	▪ Abnormal shaped breastbone ▪ Seizures ▪ Kidney malformations

Nullisomy refers to loss of both homologous chromosomes (2n–2).

- Aneuploidy is defined as any deviation from the normal number of chromosomes in a cell nucleus arising from aberrations in cell division. Cell nucleus has too many (47) or too few (45) chromosomes instead of the usual 46 chromosomes.
- The most common aneuploid disorders that can result in live births are trisomies of sex chromosomes (Klinefelter syndrome, XXY syndrome; Jacob syndrome, XYY syndrome); **trisomies** of autosome chromosome (13, 18 and 21) associated with advanced maternal age; and monosomy X (Turner syndrome 45, X).
- The chromosomal abnormality may be present in some cells in a case, and is known as Turner syndrome with mosaicism.
- Aneuploidy is detected by prenatal screening. Ultrasonography is a screening test to evaluate aneuploid fetuses. However, invasive testing with chorionic villus sampling or amniocentesis is performed for establishing diagnosis.
- Nondisjunction of chromosome is the most common mechanism of aneuploidy. Failure of separation of pair of chromosomes or chromatids occurs during cell division (meiosis I, meiosis II and mitosis).
- Nonjunction of chromosome is defined as failure of a pair of homologous chromosomes to separate in meiosis I, failure of sister chromatids to separate during meiosis II, and failure of sister chromatids during mitosis. Nondisjunction results in daughter cells with abnormal chromosome numbers (aneuploidy).
- Trisomy and monosomy chromosomal abnormalities are shown in Fig. 5.21. Differences between trisomy and monosomy are given in Table 5.27.

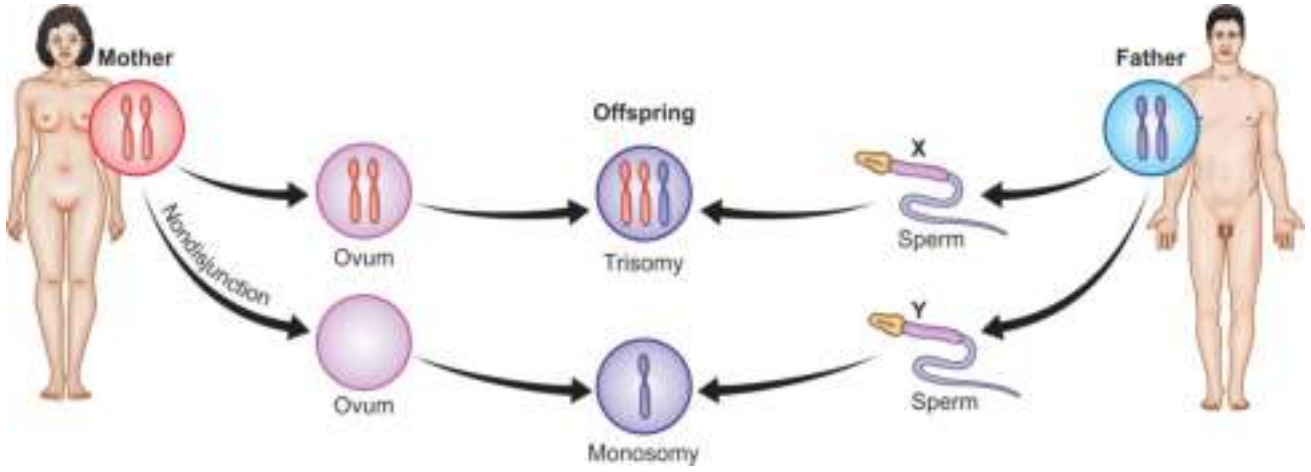


Fig. 5.21: Trisomy and monosomy chromosomal abnormalities. Trisomy is the presence of a third, extra copy of a chromosome, like in the case of Down syndrome. Monosomy is a form of aneuploidy with the presence of only one chromosome instead of the usual two chromosomes, like in the case of Turner syndrome.

Table 5.27 Differences between trisomy and monosomy

Characteristics	Trisomy	Monosomy
Definition	Trisomy refers to the condition of having an additional copy of a chromosome is present in the genome	Monosomy refers to the condition of having a diploid chromosome complement in which one chromosome lacks its homologous partner
Number of chromosomes	Presence of an additional chromosome	Presence of a single homologous chromosome in the homologous pair
Represented as	2n +1	2n –1
Example	Down syndrome (trisomy 21)	Turner syndrome (monosomy X) (45, X)

Polyploidy

Polyploidy is a term used to denote number of chromosomes multiple of haploid genome (23) such as triploidy (69 chromosomes) and tetraploidy (92 chromosomes). Polyploidy normally occurs in megakaryocytes and dividing hepatocytes. Polyploidy occurring in somatic cells of conception leads to spontaneous abortion.

ALTERATIONS IN AUTOSOME CHROMOSOME STRUCTURE

Alterations in chromosome structure occurs during cell division, which result from a break or breaks that disrupt the continuity of the chromosomes followed by reconstitution in an abnormal combination. Alterations in chromosome structure can occur by deletion of a section of chromosome, duplication of chromosome, translocation and inversion.

Deletion in Chromosome

Loss of chromosomal fragment ('p' short arm or 'q' long arm) or entire chromosome during cell division is termed deletion. Deletion of chromosome is shown in Fig. 5.22.

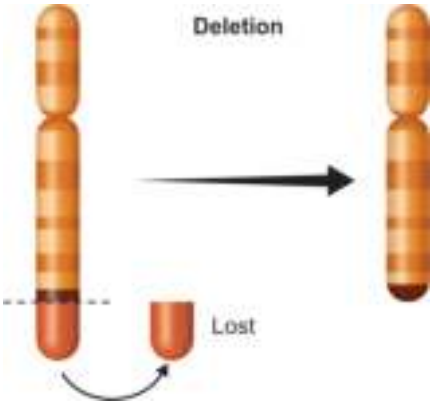


Fig. 5.22: Deletion of chromosome. Deletion of fragment of chromosome (long arm or short arm) during cell division leads to loss of genetic material and shortening of the chromosome. Deletion of the chromosome may occur in terminal or interstitial region. Deletions produce unbalanced meiotic products; thereby resulting in sterility.

- The severity of this lost genetic material depends upon the size of the deletion and the nature of the genetic material contained within it. Examples of chromosomal deletion syndromes include $5p^-$ deletion (cri-du-chat syndrome), $4p^-$ deletion (Wolf-Hirschhorn syndrome). Angelman syndrome and Prader-Willi syndrome.
- Smaller deletion in chromosome is detected by molecular techniques such as high-resolution banding, FISH and array comparative genome hybridization (array CGH).

Duplication of Chromosome

Chromosome duplication involves addition of chromosomal segment as a result of production of one or more copies of any piece of DNA or sometimes a gene or even an entire chromosome.

- A duplication of the chromosome is the opposite of a deletion. During a disease process, extra copies of the gene in the human genome can contribute to development of cancer.
- Genes can also duplicate in the evolution of human genome, where one copy of the original function and other copy of the gene produces new function. Duplication of whole chromosome causes disease.
- Chromosomal duplication happens during the crossing-over (recombination) between misaligned homologous chromosomes stage of meiosis. Duplication of chromosome results in trisomy (e.g. Down syndrome, XYY).
- Duplication of chromosome is detected by high-resolution banding, fluorescence *in situ* hybridization (FISH) and array comparative genome hybridization (array CGH).

Inversion of Chromosome

An inversion is a rearrangement in chromosome, in which a segment of a chromosome breaks at two regions is reversed end to end position in original chromosome after rotating by 180°. Inversion occurs when a single chromosome undergoes breakage and rearrangement within itself. As many as 1% of the newborn babies may carry an inversion that can be detected by G-banded

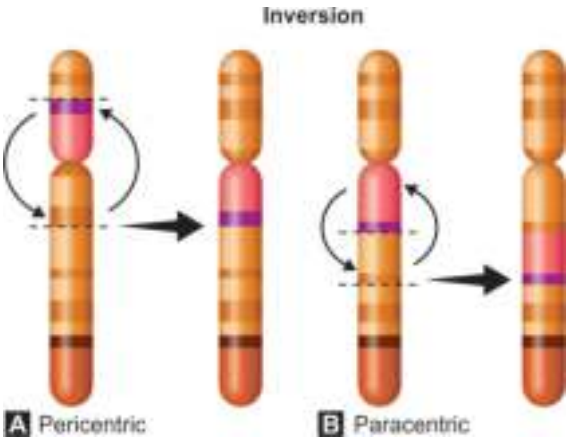


Fig. 5.23A and B: Inversion of chromosome. Inversion of chromosome requires two breaks in a single arm of chromosome. Inversions are of two types: pericentric (includes centromere) and paracentric (does not include centromere) turned through 180°.

chromosome karyotyping. Inversion produces unbalanced meiotic products; thereby resulting in sterility. There are two types of inversion: paracentric and pericentric. Inversion of chromosome is shown in Fig. 5.23A and B.

- **Differences between paracentric and pericentric inversions:** Paracentric inversion does not include the centromere and both breaks occur in one of the chromosomes. On the other hand, pericentric inversion includes the centromere and breaks occurs in both arms of chromosome. Differences and similarities between paracentric and pericentric inversions are given in Table 5.28.
- **Similarities between paracentric and pericentric inversions:** Both paracentric and pericentric inversions are large-scale chromosomal mutations occurring within a single chromosome. Both types of inversions do not cause a loss of genetic information. Both paracentric and pericentric inversions simply rearrange the linear gene sequence of a chromosome.

CHROMOSOMAL TRANSLOCATION

Chromosomal translocations involve breaks in two different nonhomologous chromosomes with exchange of chromosomal segments. Translocation may be balanced (reciprocal translocation in chronic myelogenous leukemia without loss of genetic material), or interstitial translocation (loss of segment of chromosome and reattaches to non-homologous chromosome).

Reciprocal or Balanced Chromosomal Translocation

A 'reciprocal' translocation occurs when there are break points in two chromosomes, with exchange of the fragments between two nonhomologous chromosomes, without loss of chromatin. Balanced translocation is often clinically silent. Philadelphia chromosome t(9; 22) discovered by Hugo de Vries in chronic myelogenous leukemia is an example of reciprocal translocation. Positivity of this Philadelphia chromosome t(9;22) indicates good prognosis in these patients. Reciprocal chromosomal translocation is shown in Fig. 5.24.

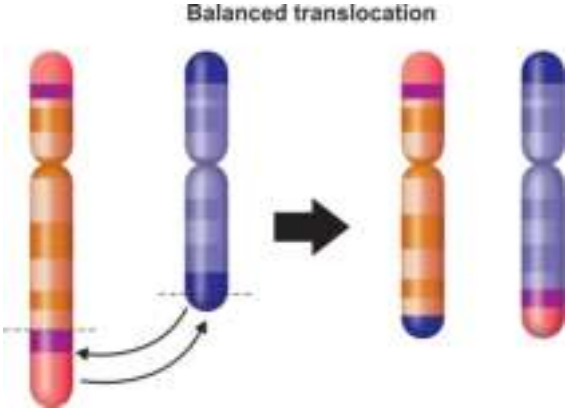


Fig. 5.24: Reciprocal chromosomal translocation. Reciprocal translocation is a chromosome rearrangement involving the exchange of chromosome segments between two chromosomes that do not belong to the same pair of chromosomes.

Table 5.28 Differences and similarities between paracentric and pericentric inversions

Parameters	Paracentric Inversion	Pericentric Inversion
Differences between paracentric and pericentric inversions		
Definition	Paracentric inversion is a type of chromosomal inversion in which a chromosomal segment that does not contain the centromere region rearranges in reverse orientation	Pericentric inversion is other type of inversion, in which a chromosomal segment containing the centromere segment containing the rearranges in reverse orientation
Breakpoint segment	Does not include a centromere	Centromere is included
Involvement of chromosome arms	Occurs in one arm of chromosome	Occurs in both arms of chromosome
Similarities between paracentric and pericentric inversions		
Mutation	Large scale mutation within same chromosome	Large scale mutation within same chromosome
Loss of genetic material	Nil	Nil

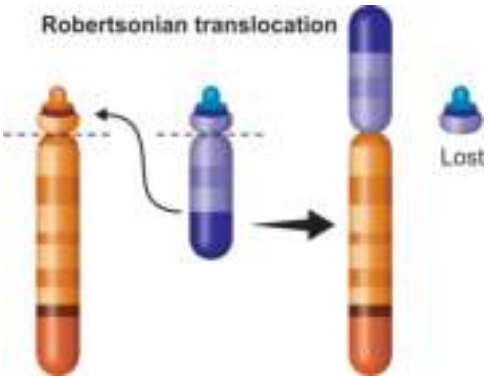


Fig. 5.25: Robertsonian translocation. In Robertsonian translocation, two nonhomologous acrocentric chromosomes break near centromeres, after which the long arms fuse to form one large metacentric chromosome. It is the most common type of chromosomal translocation in human beings.

Robertsonian Translocation

Robertsonian translocation is centric fusion of two acrocentric chromosomes resulting in the formation of one large metacentric chromosome and one small fragment. Robertsonian translocation is shown in Fig. 5.25.

- Acrosomal chromosomes with very short 'p' arms break very close to centromere leading to subsequent fusion of long 'q' arms. The human genome includes five acrocentric chromosomes: 13, 14, 15, 21, 22.
- The Y chromosome is also acrocentric chromosome. Robertsonian translocation predisposes to Down syndrome (14q;21q). Chromosome 21 is joined to a second acrocentric chromosome, commonly chromosome 14 or 22. The union of a gamete with this translocation with a gamete from an unaffected person can result in trisomy 21. There is high incidence of spontaneous abortion of fetuses.

Isochromosome

An isochromosome is an unbalanced structural abnormality created when the area surrounding the centromere in the pericentric region is divided transversely or perpendicular to the long axis of the chromosome. Isochromosome is shown in Fig. 5.26.

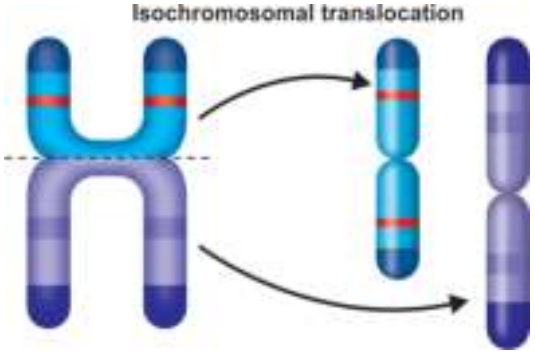


Fig. 5.26: Isochromosome arises from faulty centromere division, which leads to duplication of the long arm and deletion of the short arm, or the reverse.

- Isochromosome is formed by the duplication of one arm of a normal chromosome with deletion of the other arm. Both arms of the isochromosome are thus genetically identical.
- The chromosome consists of two copies of either the long (q) arm or the short (p) arm because isochromosome formation is equivalent to a simultaneous duplication and deletion of genetic material.
- As a result, there is partial trisomy of the genes present in the isochromosome and partial monosomy of the genes in the deleted arm. Example involving isochromosome (X chromosome) is observed in 15% cases of Turner's syndrome.

Ring Chromosome

Ring chromosome is formed by a break at both terminal (telomeric) ends of a chromosome followed by fragments and end to end fusion of fragments. The consequences depend on the amount of loss of genetic material as a result of break. Ring chromosome may form in cells following DNA damage by mutagens like radiation, but it may also arise spontaneously during development. Ring chromosome is shown in Fig. 5.27.

SOMATIC MOSAICISM

Somatic mosaicism is caused by an error (mutation) in the cell division very early in the development of the fetus, which occurs in two genetically distinct populations of cells with an individual derived a postzygotic mutation.

- In contrast to inherited mutations, somatic mutations can affect only a portion of the body and are not transmitted to the progeny. These mutations can affect varying genome size ranging from single nucleotide base pair to entire chromosome, which have been implicated in disease.
- Examples of mosaicism include mosaic Down syndrome, mosaic Klinefelter syndrome and mosaic Turner syndrome.

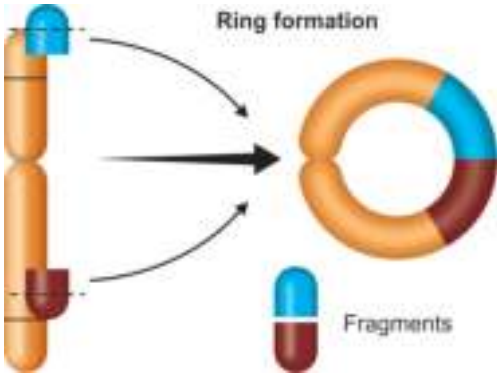


Fig. 5.27: Ring chromosome. A ring chromosome is an aberrant chromosome whose ends have fused together to form a ring as a result of DNA damage by mutagens or spontaneously during development.

Mosaic Down Syndrome

Persons with mosaic Down syndrome have a mixture of cells. Some cells have two copies, and some have three copies of chromosomes.

- A baby would be said to have mosaic Down syndrome if 5 of the 20 cells contain typical number of 46 chromosomes. The other 15 cells have a total of 47 chromosomes due to an extra chromosome 21.
- People with mosaic Down syndrome do not exhibit the physical features associated with Down syndrome individuals often go undiagnosed throughout their adulthood of this chromosome anomaly.

Mosaic Klinefelter Syndrome

Mosaic Klinefelter syndrome (46, XY/47, XXY) is not inherited, which results from random error during cell division early in the development of the fetus. As a result, there are two cell lines within the body. Some of the body's cells have usual one Y chromosome (46, XY), and other cells have an extra copy of the X chromosome (47, XXY). Persons with **mosaic Klinefelter syndrome** may have less severe signs and symptoms.

Mosaic Turner Syndrome

Mosaic Turner syndrome is also not inherited, which occurs as a random event during cell division in early fetal development. As a result, some of body's cells have the usual two sex chromosomes (46, XX) and other cells have only one copy of the X chromosome (45, X0).

- Girls and women with mosaic Turner syndrome tend to have fewer signs and health problems than those with typical Turner syndrome. It is because only some cells lack the second X chromosome in mosaic Turner syndrome.
- On the other hand, all cells in the body lack second X chromosome. The loss or change of the X chromosome happens very early in pregnancy by chance.

Confined Placental Mosaicism

Confined placental mosaicism (CPM) is defined as the presence of chromosomal abnormalities in the extraembryonic tissue (placenta), whereas cells in the fetus have normal karyotype. Therefore, placenta may be smaller in size than normal, which accounts for stillbirth or fetus growth retardation with normal karyotype. Conversely, a normal placental karyotype may allow longer survival of a fetus with abnormal karyotype. Confined placental mosaicism was first described by Kalousek and Dill in 1983.

MARKER CHROMOSOMES

Marker chromosomes are small additional chromosomes along with a normal set of chromosomes found during chromosome karyotyping that are usually derived from a structural rearrangement of chromosome X, 15, and 22. These are also referred to as supernumerary marker chromosome.

- Marker chromosomes may be in the form of ring or biosatellite chromosomes with or without centromeres. Marker chromosome with a centromere is relatively stable. However, marker chromosome without a centromere tends to be smaller with mitotic instability, which contain variable amount of heterochromatin or euchromatin.
- If marker chromosome arises *de novo*, then there is an increased risk for development of fetus mental retardation. However, if a parent has the marker as well, then it is familial and unlikely to be associated with mental retardation. Identification of the marker chromosome can be achieved by fluorescence *in situ* hybridization (FISH) or microarray chip assay.

DISORDERS OF AUTOSOMAL CHROMOSOME

Any chromosome except for the sex chromosome is known as autosome. Human beings have 44 autosomal chromosomes or autosomes. Chromosomal abnormalities usually happen due to one or more errors in cells: (a) errors during dividing of autosomes, (b) errors during dividing of sex cells, and (c) exposure to teratogenic substances resulting in birth defects.

DOWN SYNDROME (TRISOMY 21)

Down syndrome (trisomy 21) is a disorder, in which a person has an extra chromosome, i.e. three copies of a chromosome 21 instead of the usual two in every cell of the body as a result of error in cell division called 'nondisjunction' (failure of separation) of chromosome 21 in spermatozoa or ovum prior or at conception.

- The extra genetic material results in developmental changes and physical features of baby, as it grows during pregnancy and after birth. Baby is born with 46 chromosomes with extra copy of one of these chromosomes.
- Other common mechanisms that can give rise to Down syndrome include: Robertsonian translocation, isochromosome, or ring chromosome. Incidence of Down syndrome (trisomy 21) is 1 in every 1000 live births.
- There are three types of Down syndrome: (a) trisomy 21 (nondisjunction), (b) translocation type, and (c) mosaicism type. Schematic representation of Down syndrome (trisomy 21) is shown in Fig. 5.28.
  - **Down syndrome (trisomy 21):** Down syndrome (trisomy 21) accounts for 95% of all cases of Down syndrome, which occurs due to maternal meiotic nondisjunction of chromosomes. Patient has three copies of chromosome 21 rather than two copies

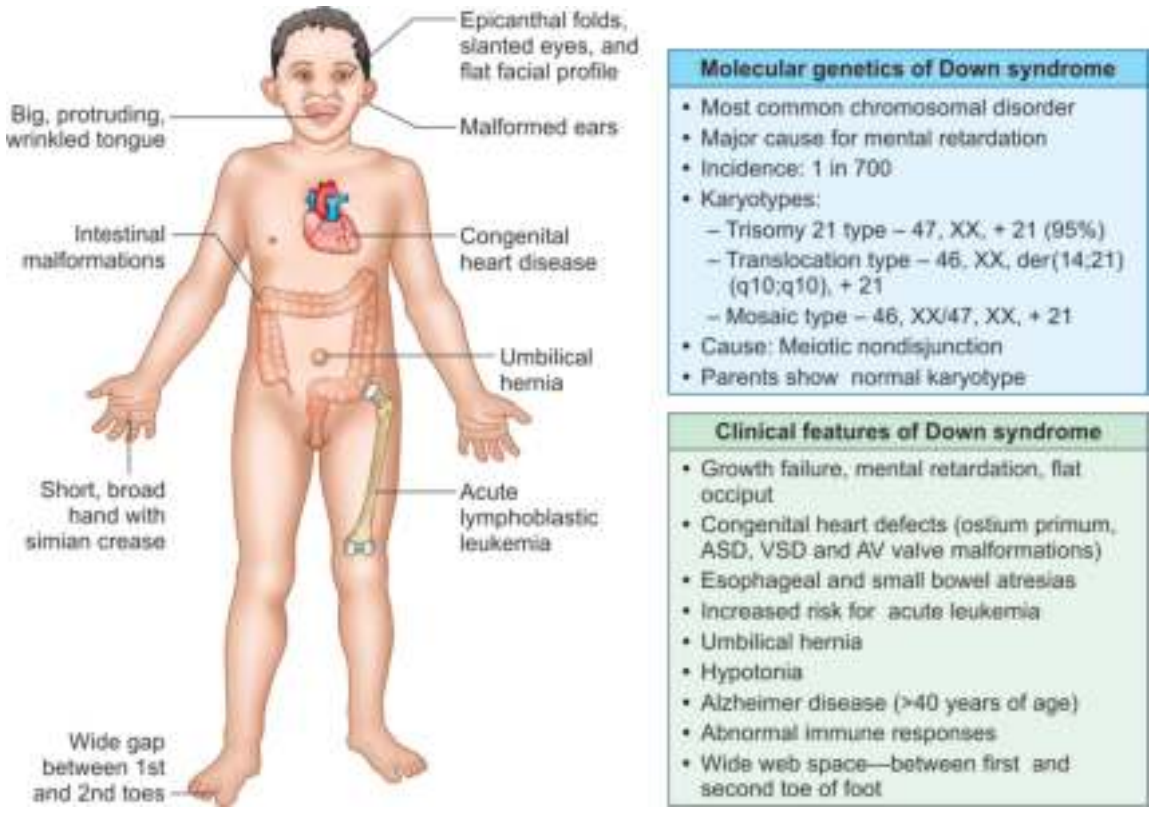


Fig. 5.28: Schematic representation of Down syndrome (trisomy 21). Patient with Down syndrome shows molecular genetic defects and clinical features.

of chromosome 21 in every cell of the body with chromosomal pattern 47, XX, +21. Incidence of Down syndrome increases with maternal age. When the cause is paternal nondisjunction, there is no relation to paternal age.

- **Down syndrome (chromosomal translocation type):** Down syndrome (chromosomal translocation type) accounts for 4% of all cases. Translocation of an extra-long arm of chromosome 21 to another acrocentric chromosome causes 46, XX, der(14; 21) (q10;q10), +21 pattern in the patient. There is no relation to maternal age. There is increased risk of Down syndrome in subsequent children.
- **Down syndrome (mosaic pattern type):** Persons with Down syndrome (mosaic pattern type) have a mixture of cells. Some of cells have extra copy of chromosome 21 and some cells lack extra copy of chromosome 21. People with Down syndrome (mosaic pattern type) do not exhibit the physical features of Down syndrome. These individuals often go undiagnosed throughout their adulthood.

Clinical Features

Patient suffering from Down syndrome presents with low intelligence quotient (IQ) ranging between 20 and 70, poor muscle tone, Mongolian idiocy (flat facial profile, oblique palpebral fissures and epicanthic folds), short neck with excess skin at the back of neck, large forehead, broad nasal bridge, wide-spaced upward slanting eyes often with a skin fold that comes out from the upper eyelid and covers the inner corner of the eye, large protruding tongue and small low-set ears.

- Small white spots are present at the periphery of iris known as Brushfield spots. Virtually all males are sterile but some females can reproduce.
- Musculoskeletal abnormalities in Down syndrome include short, broad hands with curvature of the fifth finger; simian crease, a single palmar crease; and an unusually wide space between the first and second toes. Clinical features and complications of Down syndrome are given in Table 5.29.

Maternal Screening

Maternal screening is performed by ultrasonography and hormonal assay. Ultrasonography reveals physical defects such as increased nuchal fold. The mother has high levels of serum human chorionic gonadotropin (hCG) and low levels of α-fetoprotein, and unconjugated estriol ('triple screen'). When mother has high levels of serum inhibin A, it is referred to as

Table 5.29 Clinical features and complications of Down syndrome (trisomy 21)

Body System	Physical Findings and Complications
Down syndrome: types	
Down syndrome (trisomy 21)	▪ Trisomy 21 (maternal meiotic nondisjunction of chromosome 21) ▪ Robertsonian translocation (14;21) ▪ Mosaic 21 (presence of two lines of cells)
Clinical features	
Intelligence quotient (IQ)	Low IQ ranging between 20 and 70
Facial features	▪ Mongolian idiocy (flat facial profile with large protruding tongue) ▪ Short neck with excess skin at the back of neck, large forehead and small low-set ears ▪ Wide-spaced upward slanting eyes often with a skin fold that comes out from the upper eyelid and covers the inner corner of the eye ▪ Oblique palpebral fissures and epicanthic folds broad nasal bridge ▪ Small white spots are present at the periphery of iris known as 'Brushfield spots'
Reproductive system	▪ Virtually all males are sterile ▪ Some females can reproduce
Musculocutaneous system	▪ Short, broad hands with curvature of the fifth finger; simian crease, a single palmar crease; and an unusually wide space between the first and second toes ▪ Poor muscle tone
Complications	
Congenital heart disease	▪ Atrial septal defect (ASD) ▪ Ventricular septal defect (VSD) ▪ Atrioventricular valve malformation (prolapse)
Gastrointestinal tract disorders	▪ Atresia of esophagus ▪ Atresia of small bowel ▪ Hirschsprung disease
Hematological disorder	▪ Acute lymphoblastic leukemia (ALL) ▪ Acute megakaryoblastic leukemia
Abdominal defects	Umbilical hernia
Central nervous system disorder	Alzheimer disease
Immune system disorder	Susceptibility to infection

Approximately 75% of fetuses with Down syndrome abort spontaneously. About 20% of patients die before the age of 10 years. Rest may have life expectancy of 60 years.

the 'quadruple' screen. There is also reduced levels of plasma protein A during pregnancy.

EDWARD SYNDROME (TRISOMY 18)

Edward syndrome (trisomy 18) is the second most common type of trisomy syndrome after Down syndrome (trisomy 21). Incidence of trisomy 18 is 1 in every 5,000 live births, which is more prevalent in female offspring. Karyotyping reveals trisomy 18 type (47, XX, +18) and mosaic type (46 XX, 47, XX, +18). Trisomy 18 is most often caused by extra copy of chromosome 18 in each cell in the body, instead of two copies. Edward syndrome (trisomy 18) is shown in Fig. 5.29.

Clinical Features

Patient suffering from Edward syndrome (trisomy 18) presents with mental retardation, prominent occiput, micrognathia (small lower jaw), low-set ears, rocker-bottom feet, flexion deformities of the fingers (index overlapping third and fourth), and congenital heart disease. Many babies cannot survive past the second or third trimester of pregnancy.

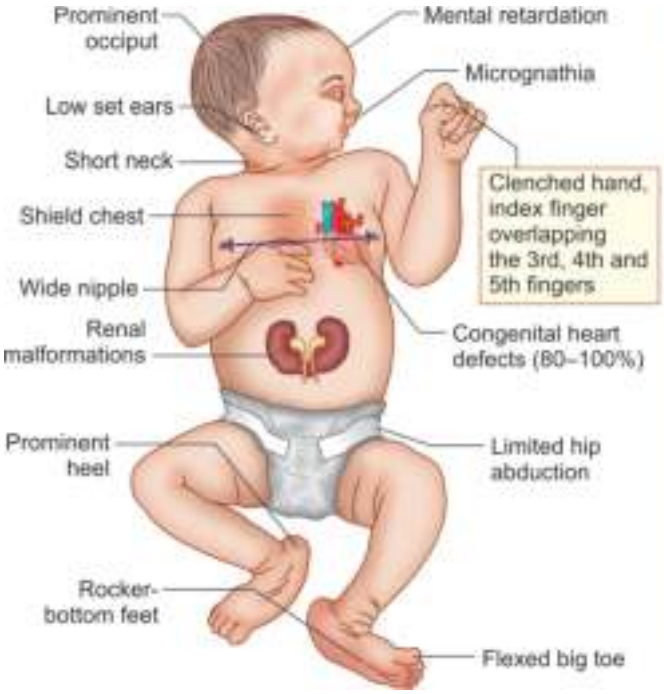
Laboratory Diagnosis

Ultrasonography is not accurate to diagnose Edward syndrome (trisomy 18) during pregnancy. More precise diagnostic methods include chorionic villus sampling and amniocentesis.

PATAU SYNDROME (TRISOMY 13)

Patau syndrome (trisomy 13) incidence is 1 in every 15,000 live births. Each cell in the body has three copies of chromosome 13, instead of the usual two in Patau syndrome.

- Most cases of Patau syndrome (trisomy 13) are not inherited. Patau syndrome results from errors in cell division, i.e. nondisjunction of chromosomes in cells with abnormal number of chromosomes.
- There are three types of Patau syndrome: (a) severe type trisomy 13 (nondisjunction, i.e. 47, XX, +13), (b) translocation (46, XX/47, XX, +13) and (c) mosaic type with some cells with extra copy of chromosome 13 (46, XX/47, XX, +13). Patau syndrome (trisomy 13) is shown in Fig. 5.30.



Edward syndrome (trisomy 18)

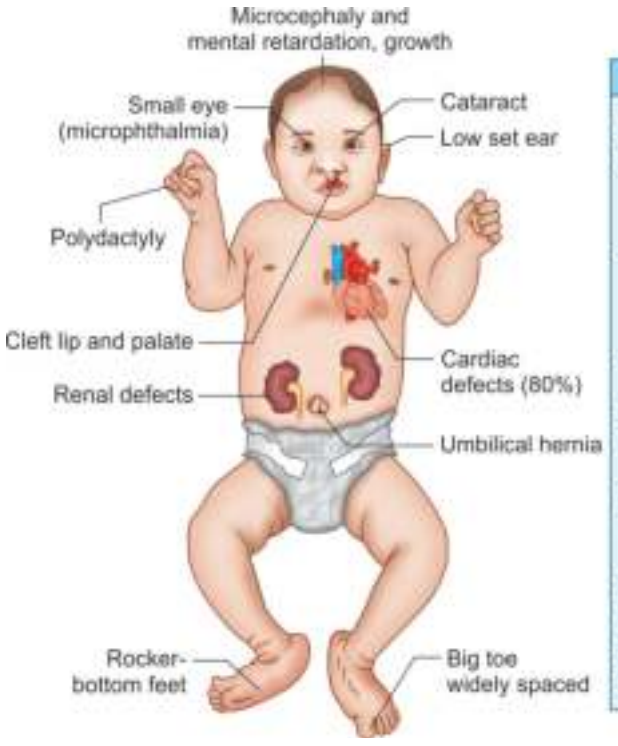
General considerations
- Incidence 1 in 8,000 births, higher in females
- Severe type (95%)
  - Trisomy 18 (47, XX, + 18)
- Mosaic type [less severe form (5%)]
  - 46, XX/47, XX, + 18
  - Some cells with two copies of chromosome 18
  - Some cells with three copies of chromosome 18

Cardiovascular disorders (80–100%)
- Ventricular septal defect
- Atrial septal defect
- Multivalvular disease
- Patent ductus arteriosus

Respiratory system disorders
- Upper airway obstruction
- Apnea

Gastrointestinal tract disorders
- Omphalocele
- Pyloric stenosis
- Tracheoesophageal fistula
- Imperforate anus

Fig. 5.29: Edward syndrome (trisomy 18) shows clinical features and genetic abnormalities.



Patau syndrome (trisomy 13)

General characteristics
- Incidence 1 in 15,000 births
- Severe type (trisomy 13.47 XX, + 13), infants die within one year of age
- Mosaic type: 46, XX/47, XX, +13 (some cells with two copies and some cells with three copies of chromosome)
- Translocation type (46, XX/47, XX, + 13)

Head and neck findings
- Microcephaly and mental retardation
- Broad flat face
- Slanting small eyes (microphthalmia)
- Short nose
- Cleft lip and palate
- Epicanthic eye folds
- Big wrinkled tongue
- Flat back

Upper limb findings
- Polydactyly (extra finger and toe)
- Overlapping of fingers over thumb
- Strange palm pattern (simian line)

Lower limb findings
- Prominent heel
- Deformed feet (rocker-bottom feet)
- Big toes widely spaced

Cardiac, renal and other defects
- Cardiac defects (80%)
- Renal defects
- Umbilical hernia

Fig. 5.30: Patau syndrome (trisomy 13) shows clinical features and genetic abnormalities.

Clinical Features

Patient suffering from Patau syndrome (trisomy 13) presents with mental retardation, microcephaly, microphthalmia, brain abnormalities, sloping forehead, cleft lip and cleft palate, low set ears, polydactyly, rocker-bottom feet, and congenital heart disease but with normal weight. It is difficult to predict the life expectancy of a baby with trisomy 13.

- Life span varies from one month, one year of 10 years.
- Ultrasonography is not 100% accurate to diagnose Patau syndrome (trisomy 13) during pregnancy.

Laboratory Diagnosis

Diagnosis of Patau syndrome (trisomy 21) can be confirmed prenatally with better than 99% accuracy through chorionic villus sampling and amniocentesis.

CRI-DU-CHAT (5p MINUS, CAT-CRY) SYNDROME

Cri-du-chat syndrome is also known as 5p– (5p minus) syndrome or cat-cry syndrome. It is a genetic disorder present since birth, that affecting both males (46, XY/5p minus) and females (46, XX/5p minus). Cri-du-chat syndrome is caused by the deletion of genetic material on the small arm (p arm) of chromosome 5.

- Infants with this disorder often has severe mental retardation, microcephaly, a high-pitched cry sounds that of a cat.
- On clinical examination, patient have low birth weight, round face, hypertelorism (wide-set eyes), low-set ears, and epicanthal folds. Clinical features of cri-du-chat syndrome in 80–90% of patients are given in Table 5.30.

DIGEORGE SYNDROME

DiGeorge syndrome is also known as microdeletion of 22q11 syndrome or velocardiofacial syndrome caused by the deletion of a small segment of chromosome 22q11.2.

- During intrauterine life, aberrant embryonic development of third and fourth branchial arches results in hypoplasia of thymus and parathyroid glands as well as anomalies of aortic arch, mandible and ear.
- Patient develops failure of maturation of T cell, but normal B cells functions resulting in recurrent infections. **CATCH-22 syndrome** also occurs due to microdeletion in chromosome 22q11.2.

Table 5.30 Clinical features of cri-du-chat syndrome in 80–90% of patients

Cri-du-chat syndrome is also known as 5p– (5p minus) syndrome or cat-cry syndrome
Epicanthus (prominent eye folds)
High-pitched voice
Intellectual disability (early severe mental retardation)
Low set posteriorly rotated eyes
Microcephaly (abnormally small skull, decreased circumference of cranium)
Microretrognathia (small protruded tongue)
Muscular hypotonia (low or poor muscular tone)
Round face
Severe global developmental delay
Wide nasal bridge

- Patient suffering from CATCH-22 syndrome presents with cardiac defects, abnormal facial features, thymic hypoplasia, cleft palate, hypoparathyroidism, hypocalcemia, tetany and recurrent infections. About 33% of cases develop behavior disorders and psychosis (bipolar disorder and schizophrenia) during adolescence.

SEX CHROMOSOME DEVIATION

Extreme karyotype deviations in the sex chromosomes are compatible with life; this is believed to be due to X chromosome inactivation (**lyonization**) and the relatively scanty genetic information carried by the Y chromosome.

Key Facts: Mary Lyon's Hypothesis of X Chromosome Inactivation

- Mary Lyon hypothesized that one of two X chromosomes in female cells derived from paternal or maternal is inactivated at random during early embryogenesis and that the inactive X chromosome is then maintained in a stable manner through all subsequent cell divisions. This inactivation of X chromosome is transmitted to all somatic cells except germ cells.
- Ovaries will always possess active chromosomes. In females, the inactive X chromosome in the somatic cells becomes condensed in the nucleus is called nuclear Barr bodies.
- Smears stained by scrapping of oral mucosa or circulating neutrophils demonstrate 'Barr bodies', which appear as drumstick appendage attached to one of the nuclear lobes.
- A minimum of 30% cells positive for sex chromatin (Barr body) indicates female genetic constitution.

BARR BODIES

Barr body is also known as sex chromatin, which appears as clumps of chromatin in the interphase nuclei of all somatic cells in females. Barr bodies are shown in Fig. 5.31.

- According to 'Lyon hypothesis', each Barr body represents one inactivated X chromosome. One Barr body is present in normal female cells (XX).
- Barr bodies are absent in males (XY). The number of Barr bodies is always one less than the number of X chromosomes.
- Barr body is absent in Turner syndrome with 45, XO karyotyping. One Barr body is present in Klinefelter syndrome with 47, XXY karyotyping. Two Barr bodies are demonstrated in 47, XXX (triple X) syndrome. Three Barr bodies are present in poly-X female with 48, XXXX karyotyping.
- Assessment of Barr bodies was considered as diagnostic tool in past. Now it has been supplanted by more definitive and sophisticated analytical

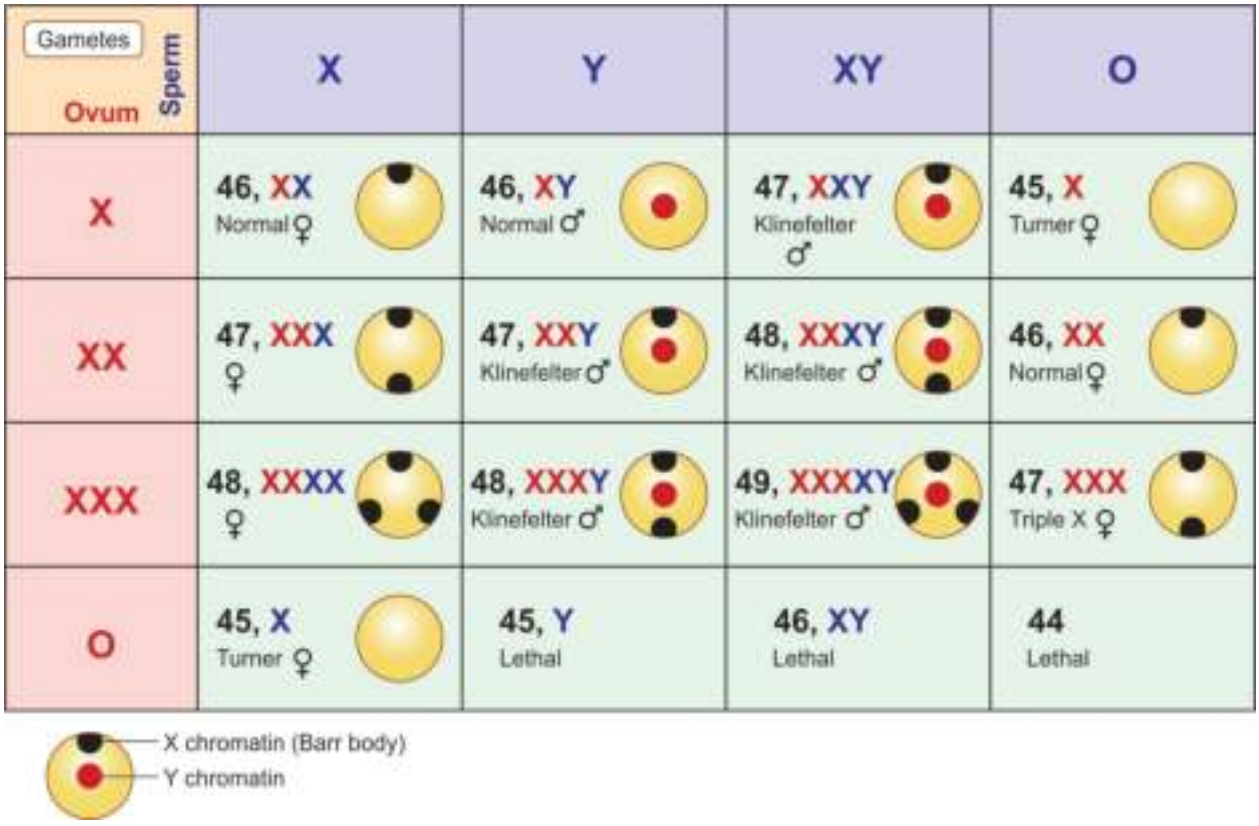


Fig. 5.31: Barr bodies. Barr body is a small, densely staining structure in the cell nuclei of females, consisting of a condensed, inactive X chromosome. It is regarded as diagnostic of genetic femaleness.

procedures. Number of different Barr bodies in human cells in different disorders related to sex chromosomes are given in Table 5.31.

X CHROMOSOME INACTIVATION

Females have two X chromosomes (XX), while males have only one X plus a Y chromosome. X chromosome inactivation is the process by which all X chromosomes except one are randomly inactivated at an early stage of embryonic development.

- X chromosome inactivation results in all normal females being mosaics, with two distinct cell lines, one with an active maternal X, and another with an active paternal X. It can be demonstrated if the female is heterozygous for an X-linked gene; if individuals demonstrate inheritable differences that distinguish the protein products of one X chromosome from the other, then members of the two cell lines can be identified.
- The X-inactive-specific transcript (Xist) is a large untranslated RNA molecule associated with 'coating' and inactivating of one of the two X chromosomes. All chromosomes except a single remaining X chromosome are inactivated. The phenotypic differences between XO, XX, and multiple X genotypes are thought to be caused by residual genes on the X chromosome that escape inactivation.

Table 5.31 Number of different Barr bodies in human cells in different disorders related to sex chromosomes

Sex Chromosomes	Disorder	Number of Barr Bodies
46, XX karyotyping in female	None	1
46, XY karyotyping in male	None	0
45, XO karyotyping	Turner syndrome	0
47, XXY karyotyping	Klinefelter syndrome	1
47, XXX karyotyping	Triple X syndrome	2
XXXX	Poly-X female	3

DISORDERS OF SEX CHROMOSOME

Classical disorders of sex chromosome are Klinefelter's syndrome, Turner syndrome, XYY syndrome, XXX syndrome (47, XXX), XY female gonadal dysgenesis (known as Swyer syndrome) with normal external genitalia; and other multi-X chromosome anomalies. Majority of disorders such as true hermaphroditism, mixed gonadal dysgenesis, and pure gonadal dysgenesis have abnormal sex chromosome.

KLINEFELTER SYNDROME (TRISOMY 47, XXY)

Klinefelter syndrome (trisomy 47, XXY) or testicular dysgenesis is the most common sex chromosome disorder in males in which an extra copy of the X chromosome is present, resulting in an XXY sex chromosome karyotype.

- The additional X chromosome(s) arises as a result of nondisjunction during gametogenesis. Incidence of this disorder is 1 in 1000 live births.
- Klinefelter syndrome is the most common genetic cause of male infertility, but many cases remain underdiagnosed because of substantial variations in Klinefelter syndrome such as 48, XXYY or 49, XXXXY or mosaic types. Klinefelter syndrome can shorten life expectancy up to two years. Schematic representation of Klinefelter's syndrome is shown in Fig. 5.32.

Clinical Features

Patient suffering from Klinefelter's syndrome presents with hypogonadism (atrophic testes), infertility and gynecomastia. Other manifestations include tall height, long arms, long legs, eunuchoid body status appearance, small penis, cryptorchidism, hypospadias, radioulnar synostosis, delayed puberty and lack of secondary male characteristics such as deep voice, beard, and male distribution of pubic hair.

Laboratory Diagnosis

Klinefelter syndrome may be suspected during a noninvasive prenatal screening blood test. Invasive prenatal amniocentesis and chorionic villi sampling are done to confirm the diagnosis.

- Blood or urine specimens can reveal abnormal hormone levels in Klinefelter syndrome. Plasma levels of hormones are elevated such as follicular

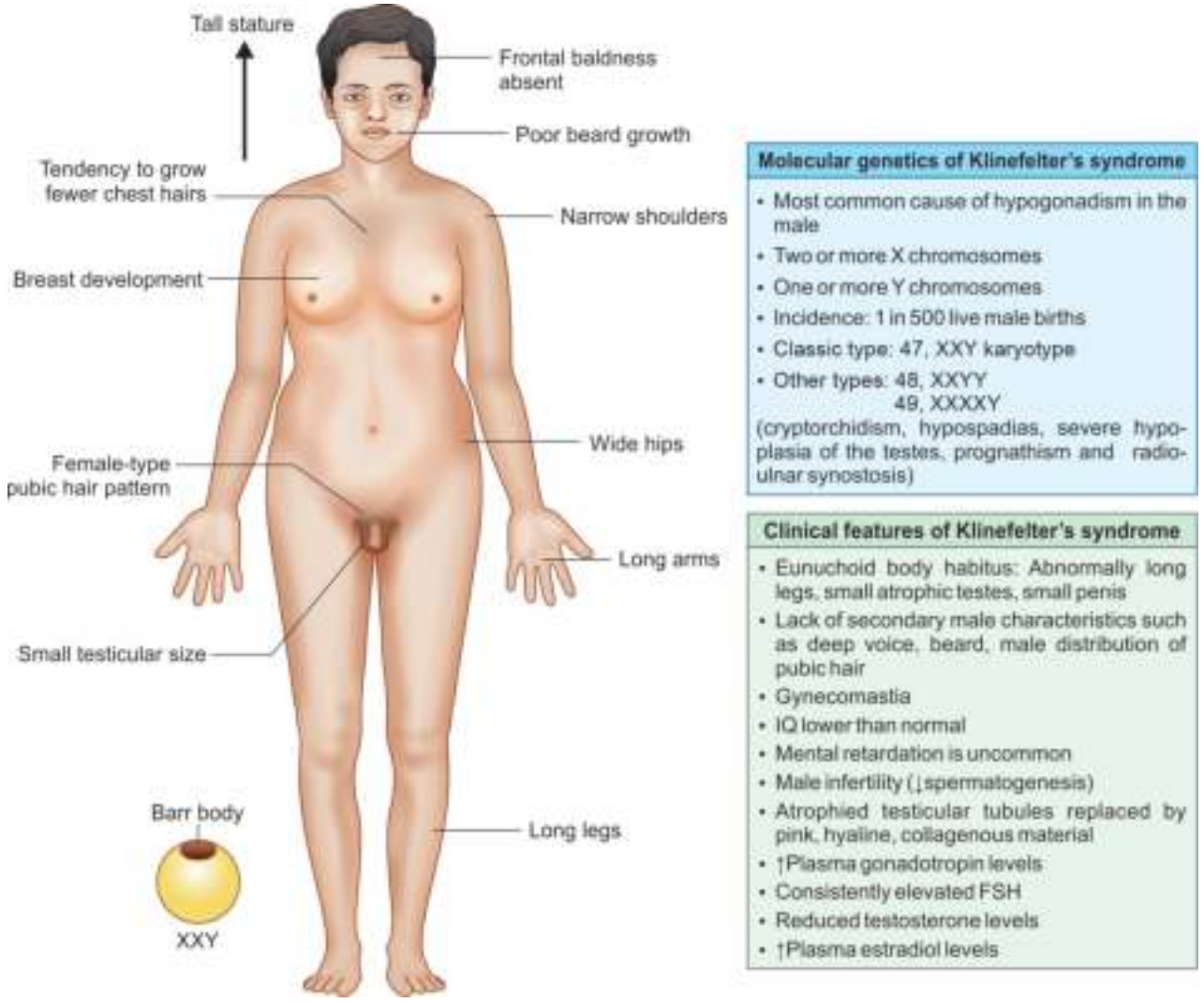


Fig. 5.32: Schematic representation of Klinefelter's syndrome. It shows molecular genetic abnormalities and clinical features such as short stature, pubertal delay and gynecomastia.

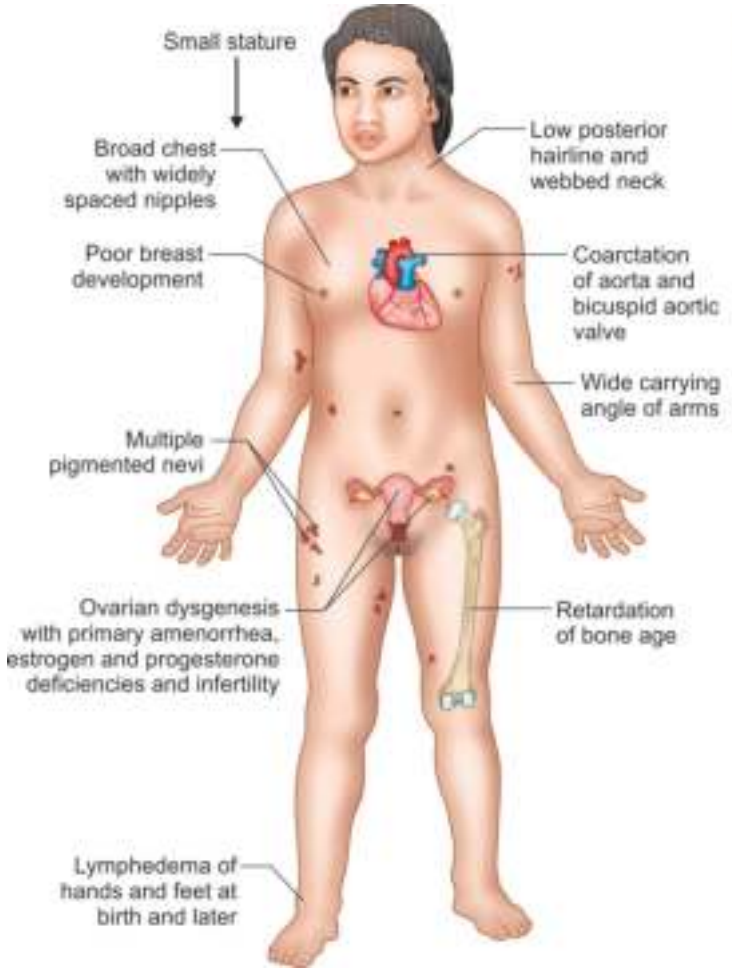
stimulating hormone (FSH), luteinizing hormone (LH), plasma pituitary gonadotropin and estradiol. Plasma testosterone level is markedly reduced due to loss of feedback inhibition in affected males.

- Karyotype analysis is done to confirm the diagnosis. Normal female (XX) has one Barr body. Normal male (XY) lacks Barr body. In **Klinefelter syndrome (XXY)**, patient shows **one Barr** body.

TURNER SYNDROME (MONOSOMY 45, X/XO)

Turner syndrome, also known as congenital ovarian hypoplasia syndrome or gonadal dysgenesis, is usually not inherited, but it is genetic chromosomal disorder in females called **monosomy** caused by a random error that leads to a complete or partial missing X chromosome in the sperm or ovum of a parent.

- Main clinical manifestations of Turner syndrome include infertility, rudimentary ovaries, early primary ovarian insufficiency, lack of menstrual cycle at menarche, short stature, lymphedema over dorsum of the hands, feet and nape of the neck, cardiovascular abnormalities and neurocognitive difficulties. **Infertility** is the most troubling problem of Turner syndrome. These females are unable to conceive even with years of estrogen replacement therapy. Turner syndrome is shown in Fig. 5.33.
- Turner syndrome demonstrates three patterns on karyotyping, classic pattern, mosaic pattern and structural pattern.
  - **Classic type of Turner syndrome:** There is complete or partial monosomy of the short arm of X chromosome (Xp), an XO karyotype (45, X) is characteristic in 57% of cases.
  - **Mosaic type of Turner syndrome:** A mosaic person has two lines of cells (one normal chromosome and another with monosomy single residual abnormal chromosome) for the affected chromosome pair due to anaphase lag. Turner's mosaicism is associated with 45, X/46, XX or 45, X/46, XY, 45, X/47, XXX, 45, X/46, X, i(X)(q10). A deletion of the SHOX gene can cause an identical phenotype and may be considered to be a variant of Turner syndrome.
  - **Structural abnormalities in second X chromosome of Turner syndrome:** These include 46, X, i(Xq) or 46, X, del(Xp), or 46, X, del(Xq) or 46, X, r(X) in 14% of cases.



Molecular genetics of Turner's syndrome

Incidence: 1 in 3000 female births

Karyotypes:

• Classic	45, X	(57%)
• Structural abnormalities in second X chromosome	46, X, i(Xq) 46, X, del(Xp) 46, X, del(Xq) 46, X, r(X)	(14%)
• Mosaic type:	45, X/46, XX 45, X/46, XY 45, X/47, XXX 45, X/46, X, i(X) (q10)	(29%)

Clinical features of Turner's syndrome

- During infancy (edema of the dorsum of the hand, foot, and nape of the neck)
- During puberty:
  - Failure to develop normal
  - Secondary sex characters: Infantile genitalia, breast development (inadequate, a little pubic hair)
- During adult life (short stature, amenorrhea)
  - Low posterior hairline
  - Bilateral neck webbing
  - Congenital heart disease (coarctation of aorta, bicuspid aortic valve)
  - Broad chest and widely spaced nipples
  - Cubitus valgus, streak ovaries, infertility
  - Pigmented nevi
  - Mental status: Normal/retardation
  - Hypothyroidism, glucose intolerance

Fig. 5.33: Turner syndrome shows molecular genetic abnormalities and clinical features such as amenorrhea, short stature, pubertal delay and obesity.

Clinical Features

Patient suffering from Turner syndrome presents with lymphedema on dorsum of the hands, feet and nape of the neck during infancy. At puberty, features include infantile genital organs, inadequate breast development, and little pubic hair.

- An adult female has short stature, webbed neck, shield-like chest with widely spaced nipples, bilateral neck webbing, and wide carrying angle of the arms, multiple nevi and cubitus valgus.
- Turner syndrome is also often associated with autoantibody-mediated hypothyroidism. Patient may have coarctation of the aorta and bicuspid valve congenital malformations.
- Most girls and women with Turner syndrome have normal intelligence. Most women with Turner syndrome cannot become pregnant naturally.

Laboratory Diagnosis

A diagnosis of Turner syndrome may be suspected when patient has physical features such as webbed neck, a broad chest and widely spaced nipples. Chromosomal composition of the female is analyzed by karyotyping technique. A standard 30-cell karyotype analysis is required for diagnosis of Turner syndrome, in order to exclude mosaicism. Diagnosis of Turner syndrome is confirmed by the presence of 45, X cell line or a cell line with deletion of the short arm of the X chromosome (Xp deletion).

JACOB SYNDROME (TRISOMY XYY)

Jacob syndrome (Trisomy XYY) is a rare sex chromosome disorder of a male infant born with an extra Y chromosome. Sex chromosome abnormality with XYY is also called Jacob syndrome. Incidence of this disorder is 1 per 1000 live births.

- Most cases are not inherited, and XYY syndrome occurs randomly in pregnancies of women from all ages and ethnic background. Normal female has two X chromosomes (XX), while normal male has one X chromosome and one Y chromosome (XY). However, male newborns with XYY syndrome have an extra Y chromosome in each cell of their body.
- Most of these cases occur due to a cell division error in the sperm prior to conception known as 'nondisjunction'(failure of separation). The exact cause of this nonjunction is not known.

Clinical Features

Male newborns suffering from Jacob syndrome (XYY syndrome) present with low-weight relative to tall height, large head dimensions, severe acne in adolescence, behavioral and emotional difficulties, learning disabilities, and delayed development of speech and language skills, delayed sitting and walking, hypotonia, hand tremors or motor tics, and slightly lower IQ than normal. Small percentage of cases of XYY syndrome have autistic spectrum of disease.

Complications

Patient with Jacob syndrome (XYY syndrome) experiences puberty at expected times, as well as normal development of sex organs and secondary sex characteristics. These cases may have decreased sperm quality with 'immature' spermatozoa often associated with male infertility.

Laboratory Diagnosis

Jacob syndrome (XYY) is usually diagnosed when prenatal karyotyping is done to detect Down syndrome by amniocentesis and chorionic villus sampling. A blood test can accurately diagnose Jacob syndrome (trisomy XYY).

TRIPLE X (47, XXX SYNDROME) AND OTHER MULTI-X CHROMOSOME ANOMALIES

Triple X (47, XXX syndrome) and other multi-X chromosome anomalies are usually unaccompanied by any clinical abnormalities, although they may be marked by menstrual irregularities. Additional X chromosomes beyond XXX are marked by progressively increasing mental deficiency, depending on the number of additional X chromosomes.

HUMAN GENE STRUCTURE AND MUTATIONS

GENE STRUCTURE

A gene is the basic physical and functional unit of heredity. Genes are made up of deoxyribonucleic acid. Each gene contains a 5′-regulatory or untranslated region (5′-UTR), where the transcription factors bind and initiate transcription of the primary transcript. Gene also contains three specific sequences of nucleotides: **exons** (coding sequence), intervening regions called **introns** (noncoding sequence) and regulatory sequences. Structure of human gene is shown in Fig. 5.34.

EXONS (CODING SEQUENCE) AND INTRONS (NONCODING SEQUENCE) IN GENE

Exons and **introns** are nucleotide sequences within a gene. An **exon** is the segment of a gene that encodes

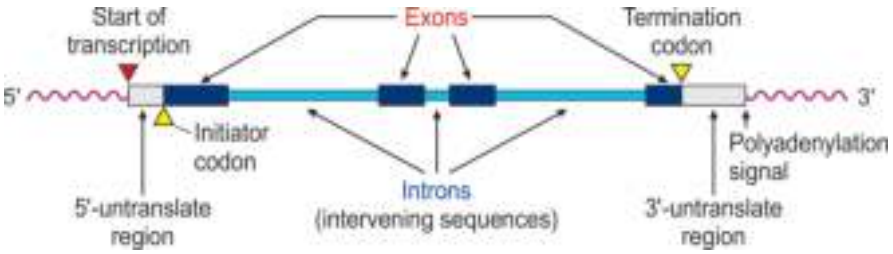


Fig. 5.34: Structure of human gene. Gene contains 5'→3'-untranslated regions, promoter, enhancer/silencer, exons (coding sequences), introns (noncoding sequences) and transcriptional and translational start and stop codon sites. Gene is composed of deoxyribonucleic acid (DNA).

the information for transcription and translation of sequence of amino acids to produce protein.

- Exons are separated by intervening sections of DNA known as introns that do not code for proteins.
- Following transcription, new immature strand of messenger RNA, called pre-mRNA. The pre-mRNA contains both exons and introns.
- The pre-mRNA molecule goes through modification process in the nucleus called **splicing** during which the noncoding introns are excised and only the coding exons remain with addition of 5′ cap and poly(A) tail.
- Splicing produces a mature messenger RNA molecule that is then translated into protein. Schematic representation of primary RNA transcript splicing and formation of messenger RNA is shown in Fig. 5.35.

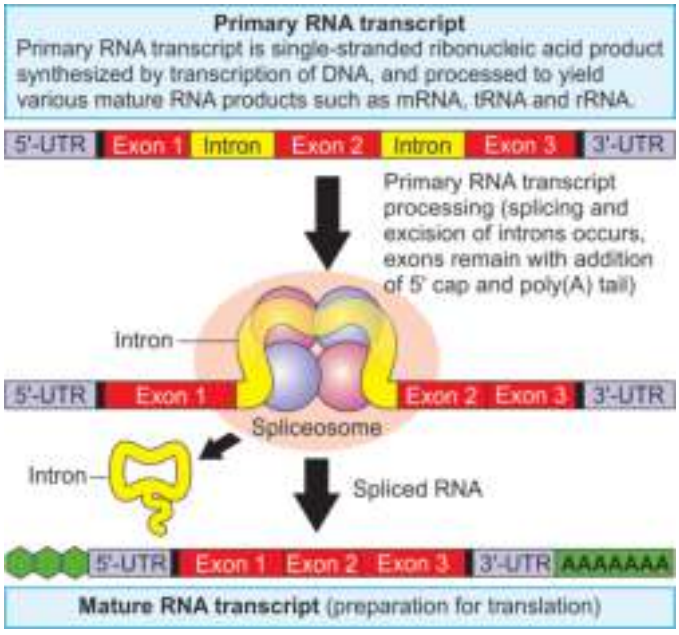


Fig. 5.35: Schematic representation of primary RNA transcript splicing and formation of messenger RNA. A primary transcript is the single-stranded ribonucleic acid product synthesized by transcription of DNA, and processed to yield various mature RNA products such as messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA).

REGULATORY REGIONS IN DNA: PROMOTERS, ENHANCERS AND SILENCERS

A regulatory sequence is a segment of deoxyribonucleic acid molecule which is capable of increasing or decreasing the expression of specific genes within an organism. Regulation of gene expression is an essential feature of all organisms. Schematic representation of regulatory regions in DNA is shown in Fig. 5.36.

- **Human gene** contains 5′→3′ untranslated regions, promoters, enhancers and silencers.
  - **Promoters** are DNA regions of 1–2 kilobases (kb) of a gene transcription start site, they contain short regulatory elements (DNA motifs) necessary to assemble RNA polymerase transcriptional machinery.
  - Enhancers are found in the same region of a gene. An enhancer is a sequence of DNA that functions to enhance transcription.

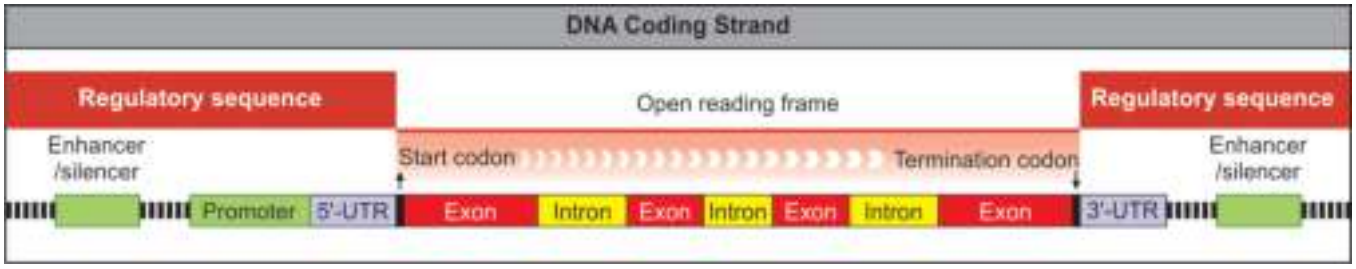


Fig. 5.36: Schematic representation of regulatory regions in DNA. Human gene is composed of deoxyribonucleic acid (DNA). The gene contains 5'→3'-untranslated regions, promoters, enhancers and silencers. Promoters are DNA regions within 1–2 kilobases (kb) of a gene transcription start site, they contain short regulatory elements (DNA motifs) necessary to assemble RNA polymerase transcriptional machinery. An enhancer is a sequence of DNA that functions to enhance transcription. A promoter has to be close to the gene that is being transcribed while an enhancer does not need to be close to the gene of interest.

- A promoter is a sequence of DNA that initiates the process of transcription. A promoter has to be close to the gene that is being transcribed while an enhancer does not need to be close to the gene of interest.

- Promoter and enhancer regions regulate the transcription of a gene into a pre-mRNA, which is modified to remove intervening introns and add a 5′ cap and poly(A) tail. It is the binding site for micro-RNAs (mRNAs).
- The mature messenger RNA (mRNA) 5′→3′-untranslated regions regulate translation into the final protein product. Each three-letter sequence of mRNA nucleotides corresponds to a specific amino acid, or to a stop codon. UGA, UAA and UAG are stop codons.
- Genetic code is universal. Silencers are noncoding regions, which can inhibit transcription.

Promoter Regulatory Region of DNA

A promoter is a regulatory region of DNA located upstream towards the 5′ region of a gene. A promoter contains specific DNA sequences that are recognized by proteins known as transcription factors (i.e. enhancers and silencers). A promoter regulates RNA polymerase, which binds and transcribes DNA to mRNA, which is ultimately translated into a functional protein.

Enhancer and Silencer Regulatory Region of DNA

Enhancer and silencer of regulatory region of DNA are located upstream towards 5′ region of a gene. Enhancer functions as a '**turn on**' **switch** in gene expression and activates the promoter region of a particular gene. Enhancer also functions with transcription factors to enhance the transcription of an associated gene. **Silencer** acts as the '**turn off**' **switch** in gene expression.

NONCODING REGULATORY RNA

A noncoding RNA (ncRNA) is a functional RNA molecule that is transcribed from DNA but not translated into proteins. Abundant and functionally important types of noncoding RNAs include transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), as well as small micro-RNAs such as micro-RNAs, siRNAs, piRNAs, snoRNAs, snRNAs, exRNAs, scaRNAs and the long ncRNAs such as Xist and HOTAIR. Schematic representation of noncoding regulatory RNAs is shown in Fig. 5.37.

MicroRNA (miRNA)

Human genome contains about 1000 microRNAs constituting 5% of total genome. MicroRNAs are mature oligomers of 22 nucleotides in length, which do not code for proteins. MicroRNAs participate in silencing

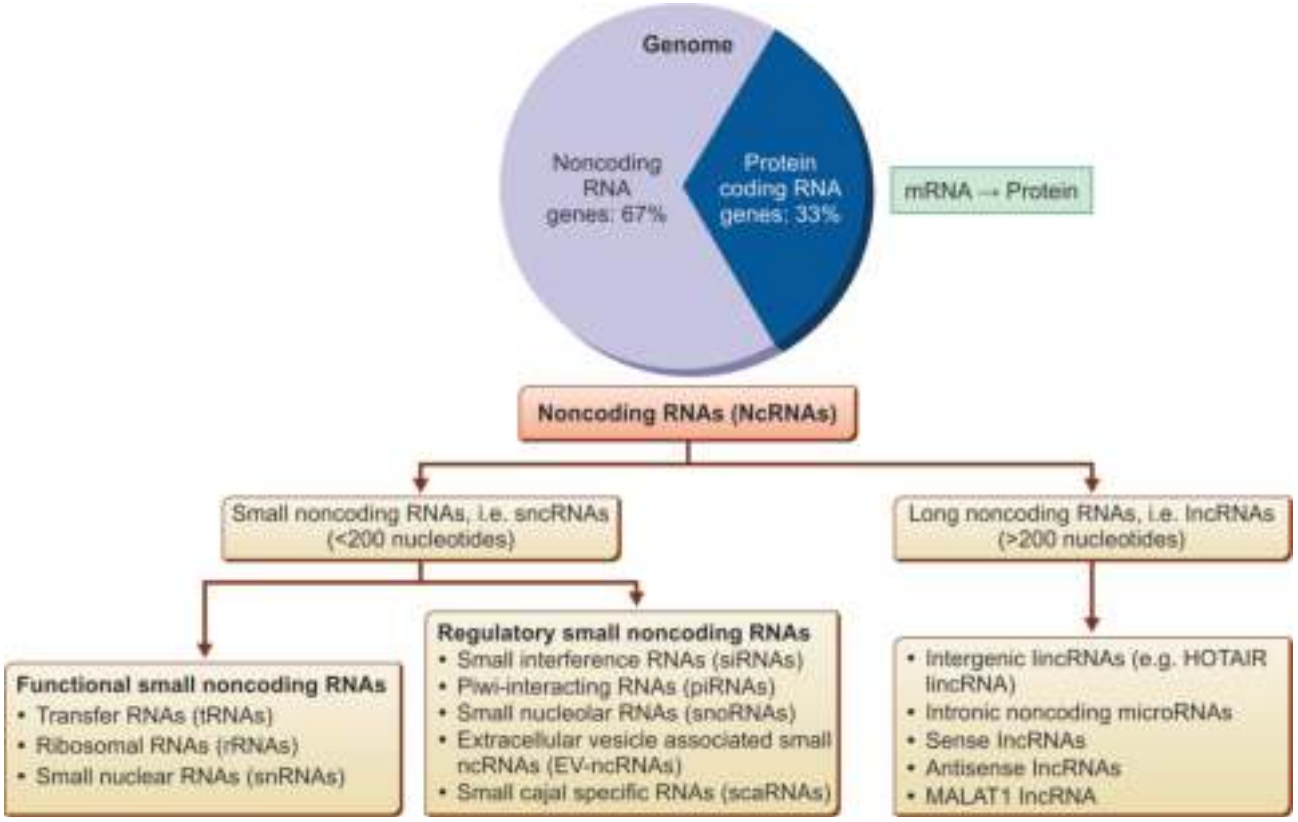


Fig. 5.37: Schematic representation of epigenetic-related small and long non-coding microRNAs(ncRNAs). Abundant and functionally important types of noncoding RNAs include transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), as well as small ncRNAs such as micro-RNAs, siRNAs, piRNAs, snoRNAs, snRNAs, exRNAs, scaRNAs and long lncRNAs (i.e.; intergenic, intronic (HOTAIR), sense, antisense and MALAT1)

post-transcriptional genes. All microRNAs comprise seed sequence in their 32 untranslated regions (UTR), which determine the specificity of miRNA binding and gene silencing.

Long Noncoding RNA (LncRNA)

Long noncoding RNA (LncRNA) participates in activation of gene, which inhibit gene transcription. Example of repressive function involves Xist (X inactive specific transcript). Xist inactivates X chromosomes resulting in Barr body formation. LncRNA stabilizes secondary or tertiary structure of protein that influence gene activity. It is linked to atherosclerosis and cancer.

START AND STOP CODONS

Cells decode mRNAs by reading their nucleotides in a group of three, called codons. Most codons specify an amino acid. Codons in an mRNA are read during translation, beginning with a start codon and continuing until stop codon is reached.

- Messenger RNA (mRNA) codons are read from 5′→3′, and these specify the order of amino acids in a protein from N-terminus (methionine) to C-terminus.
- The start codon marks the site at which translation into protein sequence begins, and the stop codon marks the site at which translation ends. The start codon is the first codon of a messenger RNA (mRNA) transcript translated by a ribosome, that initiates translation of the first amino acid in the polypeptide chain.
- The start codon always codes for methionine amino acid specified by one codon AUG. The three stop codons in messenger RNA are UAA, UAG, and UGA (U = uracil, A = adenine; G = guanine). The three **stop codons** mark the end of a protein, which are also called **termination codons** or **nonsense codons**. The genetic code is always universal.

GENE MUTATIONS

A gene mutation is a permanent structural alteration in the nucleotide sequence in genome DNA, which can be transmitted from a cell to its daughter cells. Somatic mutation is present in somatic tissues. Germline mutation is present in the gametes (eggs or sperms).

- Gene mutation results in abnormal transcribed DNA sequence (mRNA), abnormal translated proteins and lethal phenotype due to mutated RNA and protein. Gene mutations range in size, which can affect anywhere from a single DNA nucleotide base pair to a large segment of a chromosome that includes multiple genes.
- Gene mutations occur in two ways: spontaneous or induced by mutagens (ultraviolet radiation, ionizing radiation, chemical agents). Intercalating agents insert into the DNA molecule and cause single-nucleotide insertions and deletions. Oxidative reactions alter the chemical structures of nucleotide base pairs. Ionizing radiation alters nucleotide base pairs and breaking phosphodiester bonds. Ultraviolet produces light pyrimidine dimers, which block DNA replication.
- The DNA sequence of a gene can be altered in a number of ways. Gene mutations alter the function of essential proteins, which adversely affect the health of individuals. The types of gene mutations include: nonsense, missense, insertion, deletion, duplication, frameshift mutations and trinucleotide repeat expansion. Characteristics of different types of gene mutations are given in Table 5.32.

Table 5.32 Characteristics of different types of gene mutations

Gene Mutation	Comments
Mutations	Mutations are heritable changes in genetic information, which mutations include the mispairing of base in DNA replication and spontaneous depurination and deamination. Alkylating agents, deaminating chemical and hydroxylamine result in mutations by modifying the chemical structure of bases
Mutation rate	Mutation rate is the frequency with which a particular mutation arises, which is influenced by genetic and environmental factors. Some mutations occur spontaneously
Mutagenic agents	Intercalating agents insert into the DNA molecule and cause single-nucleotide insertions and deletions. Oxidative reactions alter the chemical structures of nucleotide bases. Ionizing radiation alters base structures and breaking phosphodiester bonds. Ultraviolet light produces light pyrimidine dimers, which block DNA replication
Somatic mutations	Somatic mutations occur in somatic cells
Germline mutations	Germline mutations occur in the cells that give rise to gametes (sperms or ova)
Base substitution mutation	Base substitution mutation changes the base of a single DNA nucleotide

Contd...

Table 5.32 Characteristics of different types of gene mutations (*Contd...*)

Gene Mutation	Comments
Transition	Base substitution in which a purine replaces a purine or a pyrimidine replaces a pyrimidine
Transversion	Base substitution in which a purine replaces a pyrimidine or a pyrimidine replaces a purine
Insertion	Addition of one or more nucleotides alters the number of DNA bases in a gene by adding a piece of DNA resulting in protein made by the gene, that may not function proper. Insertion can arise from strand slippage in DNA replication or from unequal crossing over. Insertion of intercalating agents into DNA molecule causes single-nucleotide insertion
Deletion	Deletion of one or more nucleotides alters number of DNA bases as well as the function of the resulting protein(s). Small deletion removes one or few base pairs within a gene, while large deletion removes an entire gene or several neighboring genes. Deletion can arise from strand slippage in DNA replication or from unequal crossing over. Insertion of intercalating agents into DNA molecule causes single-nucleotide deletion
Frameshift mutation	Insertion or deletion that alters the reading frame of a gene resulting in production of nonfunctional protein. Reading frame consists of groups of three bases that each code for one amino acid. Insertion, deletions and duplications can frameshift mutations
Inframe deletion or insertion	Deletion or insertion of a multiple of three nucleotides that does not alter the reading frame
Duplication mutation	A piece of DNA is abnormally copied one or more times resulting in alteration in function of protein
Expanding nucleotide repeats	Expanding nucleotide repeats are short DNA sequences that are repeated a number of times, in which the number of copies of the sequence increases
Forward mutation	Changes the wild-type phenotype to a mutant phenotype
Reverse mutation	Changes a mutant phenotype back to the wild-type phenotype
Silent mutation	A silent mutation produces a sense codon into a synonymous codon, that encodes the same amino acid; there is no change in amino acid sequence of the protein
Nonsense mutation	A nonsense mutation changes a sense codon into a nonsense codon, which alters DNA sequence causing premature termination of translation; but there is no substitution of one amino acid for another
Missense mutation	A missense mutation alters the coding sequence (i.e. sense codon changes into different codon), so one amino acid is substituted for another amino acid in the protein made by a gene
Neutral mutation	A neutral mutation changes alters the amino acid sequence of a protein but does not change the function of the protein
Loss-of-function mutation	Loss-of-function mutation results in complete or partial loss of function
Gain-of-function mutation	Gain-of-function mutation causes the appearance of a new trait or function or causes the appearance of a trait in inappropriate tissue or at an inappropriate time
Lethal mutation	Lethal mutation causes premature death
Suppressor mutation	Suppressor mutation suppresses the effect of an earlier mutation at a different site
Intragenic suppressor mutation	Intragenic suppressor mutation suppresses the effect of an earlier mutation within the same gene
Intergenic suppressor mutation	Intergenic suppressor mutation suppresses the effect of an earlier mutation in another gene

POINT MUTATION (SUBSTITUTION IN SINGLE NUCLEOTIDE BASE PAIR) WITHIN CODING SEQUENCES

Single nucleotide base substitution is called point mutation. Substitution is a type of gene mutation involving replacement or substitution of a single nucleotide base with another in DNA or RNA mole-cule (i.e. a change in single chemical letter such as switching an A to a G). Such substitution can change a codon that encodes a different amino acid leading to a small change in the protein produced. Schematic representation of point mutation is shown in Fig. 5.38.

Single Nucleotide Base Substitution and Gene Mutation

Single nucleotide base substitution is most common type of mutation, that occurs by two ways: (a) transition occurs when a purine is substituted with another purine or when pyrimidine is substituted with another pyrimidine in DNA or RNA molecule, and (b) transversion occurs, when a purine is substituted for a pyrimidine or a pyrimidine replaces a purine in

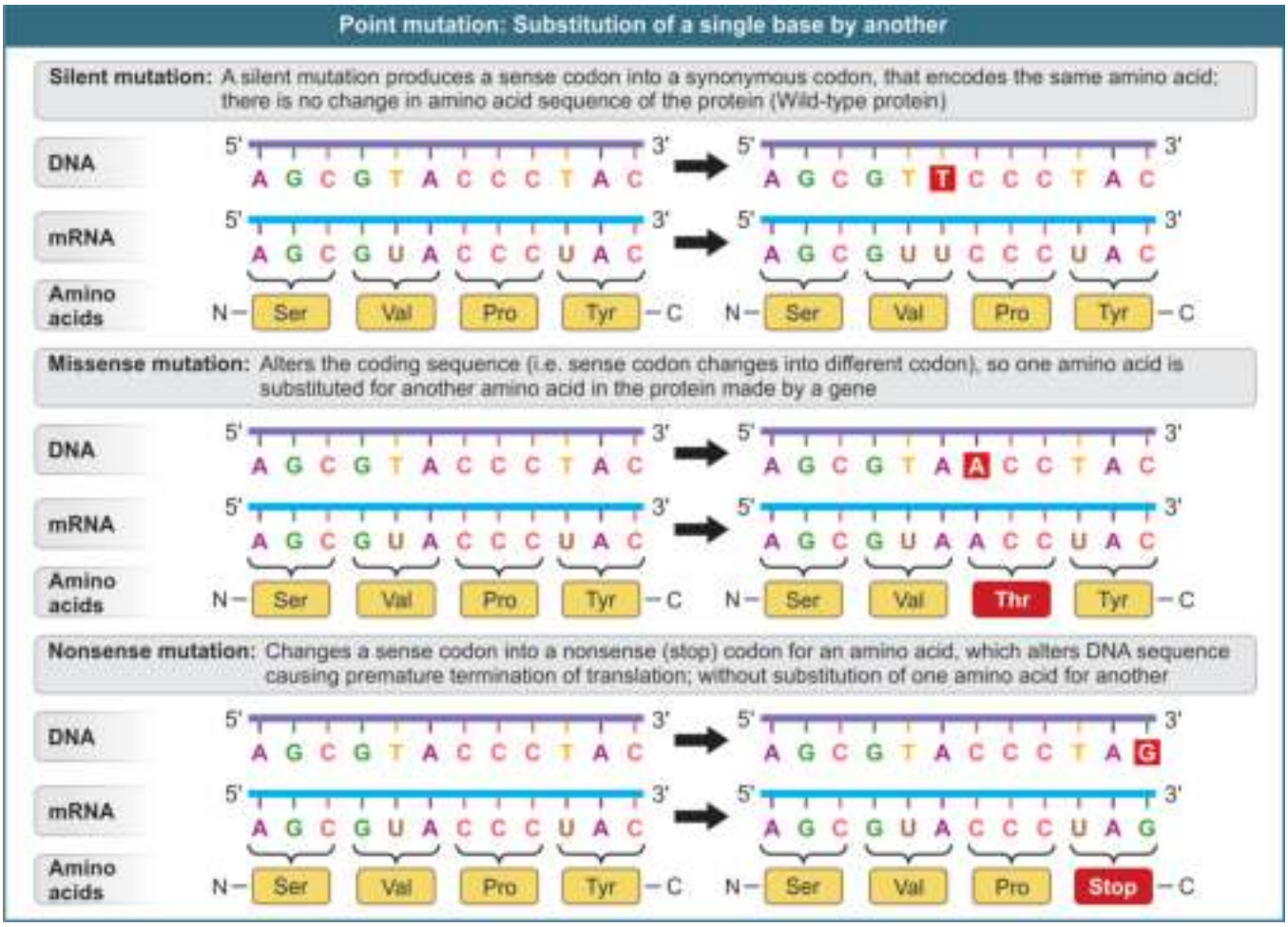


Fig. 5.38: Schematic representation of point mutation. Point mutation refers to substitution of a single base by another, that occurs by silent, missense and nonsense mutations.

DNA or RNA molecule. Comparison of transitions and transversions is given in Table 5.33.

Single Nucleotide Base Substitution and Types of Mutations

Single nucleotide base substitution induces three kinds of mutations: (a) silent mutation does not affect the sequence of amino acids during translation, (b) nonsense mutation occurs when a premature nonsense or stop codon is introduced in the DNA sequences resulting in early termination of translation, i.e. production of amino acid and protein; and (c) missense mutation changes the amino acid specified by a codon.

- It is worth remembering that frameshift mutation could allow for a partially functional protein to be made, whereas a nonsense mutation does not produce protein at all; and a missense mutation only affects a single amino acid production specified by a codon.
- Single nucleotide base substitution can cause silent, nonsense and missense point mutations at DNA, mRNA and protein level.

Silent Mutation

A silent mutation is a change in the nucleotide sequence of a gene that does not alter amino acid sequence of the encoded protein, when messenger RNA (mRNA) is translated. Thus, it has no measurable effect on the genome. For example, if the codon AAA is altered to become AAG, the same amino acid-lysine will be incorporated into the peptide chain.

Nonsense Mutation

A nonsense mutation is a genetic mutation in a DNA sequence that prematurely stops the translation (reading) of messenger RNA (mRNA) resulting in a polypeptide chain that ends prematurely and the unfinished protein product is incomplete and nonfunctional.

- DNA is a chain of many smaller molecules called nucleotide base pairs. There are three nonsense codons called amber (UAG), ochre (UAA) and opal (UGA) in the genetic code (mRNA), which do not code for an amino acid and instead signal the end of polypeptide chain during translation.

Table 5.33 Comparison of transitions and transversions

Characteristics	Transitions	Transversions
Nucleotide base replacement	▪ Purine to purine • Adenine (A) to guanine (G) • Guanine (G) to adenine (A) ▪ Pyrimidine to pyrimidine • Thymine (T) to cytosine (C) • Cytosine (C) to thymine (T)	▪ Purine to pyrimidine • Adenine (A) to thymine (T) • Adenine (A) to cytosine (C) • Guanine (G) to cytosine (C) • Guanine (G) to thymine (T) ▪ Pyrimidine to purine • Thymine (T) to adenine (A) • Thymine (T) to guanine (G) • Cytosine (C) to guanine (G) • Cytosine (C) to adenine (A)
Time of change during	DNA replication	DNA replication
DNA breakage	Not involved	Not involved
Frequency	High	Low
Effect on the nucleotide base	Yield normal nucleotide base	Yield abnormal nucleotide base
Detection of change	Both ways as above	Both ways as above
Causes	▪ Misincorporation ▪ Misreplication	▪ Misincorporation ▪ Misreplication
Reading frame of codon	Not changed	Not changed

- During protein synthesis, stop codons cause the release of the new polypeptide chain from the ribosomes.
- Examples of nonsense mutations include cystic fibrosis caused by the G542X mutation in the cystic fibrosis transmembrane conductance regulator (CFTR), β-thalassemia, Hurler syndrome and Dravet syndrome.

Missense Mutation

Missense mutation is a point mutation in which a single nucleotide change results in a codon that codes for different amino acids. If the properties of amino acid remain the same, the mutation is called '**conservative**', in which case the protein function is not altered. On the other hand, the mutation is called 'nonconservative', in which case the protein function is altered resulting in disease.

INSERTIONS AND DELETIONS WITHIN CODING SEQUENCES

Insertion, deletions and duplications can cause frameshift mutations. Insertions and deletions involve adding or subtracting a pair of nucleotide bases respectively. Insertions and deletions can alter a gene so that its message is no longer correctly analyzed. These changes are called frameshifts. Schematic representation of frameshift mutation is shown in Fig. 5.39.

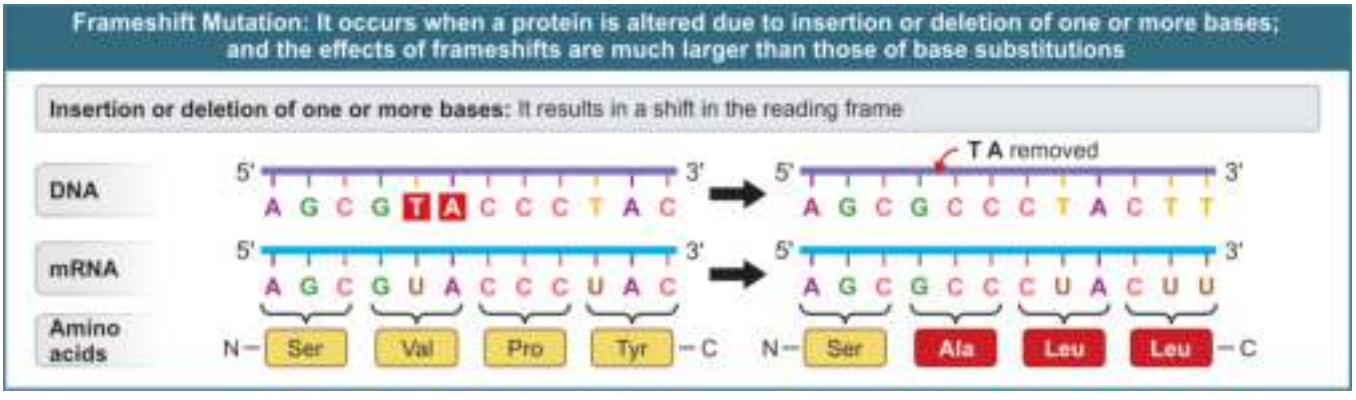


Fig. 5.39: Schematic representation of frameshift mutation. It occurs when a protein is altered due to insertion or deletion of one or more bases; and the effects of frameshifts are much larger than those of base substitutions.

- An insertion is a point mutation in one or more nucleotide base pairs is/are added to a DNA sequence. Deletion is a point mutation in which one or more base pairs is/are removed from a DNA sequence. Because the genetic code is read in codons (three nucleotide base pairs at a time), insertions and deletions may change the 'reading frame' of DNA sequence. These mutations are called frameshift mutations.
- Insertion and deletion result in a frameshift mutation that alter the reading of subsequent group of mRNA codons and, therefore, change the entire amino acid sequence that follows the mutation resulting in synthesis of abnormal translated protein. A codon is a group of three nucleotides that corresponds with a specific amino acid.
- Insertions and deletions that are multiple of three nucleotides will not cause frameshift mutations.
- Insertion and deletion are usually more harmful than a substitution in which a single amino acid is altered. Frameshift mutations occur when the number of deleted or inserted nucleotide base pairs is a multiple of three base pairs, which results in a change in only a few amino acids; it may be possible for the gene product (protein) to function, even though its sequence may be slightly different. Examples of frameshift mutations associated disorders are given in Table 5.34.

Table 5.34 Examples of frameshift mutations associated disorders

Frameshift Mutation	Cancer
BRCA1 frameshift mutation	Breast cancer, ovarian cancer
HEXA frameshift mutation	Tay-Sachs disease
CFTR frameshift mutation	Cystic fibrosis
NFKB1 frameshift mutation	Ulcerative colitis
MEN1 frameshift mutation	Multiple endocrine neoplasia 1 (MEN1)

TRINUCLEOTIDE REPEAT MUTATIONS

DNA (deoxyribonucleic acid) is a molecule that makes up our genes. Trinucleotide repeats in DNA molecule have eight copies of a CAG repeat. Two strands of DNA separate and replicate.

- In the course of DNA replication, a hairpin forms on the newly synthesized DNA strand, causing part of the template strand to be replicated twice and increasing the number of nucleotides repeat on the newly synthesized DNA strand. The two strands of the new DNA molecule separate and the strand with extra CAG copies serves as a template for DNA replication.
- When the number of trinucleotide repeats increases to a larger than normal number of copies, that alters the DNA. Each gene affected by trinucleotide repeat expansion has a different number of trinucleotide repeats that results in manifestation of disease.
- Examples of trinucleotide repeats genetic disorders include X-linked spinal and bulbar muscular atrophy, fragile X syndrome of mental retardation, Jacobsen syndrome, spinocerebellar ataxia (several types), autosomal dominant spinocerebellar ataxia, myotonic dystrophy, Huntington's disease, Friedreich ataxia, dentatorubral-pallidoluysian atrophy, myoclonus epilepsy of the Unverricht-Lundborg type and amyotrophic lateral sclerosis.
- There are many genetic disorders caused by repeat expansion to four (tetranucleotide), five (pentanucleotide) or 10 (dodecanucleotide) in length. The trinucleotide repeat genetic disorders are divided into three categories determined by the type of trinucleotide repeat. Disorders of trinucleotide repeat expansion mutation are given in Table 5.35.

Table 5.35 Disorders of trinucleotide repeat expansion mutation

Disorder	Trinucleotide Repeat Expansion	Disease Range	Normal Range
X-linked spinal and bulbar muscular atrophy	CAG	40–62	11–33
Fragile X syndrome with mental retardation	CGG	50–1500	6–54
Jacobsen syndrome	CGG	100–1000	11
Spinocerebellar ataxia (several type)	CAG	21–130	4–44
Autosomal dominant spinocerebellar ataxia	CAG	37–220	7–19
Myotonic dystrophy	CTG	40–3000	5–37
Huntington's disease	CAG	37–121	9–37
Friedreich ataxia (expansion of frataxin gene)	GAA	200–900	6–29
Dentatorubral-pallidoluysian atrophy	CAG	49–75	7–26
Myoclonus epilepsy of the Unverricht-Lundborg type	CCCCGCCCCGCG	12–13	2–3
Amyotrophic lateral sclerosis	GGGGCC	700–1600	2–23

Polyglutamic (PolyQ) Disorders

The most common trinucleotide repeat is CAG, when it is present in the coding region of a gene, which codes for amino acid glutamine (Q). Therefore, these genetic disorders are called polyglutamine (polyQ) disorders.

Nonpolyglutamic Disorders

Trinucleotide repeats do not involve the CAG in the coding region of the gene. Hence, trinucleotide repeat disorders are referred to as nonpolyglutamic disorders. Trinucleotide repeats are present in the promoter region of the affected gene, with the 5′-untranslated regions (5′-UTR) within introns, or within the 3′-UTR.

Polyalanine (Poly-Ala) Tract Disorders

Trinucleotide repeat expansion consists of genetic disorders associated with expansion of a polyalanine tract, or alterations in genes harboring a polyalanine tract, that may cause protein folding and aggregation.

- Polyalanine (poly-Ala) tract expansions are linked to nine inherited human diseases, in which about 500 genes encoding proteins, that harbor stretches of consecutive alanine residues ranging from 4 to 2000 amino acids in length.
- Key feature of the polyalanine repeat is due to imperfect repeat consisting of the nucleotides GCN, where N designates any nucleotide.

MUTATIONS WITHIN NONCODING SEQUENCES (INTRONS)

Most human genes contain segments of coding sequences (exons) interrupted by noncoding sequences (introns). Both exons and introns are transcribed to yield a long primary RNA transcript. The introns are then removed in order for the messenger RNA (mRNA) to encode a protein with the right sequence. Noncoding sequences (introns) in DNA do not provide instructions for making proteins.

Introns

The introns are noncoding sequences, that may affect a gene function/expression, which bind additional transcriptional enhancers or silencers; so, mutation in these regions can affect transcription. Introns can also be important in a process called alternate splicing, which can produce multiple types of small regulatory RNAs (miRNAs, incRNAs) from a single-gene.

Regulatory Noncoding RNAs

Regulatory RNAs are noncoding RNA molecules, that play a role in cellular processes such as activation or inhibition processes.

Table 5.36 Summary of direct and indirect intron functions

Direct Intron Functions
▪ Introns regulate alternate splicing
▪ Intron sequences control mRNA transport or chromatin assembly
▪ Introns in the 5'→3'-UTR (untranslated regions) affects nonsense-mediated decay of gene product
Indirect Intron Functions
▪ Different ordinal position of introns within a gene may have different functional role
▪ Introns length matters in the efficiency of natural selection
▪ Introns can provide a source of new genes
▪ Traits associated-SNPs (single nucleotide polymorphism) are enriched in introns
▪ Introns harbor several kinds of noncoding functional RNA genes

- When the mutations change the processing or sequence of these regulatory RNAs, which can alter the quantity produced of gene product.
- Mutations in the introns can lead to differentially truncated or spliced gene products, that may not be functional or even have more or less different functions.
- Finally, mutations in introns in the 5′→ 3′-UTR (untranslated regions) may affect the amount of protein produced. Summary of direct and indirect intron functions is given in Table 5.36.

SOMATIC MUTATIONS

Genes and chromosomes can undergo mutation either in somatic tissue (nongerm cell) or germline tissue (i.e. sperm and ovum), and these changes are called somatic or germline mutations respectively.

- Somatic mutations differ from the germline genetic alterations that occur in the germ cells (i.e. sperm and ovum) and can be passed onto offspring in every cell in the entire body.
- Somatic mutations are acquired mutations and frequently caused by environmental factors such as exposure to ultraviolet radiation or to certain chemical agents.
- Somatic mutations can be passed to the progeny of the mutated cell in the course of cell division, which accumulate in the cells despite proficient DNA repair mechanisms. Somatic genetic alterations can cause cancer or other diseases.
- **Somatic mutations** can be identified from parallel DNA sequencing by directly comparing the DNA sequence from the tumor tissue samples with their normal counterparts.

HUMAN GENETIC DISORDERS

Many human disorders have a genetic component, which can be caused by a mutation in one gene (monogenic disorder), by a combination of gene mutations and environmental factors, or by damage to chromosomes (changes in the number or structure of entire chromosomes, the structures that carry genes).

DISORDERS RELATED TO SINGLE-GENE MUTATION

Single-gene mutation disorders are caused by DNA changes in one particular gene, and often have predictable inheritance patterns. Most common single-gene mutation disorders include cystic fibrosis, primary hemochromatosis, sickle cell disease, fragile X syndrome, Duchenne muscular dystrophy, Huntington disease, phenylketonuria, color blindness, familial hypercholesterolemia and Tay-Sachs disease. Single gene mutation disorders induced due to various defects are given in Table 5.37. Single-gene mutation disorders and inheritance pattern are given in Table 5.38.

- Even though diseases are primarily caused by a single-gene mutation or different gene mutations can cause same disease, but with varying degrees of severity and phenotype. For example, other genes have been shown to modify the clinical course of cystic fibrosis in children, who carry the same CFTR gene mutation. In addition, for some disorders such as galactosemia, mutations in different genes can result in similar phenotype.
- Genetic testing is now available for many single-gene mutation disorders; however, the clinical examination is very important in the differential diagnosis particularly with no family history.
- Neonatal screening is useful in management of these disorders. Despite advancements in the understanding of genetic etiology and improved diagnostic techniques, treatments are not available to prevent the onset and progression of single-gene mutation disorders.
- There are a number of inheritance patterns of single-gene mutation disorders that are predictable such as autosomal dominant, autosomal recessive and X-linked recessive disorders.

Table 5.37 Single-gene mutation disorders induced due to various defects

Enzyme Defect
Phenylketonuria (accumulation of substrate, lack of product, failure to inactivate a protein which causes damage)
Receptor/Transport of Protein Defect
Familial hypercholesterolemia
Structural Protein Defects
▪ Marfan's syndrome ▪ Ehlers-Danlos syndrome
Enzyme Defect that Increases Drug Susceptibility
G6PD deficiency

COMPLETE MUTAGENIC DISORDERS

Human genome contains DNA molecule that encodes genetic makeup of the cell. DNA is subjected to several extrinsic and intrinsic insults (mutagens such as radioactive substances, X-rays, ultraviolet radiation, certain chemical and biological agents) and renders incorporation of faulty base pairing among the nucleotides.

- Mutagenesis is defined as the change in the genetic information of an organism in a stable manner, that may occur spontaneously in nature or as a result of exposure to mutagens.
- Researchers also use a number of techniques to create mutations including transposon mutagenesis to generate random gene knockouts at random position, and site-directed mutagenesis, which utilizes the polymerase chain reaction to introduce specific mutations.

Table 5.38 Single-gene mutation disorders and inheritance pattern

Disorder	Gene (Chromosome Location)	Inheritance Pattern
Familial hypercholesterolemia	LDL receptor (19p13)	Autosomal dominant disorder
Huntington disease	Huntington (4p16)	Autosomal dominant disorder
Sickle cell anemia	Beta-globin (11p15)	Autosomal recessive disorder
Cystic fibrosis	CFTR (7q31)	Autosomal recessive disorder
Primary hemochromatosis	HFE (6p21)	Autosomal recessive disorder
Tay-Sachs disease	Hexosaminidase A (15q23)	Autosomal recessive disorder
Congenital deafness (nonsyndromic)	Connexin 26 gene (13q11)	Autosomal recessive disorder
Duchenne muscular dystrophy	Dystrophin (Xq21)	X-linked recessive disorder

RANDOM MUTAGENESIS

Random mutagenesis is a powerful technique for generating enzymes, proteins, entire metabolic pathways, or even entire genomes with desired or improved properties. In this technique, point mutations are introduced at random positions in a gene of interest through polymerase chain reaction employing an error-prone DNA polymerase. Randomized sequences are then cloned into a suitable expression vector and the resultant mutant libraries can be screened to detect mutants with altered properties.

SITE-DIRECTED MUTAGENESIS

Site-directed mutagenesis is a technique for altering a gene or vector sequence at a selected location. Point mutations, insertions, or deletions are introduced by incorporating primers containing the desired modification with a DNA polymerase in an amplification reaction.

MENDELIAN (MONOGENIC) DISORDERS (SINGLE-GENE MUTATION)

Gene mutation is a permanent change in the DNA sequence that makes up a gene. Mutations range in size from one DNA nucleotide base to a whole chromosome change. Single-gene mutation follows laws of inheritance described by Gregor Mendel in 1856.

- Mutation of a gene leads to synthesis of abnormal protein. Therefore, disorders caused by single-gene mutation are known as Mendelian disorders. In single-gene mutation disorders, an error occurs at a single-gene site on the DNA strand.
- An error may occur in the copying and transcribing of a single codon through additions, deletions, or excessive repetitions. Some disorders of single-gene mutation and their significance are given in Table 5.39.

Table 5.39 Some disorders of single-gene mutation and their significance

Disorder	Clinical Manifestations
Autosomal dominant inheritance	
Adult polycystic kidney disease	Numerous cysts replacing kidneys
Familial hypercholesterolemia	Premature atherosclerosis
Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome)	Recurrent hemorrhage from skin and mucous membrane telangiectasia
Hereditary spherocytosis	Hemolytic anemia
Marfan's syndrome	Abnormal elastic tissue—skeletal, cardiovascular and ocular disease
Ehlers-Danlos syndromes	▪ Abnormal collagen—skin, joint and vascular effects ▪ Ehlers-Danlos syndromes may show autosomal or sex-linked recessive inheritance
Neurofibromatosis I	Multiple nerve sheath tumors
Neurofibromatosis II	Acoustic neuroma
Huntington's chorea (chromosome 4 mutant allele)	Progressive neuronal degeneration of brain tissue in middle age
Familial polyposis coli	Multiple colonic adenomas with malignant potential
Tuberous sclerosis	Multiple hamartomas within brain face, and kidneys (rhabdomyoma of heart, and subependymal giant cell astrocytoma
von Hippel-Lindau disease	Autosomal dominant disorder characterized by retinal hemangioma, cerebellar hemangioblastoma, adenomas or cysts in liver, kidney, pancreas, and renal clear cell carcinoma (sporadic or familial)
Achondroplasia	Dwarfism
Myotonic dystrophy	Muscle weakness and wasting. These neurological syndromes are known to be the result of inserts of multiple triple repeats
Osteogenesis imperfecta	Brittle bones, fracturing with minimal trauma

Contd...

Table 5.39 Some disorders of single-gene mutation and their significance (*Contd…*)

Disorder	Clinical Manifestations
Autosomal codominant inheritance	
ABO blood group antigens	Blood group disorders
α_1-Antitrypsin deficiency	Inhibitor of trypsin responsible for emphysema
HLA antigens	HLA associated disorders
Autosomal recessive inheritance	
Cystic fibrosis	Abnormal ion-transport protein
Sickle cell anemia	Abnormal hemoglobin
Thalassemia	Decreased synthesis of chain hemoglobin
Glycogen storage disease	Enzyme deficiency: Anderson disease (branching enzyme), McArdle disease (phosphorylase V), Hers disease (phosphorylase VI), Forbes-Cori disease (debranching enzyme), Pompe disease (lysosomal acid maltase) and von Gierke disease (glucose-6-phosphatase)
Mucopolysaccharidoses	Enzyme deficiency involving liver, spleen, bone marrow, lymph nodes
Lipidoses	Enzyme deficiency
Phenylketonuria	Deficiency of phenylalanine hydroxylase and impaired brain development
Albinism	Enzyme deficiency
Wilson's disease	Copper accumulation
X-linked recessive inheritance	
Hemophilia A	Bleeding tendency due to factor VIII deficiency
Hemophilia B	Bleeding tendency due to factor IX deficiency
Hunter syndrome	Hepatosplenomegaly, joint stiffness, mild mental retardation, retinal degeneration and cardiac lesions
G6PD deficiency	Hemolytic anemia occurs with certain drugs
Duchenne muscular dystrophy	Progressive muscle weakness due to dystrophin deficiency
Becker muscular dystrophy	Relative dystrophin deficiency
Fabry disease	Deficiency of α-galactosidase resulting in accumulation of ceramide trihexoside in skin over the lower trunk, febrile episodes, severe burning pain in the extremities, and cardiovascular and cerebrovascular involvement
X-linked Bruton agammaglobulinemia	Decreased gamma globulins due to B cell maturation
X-linked ichthyosis	Permanently thick scaly skin due to steroid sulphatase deficiency
Fragile X syndrome	Intellectual disability
X-linked dominant inheritance	
X-linked dominant hypophosphatemic rickets	Mutation in PHEX gene located on X chromosome, resistant to vitamin D supplementation
Rett syndrome	Mutation in MECP2 located on X chromosome in 95% of cases among females
Alport syndrome (most cases)	Mutation in COL4A5 collagen gene coding collagen protein located on X chromosome
Incontinentia pigmenti	More common in females than males
Giuffré-Tsukahara syndrome	Also known as radioulnar synostosis-microcephaly-scoliosis syndrome
Goltz syndrome	Also known as facial dermal hypoplasia
X-linked dominant protoporphyria	ALAS2 gene on X chromosome affecting males
Fragile X-linked dominant syndrome	Caused by an expansion of the CGG triplet repeat with the FMR1 (fragile X mental retardation 1) gene located on the X chromosome

MODES OF INHERITANCE OF MENDELIAN DISORDERS

Five basic modes of inheritance for single-gene diseases include autosomal dominant, autosomal recessive, X-linked recessive, X-linked dominant, and mitochondrial inheritance.

- In autosomal dominant disorders, affected parents transmit the phenotype to their children (sons and daughters) with 50% chance of inheritance in each generation of the pedigree.
- Autosomal recessive disorders appear in their children (sons and daughters with 25% chance of being affected, 50% chance of being carrier and 25% without being suffering) of unaffected parents.
- In sex-linked recessive disorders, genes for the traits are X chromosome (not on Y chromosome), hence these disorders always affect males whether X-linked recessive or dominant disorders because they have only one X chromosome.
- Mitochondria contain their own DNA (mtDNA), which is quite different from the chromosome in the nucleus. Unlike nuclear DNA (nDNA), mitochondrial DNA is maternally inherited and present as multiple copies per cell. Mitochondrial inheritance is uniparental. The paternal mitochondria are lost before sperm enters or fuses with the ovum. In others, the paternal cytoplasm is excluded from the ovum upon fertilization.

AUTOSOMAL DOMINANT INHERITANCE

Autosomal dominant inheritance that a person only requires one copy of the changed gene (genetic difference) in order to have the genetic disorder.

- Usually, the changed gene is inherited from a parent, who also suffers from the genetic disorder and every generation in the family members may suffer from the disorder.
- There are some instances in which an individual has single-gene mutation that causes the genetic disorder, but is asymptomatic; but still pass the mutated gene to his or her children.
- A person, who carries a mutated gene for an autosomal dominant inheritance has a 50% chance of passing the mutated gene to each child.

AUTOSOMAL RECESSIVE INHERITANCE

Autosomal recessive inheritance means that it is essential to have two copies of the changed gene to have the genetic disorder. Each parent contributes one changed copy of the gene to the child, who has the genetic disorder.

- The parents are called carriers of the genetic disorder, because they have only one normal copy of the gene and one changed copy of the gene, but parents do not show symptoms of the genetic disorder.
- When both parents are carriers of the changed gene, each of their children has a 25% chance of having the genetic disorder, a 50% chance of being a carrier of the genetic disorder (like their parents), and a 25% chance of neither a carrier nor having the genetic disorder. These risks remain the same for each pregnancy.
- When there is more than one person in the family suffering from the genetic disorder, these persons are most often in the same generation.

X-LINKED RECESSIVE INHERITANCE

X-linked recessive inheritance is usually seen in males, which is more common than X-linked dominant inheritance. Person with X-linked recessive inheritance lacks any normal copy of the gene. X-linked (sex-linked) recessive disorders are given in Table 5.40.

- Male child only has one X chromosome, so if a male inherits a changed gene on the X chromosome (which is always inherited from the mother), then male child lacks another copy of the working gene to compensate.
- Female child with one copy of a changed gene on X-chromosome is called carrier of X-linked inheritance. It is rare for a female child to have the changed gene on both her X chromosomes.
- In most cases, females, who are carriers do not show symptoms because the working of gene compensates for the non-working copy of the gene.
- Carrier females have 25% chance of having a son with the X-linked recessive disorder, 25% chance of having a son without the genetic order, 25% chance of having a carrier daughter and 25% chance of having a daughter, who is not carrier.
- Males with X-linked recessive disorder cannot pass the genetic disorder to their sons, but 100% of their daughters will be carrier.

Table 5.40 X-linked (sex-linked) recessive disorders

Duchenne muscular dystrophy
Hemophilia A and B
Fragile X syndrome
X-linked Bruton agammaglobulinemia
Wiskott-Aldrich syndrome
Lesch-Nyhan syndrome
Glucose-6-phosphate dehydrogenase (G6PD) deficiency
Hunter syndrome

X-LINKED DOMINANT INHERITANCE

X-linked dominant inheritance follows an inheritance pattern similar to autosomal dominant inheritance except that females are more affected than males. However, X-linked dominant inheritance is rare.

MITOCHONDRIAL INHERITANCE

Mitochondrial inheritance results from the expression of mitochondrial DNA. Mitochondrial inheritance does not follow Mendelian pattern since it involves mitochondrial genome expression.

- Conditions caused by a mutation in the mitochondrial DNA have unusual patterns: (a) both males and females are affected, (b) the condition is transmitted through the female to her offspring, and (c) if a male has the trait and his spouse does not, their offspring would not have the trait.
- Mitochondrial disorders include mitochondrial myopathy, diabetes mellitus and deafness (DAD), Leber hereditary optic neuropathy (LHON), Leigh syndrome, subacute sclerosing encephalopathy; neuropathy, ataxia, retinitis pigmentosa and ptosis (NARP) and myoneurogenic encephalopathy (MNGIE).

CODOMINANT INHERITANCE

Codominant inheritance is a type of inheritance wherein the alleles of a gene pair in heterozygotes are fully expressed. As a result, the phenotype of the offspring is a combination of the phenotype of the parents. Thus, the final trait is neither dominant nor recessive. An example in human is the **ABO blood group**, where both A and B alleles are expressed. Therefore, if an individual inherits allele A from mother and allele B from mother, such individual has **blood group AB type**.

MENDELIAN TRAITS

Mendelian traits are dominant, recessive or codominant. Codominant inheritance is characterized by complete expression of both alleles in a heterozygote as seen in ABO blood group inheritance.

- For unknown reasons, on autosomes, one allele may be more influential than another in determining a specific trait. The more powerful, or dominant gene is more likely to be expressed in the offspring than recessive gene.
- Offspring will express a dominant allele when one or both chromosomes in a pair carry it. A recessive allele will not be expressed unless both chromosomes carry identical copies of the allele.

AUTOSOMAL DOMINANT DISORDERS

In single-gene disorders, an error occurs at a single-gene site on the DNA strand. An error may occur in the copying and transcribing of a single codon through additions, deletions, or excessive repetitions.

- Autosomal dominant disorder transmission most often affects male and female offspring equally. Affected males and females appear in each generation of the pedigree.
- Affected mothers and fathers transmit the phenotype to both sons and daughters. Selected autosomal dominant disorders are given in Table 5.41. Some autosomal dominant disorders of single-gene mutation and their significance are given in Table 5.42.

Table 5.41 Selected autosomal dominant disorders

Neurological Disorders
Huntington disease
Musculoskeletal Disorders
▪ Marfan's syndrome (fibrillin 1 protein involved) ▪ Ehlers-Danlos syndrome ▪ Osteogenesis imperfecta ▪ Achondroplasia ▪ Myotonic dystrophy
Hematopoietic Disorders
▪ Hereditary spherocytosis (ankyrin, spectrin, band 4.1, band 3 proteins involved) ▪ von Willebrand disease
Renal Disorders
Adult polycystic kidney disease
Metabolic Disorders
Familial hypercholesterolemia (LDL receptor protein involved)
Tumor Syndromes
▪ Neurofibromatosis type 1, also called von Recklinghausen disease (neurofibromin protein involved) ▪ Neurofibromatosis type 2, also called bilateral acoustic neurofibromatosis (merlin protein involved) ▪ Familial adenomatous polyposis coli (APC protein involved) ▪ Wilm's tumor ▪ Li-Fraumeni syndrome (i.e. early breast carcinoma associated with sarcoma and other tumors) ▪ Retinoblastoma ▪ Tuberous sclerosis (hamartin, tuberin proteins involved) ▪ von Hippel-Lindau disease
Other Autosomal Dominant Disorder
Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome)

Table 5.42 Some autosomal dominant disorders of single-gene mutation and their significance

Disorder	Clinical Manifestations
Adult polycystic kidney disease	Numerous cysts replacing both kidneys
Familial hypercholesterolemia	Premature atherosclerosis
Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome)	Recurrent hemorrhage from skin and mucous membrane telangiectasia
Hereditary spherocytosis	Hemolytic anemia
Marfan's syndrome	Abnormal elastic tissue—skeletal, cardiovascular and ocular disease
Ehlers-Danlos syndrome (autosomal or sex-linked recessive inheritance)	Abnormal collagen—skin, joint and blood vessels
Neurofibromatosis I	Multiple nerve sheath tumors
Neurofibromatosis II	Acoustic neuroma
Huntington's chorea (chromosome 4 mutant allele)	Progressive neuronal degeneration of brain tissue in middle age
Familial polyposis coli	Multiple colonic adenomas with malignant potential
Tuberous sclerosis	Multiple hamartomas within brain, face, and kidneys (rhabdomyoma of heart, and subependymal giant cell astrocytoma)
von Hippel-Lindau disease	Autosomal dominant disorder characterized by retinal hemangioma, cerebellar hemangioblastoma, adenomas or cysts in liver, kidney, pancreas, and renal clear cell carcinoma (sporadic or familial)
Achondroplasia	Dwarfism
Myotonic dystrophy	Muscle weakness and wasting. These neurological syndromes are known to be the result of inserts of multiple triple repeats
Osteogenesis imperfecta	Brittle bones, fracturing with minimal trauma

ADULT POLYCYSTIC KIDNEY DISEASE

Adult polycystic kidney disease (APKD) is an autosomal dominant disorder. Though the genetic defect is present at birth, yet it manifests between 15 and 30 years of age. APKD is most often associated with berry aneurysm of the circle of Willis resulting in subarachnoid hemorrhage.

- Patient develops numerous bilateral renal cysts that replace and ultimately destroy the renal parenchyma resulting in enlargement. Most cases are caused by mutations in the polycystic kidney disease 1 (PKD1) gene, which encodes polycystin.
- The kidneys are enlarged up to 3 or 4 kg or more. The affected kidney contains large fluid-filled cysts with areas of organized hemorrhage.
- Patient presents with sense of heaviness in the loins, bilateral flank and palpable renal masses, hypertension, blood clots in the urine (**hematuria**), progression to end-stage renal failure over a period of several years. Secondary polycythemia may occur.

FAMILIAL HYPERCHOLESTEROLEMIA

Familial hypercholesterolemia is an autosomal dominant disorder caused by mutations of the gene coding for LDL receptors.

- The gene defect affects the uptake of LDL in the liver, causing hypercholesterolemia. It leads to high levels of low-density lipoproteins in blood.
- Clinically, the disease presents with xanthomas characterized by raised yellow lesions filled with lipid-laden macrophages, in the skin and tendons. Patient develops marked atherosclerosis, ischemic heart disease and peripheral vascular disease.

HEREDITARY HEMORRHAGIC TELANGIECTASIA

Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome) is a rare autosomal dominant disorder that leads to abnormal blood vessel formation in the skin, mucous membranes, lungs, liver and brain resulting to recurrent hemorrhage from these lesions.

- A person with hereditary hemorrhagic telangiectasia (**HHT**) may form blood vessels lacking capillaries, hence, blood passes from arteries directly into the vein. HHT type 1 is caused by mutations in the ENG gene.
- HHT type 2 is caused by mutation in the ACVRL1 gene. Juvenile polyposis/HHT syndrome occurs due the mutation in the SMAD4 gene. Incidence is more in certain populations, such as in Mormon families of Utah. Pulmonary lesions are at risk of rupture and life-threatening.

HEREDITARY SPHEROCYTOSIS

The normal membrane cytoskeleton of RBCs is composed of spectrin, ankyrin, actin, protein 4.1 and protein 3, which maintain the normal biconcave shape of RBCs.

- Hereditary spherocytosis (**HS**) is an autosomal dominant disorder associated with inherited defects of erythrocyte membrane-associated cytoskeleton proteins such as spectrin, ankyrin, actin, protein 4.1 and protein 3.
- Mutation of the ankyrin gene is the most common finding, accounting for most cases of hereditary spherocytosis. Protein 4.2 deficiency is common in Japan. Patient develops hereditary spherocytosis. The spheroidal erythrocytes are sequestered and destroyed in the spleen, producing hemolytic anemia.

Clinical Features

Signs and symptoms can range from mild to severe anemia, jaundice, cholelithiasis and/or splenomegaly. Osmotic fragility is increased. Spleen is firm, deep red tissue with thin capsule, but Malpighian follicles are grossly not identifiable. Histologic examination of spleen shows marked congestion in the cords, empty sinuses containing ghost cells, prominent endothelial lined sinuses, hemosiderin deposition and erythrophagocytosis. Splenectomy prolongs the life of red blood cells, although these still have genetic defects.

MARFAN'S SYNDROME

Marfan's syndrome is an autosomal dominant systemic connective tissue disorder, that affects many parts of the body such as skeleton, eyes and cardiovascular system.

- The diagnosis of Marfan's syndrome is established with new diagnostic criteria, known as Ghent nosology, based on major and minor clinical manifestations in various organ systems and the family history.
- Aortic root aneurysm, prolapse of mitral valve and ectopia lentis (dislocated lenses) are the cardinal features. Skeletal muscle, fat, skin, fascia and respiratory tract may be affected. Major and minor diagnostic criteria of Marfan syndrome are given in Table 5.43.

Pathogenesis

Exact pathogenesis of Marfan's syndrome has not been fully elucidated. The disorder is caused by missense mutations of the fibrillin gene 1 (**FGN1**) on chromosome 15. Fibrillin is a family of connective tissue proteins analogous to the collagens.

Clinical Features

Clinical manifestations of Marfan's syndrome may include tall and slender built, disproportionate long arms, legs and fingers; deformity of the thoracic cage, striae atrophicae, mitral valve prolapse, a mid- and nonprogressive dilatation of the aortic root. Clinical manifestations of Marfan syndrome are given in Table 5.44 and schematic representation of Marfan's syndrome is shown in Fig. 5.40.

Table 5.43 Major and minor diagnostic criteria of Marfan syndrome

Major Diagnostic Criteria
▪ Enlarged aorta
▪ Tear in aorta
▪ Dislocation of ocular lens (ectopia lentis)
▪ Family history of Marfan syndrome
▪ Dural ectasia (enlargement of lining that surrounds part of the spinal cord)
Minor Diagnostic Criteria
▪ Short-sightedness (myopia)
▪ Unexplained stretch mark
▪ Loose joints

Patient has at least four of the skeletal problems such as very tall height and thin built, long arms and legs, long slender spider-like fingers (arachnodactyly), hyperextensible joints (especially thumb), anterior chest deformity (pectus excavatum) and scoliosis and lordosis.

Laboratory Diagnosis

Marfan syndrome is usually diagnosed in young persons associated with poor prognosis. Laboratory tests for Marfan syndrome can include echocardiogram, electrocardiogram, cardiac magnetic resonance imaging or computed tomography and DNA test to confirm the genetic defect.

Table 5.44 Clinical manifestations of Marfan syndrome

Skeletal Defects
▪ Vey tall height and thin built
▪ Long arms and legs
▪ Long slender spider-like fingers (arachnodactyly)
▪ Hyperextensible joints (especially thumb)
▪ Anterior chest deformity (pectus excavatum)
▪ Scoliosis and lordosis
Cardiovascular Defects
▪ Mitral valve prolapse resulting in mitral regurgitation
▪ Cystic medial degeneration of aorta causing dilatation of aortic valve and aortic regurgitation and later dissecting aortic hematoma
Ocular Defects
▪ Bilateral dislocation (subluxation) of the ocular lens (ectopia lentis)

Cause of death in Marfan syndrome is aortic dissection. Hence, hypertension should be treated at the earliest.

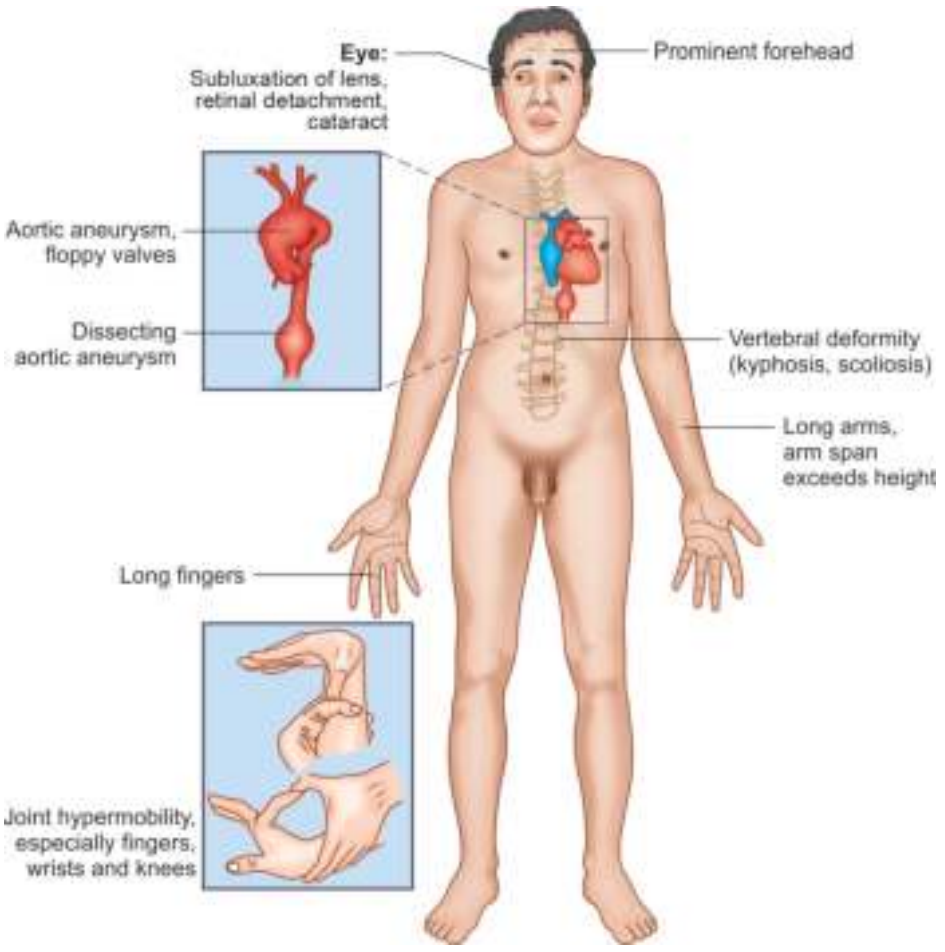


Fig. 5.40: Schematic representation of Marfan's syndrome. Marfan's syndrome shows clinical manifestations.

EHLERS-DANLOS SYNDROME

Ehlers-Danlos syndrome is rare autosomal dominant disorder of collagen fibers. Autosomal recessive and X-linked recessive varieties are also known. There is generalized defect in collagen fibers, molecular structure, synthesis, secretion, and degradation. Ehlers-Danlos syndrome is characterized by joint hypermobility, abnormalities in skin texture elasticity, vascular fragility in blood vessels and viscera.

Clinical Features

Skin, ligaments and joints of person suffering from Ehlers-Danlos syndrome are commonly affected. Generalized defects in collagen fibers lead to hyperelasticity of skin (stretching of skin to many centimeters). Bleeding diathesis occurs as a result of fragile skin. Dehiscence of surgical incisions is common because sutures do not hold well. Hypermobility of joints (unusual extension and flexion) is common finding. Clinical manifestations of Ehlers-Danlos syndrome are given in Table 5.45.

Table 5.45 Clinical manifestations of Ehlers-Danlos syndrome

Site Involved	Cardinal Features	Primary Consequences	Secondary Consequences
Joints	Hypermobility	Arthralgia, joint dislocation	Instability of joints and soft tissue trauma
Skeleton	Osteoporosis	Scoliosis, fracture	Skeletal deformities
Skin	Hyperextensibility	Thinning of skin, striae atrophicae	Bruising and hematoma, violaceous papyraceous scars, subcutaneous spheroids
Vasculature	Mitral valve involvement in type 4 of Ehlers-Danlos syndrome	Prolapse mitral valve	Subacute bacterial endocarditis
Skeletal muscles	Skeletal muscles at various regions	Hernia, urinary bladder and rectal diverticula	Rectal prolapse, uterine prolapse and neuromyopathy

Laboratory Diagnosis

Laboratory tests for Ehlers-Danlos syndrome include genetic tests, skin biopsy, and echocardiogram.

NEUROFIBROMATOSIS TYPE 1

Neurofibromatosis type 1, an autosomal disorder, is also known as von Recklinghausen disease caused by mutation of NF1 gene, a tumor suppressor gene located on chromosome 17q11.2. NF1 gene coding for abnormal protein product neurofibromin is expressed in many tissues. Neurofibromin belongs to family of GTPase-activating proteins (GAPs) that facilitates the conversion of active RAS-GTP to inactive RAS-GDP. Loss of GAP activity in cells acquiring a second hit mutation in NF1 gene permits uncontrolled RAS p21 protein activation, an effect that predisposes to the formation of benign neurofibromas. Diagnostic criteria of neurofibromatosis type 1 are given in Table 5.46.

Clinical Features

Patient suffering from neurofibromatosis type 1 presents with disfiguring neurofibromas in skin, skin pigmentation (café au lait spots), pigmented dome-shaped lesions on the surface of iris (known as Lisch nodules), meningioma, optic glioma, pheochromocytoma, Wilm's tumor, rhabdomyosarcoma, and leukemia. Approximately 3–5% of patients, benign neurofibromas can become malignant.

NEUROFIBROMATOSIS TYPE 2

Neurofibromatosis type 2 is an autosomal dominant disorder caused by mutation in NF2 tumor suppressor gene on chromosome 22q.12, which encodes abnormal **merlin**, and characterized by bilateral acoustic neuroma/vestibular schwannomas. Symptoms become apparent during childhood, adolescence, early adulthood or later in adult life. Diagnostic criteria of neurofibromatosis type 2 are given in Table 5.47.

Table 5.46 Diagnostic criteria of neurofibromatosis type 1

Patient must have at least two or more features as mentioned:
Two or more neurofibromas
One plexiform neurofibroma
Six or more café au lait macules >5 mm (pubertal) or >15 mm (post-pubertal)
Ocular iris hamartomas
Sphenoid bone dysplasia
First degree relative suffering from neurofibromatosis type 1

Table 5.47 Diagnostic criteria of neurofibromatosis type 2

- **Patient with NF2 disease should have features as mentioned:** Bilateral vestibular schwannomas
- **Family history of neurofibromatosis type 2 in first degree relative:**
  - Unilateral vestibular schwannoma in below 30 years of age
  - Any two of the lesions, i.e. meningioma, schwannoma, glioma, posterior subcapsular lenticular opacities/juvenile cortical cataract.

Clinical Features

Depending on the exact size and location of the tumor in the vestibulocochlear nerve, patient with neurofibromatosis type 2 experiences problems with balance and gait, dizziness, headache, tinnitus, and/or progressive hearing loss. Some persons may have additional abnormalities such as meningioma, astrocytoma, juvenile posterior subcapsular lens opacities.

TUBEROUS SCLEROSIS COMPLEX

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder with variable expression, and also known as Epiloia disease or Bourneville-Pringle disease. Disorder is caused by mutations in either the TSC1 or TSC2 genes, which code for the inhibitors of the central cell growth control the mechanistic target of rapamycin (mTOR) pathway most often result in early-life refractory epilepsy and autism spectrum disorder. Patient is associated with multiple hamartomas within brain (cortical tubers and subependymal hamartomas), renal angiomyolipoma (80%), cardiac rhabdomyoma, subependymal giant cell astrocytoma and hypomelanotic macules.

VON HIPPEL-LINDAU DISEASE

von Hippel-Lindau disease is an autosomal dominant disorder with high penetrance and variable phenotypic expression.

- von Hippel-Lindau disease is caused by mutation in vHL, a tumor suppressor gene located on chromosome 3p (3p12 to 3p26). vHL gene mutation occurs by loss of sequences on chromosome 3p, imbalanced translocation (3; 6, 3; 8, 3; 11) and hypermethylation (80%). Mutation of one allele of vHL gene is seen in 98% of sporadic and 2% of familial renal cell carcinomas.
- von Hippel-Lindau disease is most often associated with vascular tumors (e.g. retinal hemangioma, cerebellar hemangioblastoma, spinal cord hemangioblastoma), and less frequently, pancreatic cystic neoplasm, pancreatic neuroendocrine tumor, clear cell carcinoma of the kidney, endolymphatic sac

tumor, pheochromocytoma, paraganglioma, and liver cysts or liver adenoma.

AUTOSOMAL RECESSIVE DISORDERS

Autosomal recessive inheritance usually affects male and female offspring equally. If both parents are affected, all their offspring will be affected. If one parent is affected and the other is a carrier, 50% of their children will be affected. Autosomal recessive disorders may occur when there is no family history of the disease.

Table 5.48 Selected autosomal recessive disorders

Metabolic Disorders

- Cystic fibrosis (abnormal ion-transport protein)
- Hemochromatosis
- Lysosomal storage diseases
- Glycogen storage diseases
- Mucopolysaccharidoses
- Galactosemia
- Phenylketonuria (amino acid metabolic disorder)
- Albinism (enzyme deficiency)
- Wilsons' disease (copper accumulation)

Hematopoietic Disorders

- Sickle cell anemia (abnormal hemoglobin)
- Thalassemia (decreased synthesis of hemoglobin)

Neuromuscular Disorders

- Friedreich ataxia
- Muscular dystrophy (some forms)

Endocrinal Disorders

- Congenital adrenal hyperplasia (21-hydroxylase deficiency)

Selected autosomal recessive disorders are given in Tables 5.48 and 5.49.

SICKLE CELL DISEASE

Sickle cell disease is an autosomal recessive disorder of the hemoglobin, that affects red blood cells. Sickle cell anemia is the name of a special form of sickle cell disease, in which there are two sickle cell genes.

- Sickle cell trait is the inheritance of one sickle cell gene. Homozygous genotypes contain abnormal hemoglobin HbS/HbS (sickle cell disease). Heterozygous genotypes contain abnormal hemoglobin HbA/HbS (sickle cell trait).
- The mutant hemoglobin molecule undergoes polymerization under low oxygen tension causing the change in the shape of the RBC from biconcave to elongated sickle-like structure resulting in excessive destruction of RBCs.
- The life span of RBCs is reduced 10–20 days (normal RBCs life span is 100–120 days). Patients suffer from chronic hemolytic anemia and other clinical manifestations. Schematic representation of sickle cell anemia is shown in Fig. 5.41.

Pathophysiology

Mutant allele chromosome 11 causes substitution of valine for glutamine at the 6th codon in β-chain of globin molecule of hemoglobin due to the single base substitution at the 6th codon of the β-globin gene from GAG to GUG and synthesize abnormal HbS resulting in sickle cell anemia.

Table 5.49 Selected autosomal recessive disorders and enzyme defects

Disorder	Enzyme Deficiency	Accumulation in Tissues
Tay-Sachs disease	Hexosaminidase A (Hexa gene mutation on 15)	GM2 ganglioside
Gaucher's disease A and B	Glucocerebrosidase	Glucocerebroside
Niemann-Pick disease	Sphingomyelinase	Sphingomyelin
Hurler's syndrome	α-L-iduronidase (IDUA gene mutation on 4p16.3)	Heparan sulfate, dermatan sulfate
von Gierke disease (type 1 glycogenosis)	Glucose-6-phosphatase	Glycogen
Pompe disease (type 2 glycogenosis)	α-1,4-Glucosidase	Glycogen
Cori disease (type 3 glycogenosis)	Amylo-1,6-glucosidase	Glycogen
McArdle syndrome (type 4 glycogenosis)	Muscle phosphorylase	Glycogen
Galactosemia	Galactose-1-phosphate uridyltransferase	Galactose-1-phosphate
Phenylketonuria	Phenylalanine hydroxylase	Phenylalanine and its degradation products
Alkaptonuria	Homogentisic oxidase	Homogentisic acid
Hereditary hemochromatosis	Human homeostatic iron regulator (HFE protein)	Iron
Wilson disease	ATP-dependent Cu^{2+} transporter	Copper
Maple syrup urine disease	Branched-chain α-keto acid dehydrogenase complex	Branched-chain amino acids including leucine, isoleucine, and valine
Hunter disease	Iduronate-2-sulfatase	Heparan sulfate, dermatan sulfate

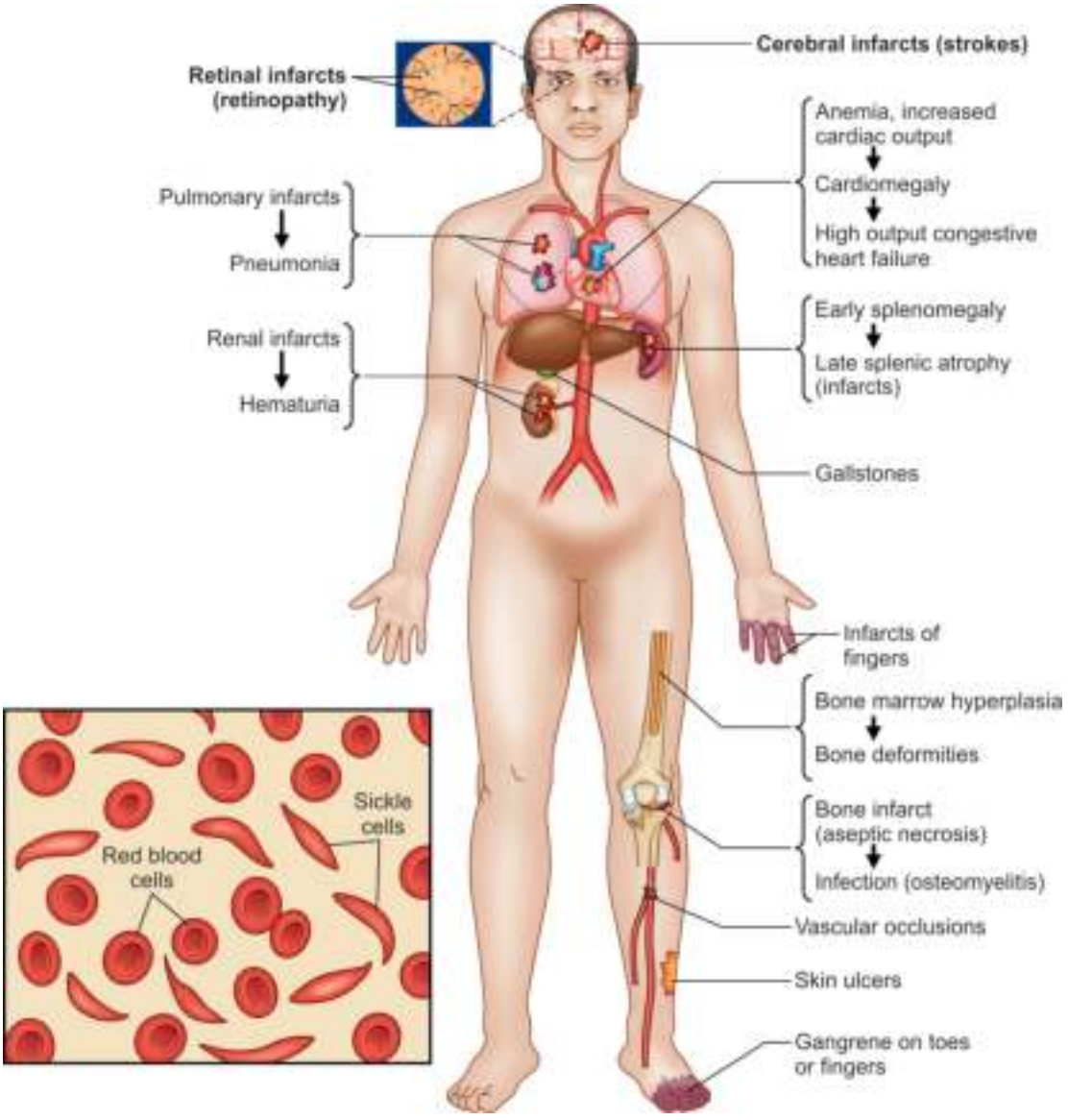


Fig. 5.41: Schematic representation of sickle cell anemia. Clinical and pathological findings, peripheral blood smear shows sickled RBCs. Sickled red cell appears in peripheral blood specimens only in patients with sickle cell disease because all of their hemoglobin is hemoglobin S. Red cells with any amount of hemoglobin S, however, can be induced to sickle as part of a laboratory test for the presence of hemoglobin S.

- As a result, red blood cells sickle in low oxygen states causing occlusion of the small blood vessels circulating through the tissues of the body.
- Impaired circulation and lack of oxygen result in tissue damage/organs and severe disability. The sickle-shaped red blood cells are prematurely removed from the circulation resulting in a chronic hemolytic anemia.

Clinical Features

Patient with sickle cell anemia develops episodes of intermittent 'crises' of variable frequency and severity depending on the degree of organ involvement. Major symptoms of sickle cell anemia include fatigue, anemia, pain, sickle cell crises (e.g. vaso-occlusive most common, sequestration, aplastic, hemolytic and bone pain crises), dactylitis (swelling and inflammation of the hands and/or feet), arthritis, sudden pooling of blood in the spleen and liver (congestion), bacterial infections, leg ulcers, aseptic necrosis of bone, heart and lung injury, renal papillary necrosis and retinopathy.

Treatment

With newborn screening and early management, the death rate among the children with sickle cell disease has declined.

- Current treatment of sickle cell anemia is directed primarily toward managing the individual

manifestations of the illness, as they occur. Blood transfusion is indicated for symptomatic anemia to prevent cerebral stroke and for acute chest syndrome. Repeated blood transfusion increases risk for excess deposition of iron in various tissues.

- In addition, due to differences in RBCs antigens between donors and recipients, these patients are at increased risk for development of RBC alloantibodies, which can complicate further blood transfusion. It is therefore, essential of prevent alloimmunization by transfusing leukoreducted red blood cells that match the patient for the C, E, and Ki antigens.
- Transplantation of human progenitor cells obtained from bone marrow, peripheral blood or umbilical cord can cure sickle cell disease.

CYSTIC FIBROSIS

Cystic fibrosis is an autosomal recessive life-threatening disorder that affects lungs and exocrine glands. Both parents carry the mutated CFTR gene located on long arm of chromosome 7, that encodes an abnormal transmembrane conductance regulator protein and transmit to their children.

- Normally, CFTR protein participates in the movement of chloride and other ions across the membranes. Genetic defect of chromosome 7 allele causes failure of chloride ions transport resulting in accumulation of chloride in sweat and tears.
- Defective CFTR protein results in production of thick and sticky mucus secretion that clog the lungs and obstructs the pancreas. Cystic fibrosis is a lethal disease among White population. Patient with cystic fibrosis exhibiting clinical manifestations is shown in Fig. 5.42.

Clinical Features

Patient of cystic fibrosis develops recurrent pulmonary infections and dysfunction of both gastrointestinal tract and accessory glands; warning sign of cystic fibrosis in a newborn is intestinal obstruction caused by thick viscous meconium known as 'meconium ileus'.

- Cystic fibrosis affects sweat glands in the skin resulting in abnormally thick salty perspiration. There is increased risk of heat stroke and salts depletion especially in infants.
- Almost all men with cystic fibrosis are **infertile** but not sterile due to defective development of the vas deferens, epididymis and seminal vesicles. Women with cystic fibrosis often have normal reproductive system and can have successful pregnancies. Systemic manifestations of cystic fibrosis are given in Table 5.50.

Laboratory Diagnosis

Sweat chloride test is an important diagnostic tool of cystic fibrosis. Secretion by sweat glands of chloride and sodium is normal, but their reabsorption by sweat

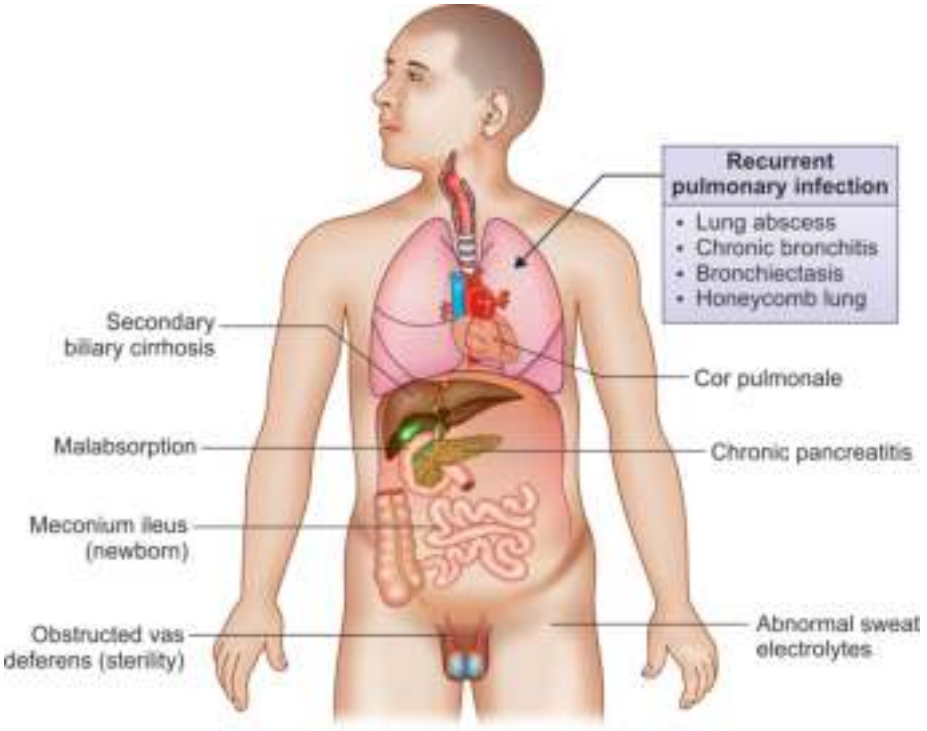


Fig. 5.42: Schematic representation of cystic fibrosis. Patient with cystic fibrosis shows clinical manifestations.

Table 5.50 Systemic manifestations of cystic fibrosis

Organs	Secondary Effects	
Skin	▪ Salty sweat results in hyponatremia ▪ Acrodermatitis	▪ Dilation of eccrine and apocrine glands
Gastrointestinal lower tract	▪ Meconium ileus warning sign in newborn resulting in obstruction, perforation, peritonitis	▪ Rectal prolapse
Pancreas	▪ Pancreatic duct obstruction causes pancreatic insufficiency (steatorrhea) ▪ Chronic pancreatitis	▪ Failure to thrive ▪ Diabetes mellitus
Liver	▪ Hepatic duct obstruction causes biliary cirrhosis resulting in esophageal varices	▪ Sclerosing cholangitis
Gallbladder	Cholecystitis	
Kidney	▪ Nephrolithiasis ▪ Diabetic glomerulopathy	▪ Immune complex-mediated glomerulonephritis
Respiratory system	▪ Bronchiectasis ▪ Recurrent infections ▪ Atelectasis	▪ Allergic bronchopneumonia ▪ Aspergillosis
Heart	▪ Cor pulmonale ▪ Left ventricular fibrosis	▪ Carotid bodies enlarged
Male genital system	Male infertility (obstructive aspermia due to vas deferens atresia in 6% and male infertility in 1–2%)	
Female genital system	▪ Multiple ovarian follicular cysts ▪ Cervicitis	▪ Reduced fertility
Musculoskeletal system	▪ Osteoporosis ▪ Muscle wasting	▪ Hypertrophic pulmonary arthropathy ▪ Clubbing of fingers
General manifestations	▪ Malnutrition ▪ Vitamin deficiencies (vitamin B_{12}, vitamin K*)	▪ Amyloidosis

**Vitamin K deficiency at birth may lead to bleeding.*

ducts is impaired. Molecular testing of CFTR gene mutation is another diagnostic tool. Screening done by using immunoreactive trypsinogen (IrT) assay shows elevated levels in infants with cystic fibrosis.

Treatment

The patient with cystic fibrosis disease is treated by inhaler bronchodilators to reduce resistance in respiratory airways. Inhaled mucolytic agents and saline help in dissolving mucus. Antibiotics are administered to cover infection.

GLYCOGEN STORAGE DISEASES

Glycogen storage diseases (**GSDs**) are the result of deficiency of lysosomal enzymes that cause the alteration of glycogen metabolism (glycogen synthesis or glycolysis) in the liver and skeletal muscle.

- The liver forms of glycogen storage diseases (types 1, 3, 4 and 6) are marked by inability to convert glycogen to glucose.
- The skeletal muscle forms of glycogen storage diseases (types 2, 3A, 5 and 7) have mild symptoms appearing during strenuous exercise owing to inability to provide energy for skeletal muscle contraction. Glycogen storage diseases are given in Table 5.51.

Glycogen Storage Disease Type 0

Glycogen storage disease type 0 (GSD type 0) is an inherited autosomal recessive lysosomal storage disorder caused due to mutation in glycogen synthase 2 (GYS2) gene that encodes abnormal glycogen synthase enzyme.

- GSD type 0 is inherited within families in an autosomal recessive fashion. Normally, glycogen synthase participates in the generate glycogen, that is stored in the liver.
- Glycogen synthase deficiency leads to low amount of glycogen in the liver resulting in hypoglycemia and hyperketonemia.

Clinical Features

Early in infancy, children usually have no symptoms. Any child with a history of needing frequent meals and with hypoglycemia and presence of ketone bodies in the urine is suggestive of GSD type 0.

Table 5.51 Glycogen storage diseases

Type	Defective Enzyme	Organ Affected	Glycogen in the Affected Organ	Clinical Features
Type 1 GSD (von Gierke disease)	Glucose-6-phosphatase or transport system	▪ Liver ▪ Kidney	Increased amount of normal structure	▪ Massive hepatomegaly, failure to thrive ▪ Severe hypoglycemia ▪ Ketosis ▪ Hyperuricemia ▪ Hyperlipidemia
Type 2 GSD (Pompe disease)	Alpha-1,4-glucosidase (lysosome)	All organs (liver, kidney, skeletal muscle)	Massive increase in amount, normal structure	▪ Hypotonia (floppy baby) ▪ Hypertrophic hypertrophy ▪ Cardiorespiratory failure causes death usually before age of two years
Type 3 GSD (Cori disease)	Amylo-1,6-glucosidase (debranching enzyme)	▪ Skeletal muscles ▪ Liver	Increased amount; short outer branch	Like type 1, but milder form
Type 4 GSD (Anderson disease)	Branching enzyme (α-1,4 $\rightarrow$ α-1, 6)	Liver and spleen	Normal amount; very long outer branches	▪ Progressive liver cirrhosis with fatal outcome before age of two years ▪ Hepatocellular failure with fatal outcome before age of two years
Type 5 GSD (McArdle disease)	Phosphatase	Skeletal muscles	Moderately increased amount; normal structure	▪ Limited ability to perform strenuous exercise due to painful muscle cramps ▪ Otherwise, patient is well-developed and normal
Type 6 GSD (Hers disease)	Phosphorylase	Liver	Increased amount	Like type 1, but milder course
Type 7 GSD	Phosphofructokinase	Skeletal muscles	Increased amount, normal structure	Like type V
Type 8 GSD	Phosphorylase kinase	Liver	Increased amount, normal structure	Mild hepatomegaly, mild hypoglycemia

Type 1 through type 7 are inherited disorder, and type 8 is sex-linked disorder.

Laboratory Diagnosis

Genetic testing on blood sample is now available. Aim of treatment for type 0 glycogen storage disease is to prevent low blood sugar (hypoglycemia) by avoiding fasting. Frequent meals and snacks should be given every 3–4 hours during the day.

von Gierke's Disease (GSD Type 1)

von Gierke disease is most common autosomal recessive lysosomal storage disorder caused due to deficiency of glucose-6-phosphatase, in which body cannot break glycogen stored in the liver and skeletal muscle. Normally, glycogen stored in the liver and skeletal muscle is broken down to glucose by glucose-6-phosphatase to generate energy. von Gierke disease is also called glycogen storage disease type 1 (**GSD 1**).

Clinical Features

Patient with GSD-1 presents with severe hypoglycemia that coincides with ketonuria, metabolic acidosis and elevated lactate (due to excessive glycolysis) and alanine. Building up of glycogen in liver results in hepatomegaly.

- Decreased glucose causes lipolysis resulting in hyperlipidemia. Uricemia is caused by competitive inhibition by lactate of renal tubular urate secretion and increased uric acid production.
- Patient has growth retardation. Epinephrine and glucagon cannot produce hyperglycemia but result in lipolysis and increased lactate concentration.

Laboratory Diagnosis and Treatment

GSD-1 disorder is diagnosed by liver biopsy and DNA testing. Patients are treated by cornstarch, allopurinol and granulocytes colony stimulating factor (**GCSF**).

Pompe Disease (GSD Type 2)

Glycogen storage disease type 2, also called Pompe disease, is an autosomal recessive lysosomal storage disorder, which damages skeletal muscle, cardiac muscle, liver and nerves throughout the body. Pompe disease occurs due to deficiency of lysosomal enzymes, α_4-glucosidase enzyme and α_4-glucosidase resulting in accumulation of glycogen in the lysosomes.

Clinical Features

Patient of Pompe disease (GSD-2) develops intractable hypoglycemia, muscle weakness and cardiomegaly with normal liver functions. Pompe disease is of two types: (a) infantile type and (b) delayed onset type (childhood and juvenile/adult types).

- Infantile type is most severe form of Pompe disease. Infants are normal at birth, and manifest within the first two to three months with rapidly progressive skeletal muscle weakness, diminished skeletal muscle tone, respiratory problems, feeding problems, hypertrophic cardiomyopathy progressing to cardiorespiratory failure before 3 years of age.
- Delayed onset childhood and juvenile/adult type of Pompe disease manifests during infancy or early childhood with delayed motor milestones. Juvenile/adult form of Pompe disease presents during in second and seventh decades of life with muscle weakness.

Laboratory Diagnosis and Treatment

Patients of Pompe disease are diagnosed by skeletal muscle biopsy, liver biopsy, lysosomal acid maltase assay and DNA testing. Patients are treated by lysosomal acid maltase replacement.

Cori Disease (GSD Type 3)

Cori disease is an autosomal recessive lysosomal storage disorder caused due to deficiency of glycogen debranching enzymes (amylo-1,6-glucosidase) resulting in storage of abnormal form of glycogen in the liver, heart, or skeletal muscle.

Clinical Features

Patient with Cori disease (GSD-3) presents with stunted growth, hepatomegaly, and hypoglycemia. Glycogen storage disease is divided into types 3A, 3B, 3C and 3D, which are distinguished by the pattern of signs and symptoms.

- GSD types 3A and 3C mainly affects the liver (hepatomegaly) and skeletal muscles (muscle weakness).
- GSD types 3B and 3D affect only the liver (hepatomegaly). GSD types 3A and 3B are the most common forms of Cori disease.

Laboratory Diagnosis and Clinical Course

Beginning in infancy, any type of GSD 3 is characterized by low blood sugar (hypoglycemia), hyperlipidemia and elevated blood concentration of liver transaminases and creatinine kinase.

- As the infant gets older, the child develops hepatomegaly. Liver size returns to normal during adolescence.
- Some affected persons develop cirrhosis, hepatocellular failure and liver adenoma in life.

Anderson Disease (GSD Type 4)

Anderson disease is an autosomal recessive trait lysosomal storage disorder caused by deficient activity of the glycogen-branching enzyme resulting in accumulation of abnormal glycogen in the liver, muscle, and/or other tissues. Synonyms of GSD type 4 are Anderson disease or glycogenosis, amylopectinosis, branching enzyme deficiency and glycogenosis type 4.

Clinical Features

Infant with Anderson disease (GSD-4) presents with failure to thrive and hepatosplenomegaly followed by cirrhosis progressing to portal hypertension and hepatocellular failure. Death occurs from heart or liver failure before 5 years of age. In addition, several neuromuscular variants of Anderson disease have been described that may be evident at birth, in late childhood or adulthood.

- Patient develops isolated skeletal muscle involvement with skeletal myopathy and/or dilated cardiomyopathy leading to muscle weakness, fatigue, exercise intolerance, shortness of breath with exertion, edema and cardiac arrhythmias.
- Neuromuscular variant of GSD type 4 in adults, known as polyglucosan body disease is characterized by dysfunction of the central nervous system (brain and spinal cord) and peripheral nervous system (motor, sensory and autonomic nervous system).

Laboratory Diagnosis and Treatment

Diagnosis of Anderson disease (GSD-4) is established by clinical evaluation, history and physical examination. Biopsy obtained from liver, skeletal muscle, heart, skin or peripheral nerve and examined under light microscope demonstrates abnormal deposition of amylopectin-like material. Patients are diagnosed by liver biopsy and DNA testing. Patients are treated by liver transplantation.

McArdle Disease (GSD Type 5)

McArdle disease (GSD-5) is the most common metabolic autosomal recessive lysosomal storage disorder of skeletal muscle carbohydrate metabolism and one of the most frequent genetic myopathies.

- McArdle disease is caused by mutations in PYGM gene (muscle glycogen phosphorylase form), located on chromosome 11q13 that codes for the phosphorylase enzyme.
- Deficiency of muscle phosphorylase results in accumulation of glycogen in skeletal muscle. Synonyms of GSD type 5 are McArdle disease, glycogenosis type 5, myophosphorylase deficiency or muscle glycogen phosphorylase deficiency.

Clinical Features

McArdle disease (GSD-5) manifests during second or third decade of life with skeletal muscle cramps, muscle stiffness and muscle weakness after strenuous exercise, hypoglycemic seizures or cardiomegaly; increased blood concentration of creatine kinase, myoglobinuria (dark burgundy-colored urine due to presence of myoglobin, a protein found in heart and skeletal muscles), exaggerated increase in ammonia and diminished activity of muscle phosphorylase. Patient is advised to take sucrose prior to strenuous activity. Patients are diagnosed by skeletal muscle enzyme assay and DNA testing.

Hers Disease (GSD Type 6)

Hers disease (GSD-6) is an autosomal recessive disorder caused by a deficiency of hepatic phosphorylase enzyme resulting in excessive accumulation of glycogen in the liver. Hepatic phosphorylase enzyme is essential to break down glycogen stored in the liver and skeletal muscle and used for energy.

Clinical Features

Infant or child suffering from Hers disease presents with failure to thrive, hypotonia, hepatomegaly, ketotic hypoglycemia, elevated hepatic transaminases, hyperlipidemia and low prealbumin level.

- Most common complications of Hers disease (GSD-6) include short stature, delayed puberty, osteopenia and osteoporosis. Liver fibrosis commonly develops in GSD-6, but cirrhosis and hypertrophic cardiomyopathy rarely develop.
- Clinical and biochemical alterations may decline with age, but ketosis and hypoglycemia can continue to occur.

Laboratory Diagnosis

Diagnosis of Hers disease (GSD-6) is established by clinical evaluation, history and physical examination; and molecular testing of PYG1 gene mutation.

Taruis' Disease (GSD Type 7)

Taruis' disease (GSD-7) is an autosomal recessive lysosomal storage disorder. It is more often in individuals of Japanese and Ashkenazi Jewish descent equally affecting males and females. Disease is caused by mutations in the muscle phosphofructokinase gene that encodes phosphofructokinase enzyme resulting in deficiency of phosphofructokinase enzyme in skeletal muscle and erythrocytes. Phosphofructokinase enzyme deficiency leads to reduced amount of energy available to skeletal muscles during exercise.

Clinical Features

Taruis' disease (GSD-7) usually begins in childhood. Patient presents with weakness, pain, stiffness of skeletal muscles during exercise; sometimes associated with nausea, vomiting and dark burgundy-colored urine due to the presence of myoglobin (myoglobinuria). GSD type 7 can rarely affect infants and adults.

Laboratory Diagnosis and Treatment

Taruis' disease is diagnosed by a **skeletal muscle biopsy** for the measurement of phosphofructokinase enzyme level or analysis of phosphofructokinase enzyme in red blood cells.

- Molecular genetic testing for mutation in phosphofructokinase gene encoding phosphofructokinase enzyme in Japanese and Ashkenazi Jewish descent.
- The patients should be advised to avoid strenuous exercise for prevention of skeletal muscle pain and cramps. Consumption of carbohydrates should be avoided. Genetic counseling is recommended for affected individuals and their families.

Glycogen Storage Disease (GSD Type 9)

Glycogen storage disease type 9 is an autosomal recessive lysosomal storage disorder. Disorder is caused by the inability to breakdown glycogen by phosphorylase enzyme in the liver. The signs and symptoms such as hepatomegaly and slow growth begin in the early childhood.

Clinical Features

Affected children may have delayed development of milestones (sitting, standing, walking) and mild skeletal muscle weakness. Adolescents may have delayed puberty and liver fibrosis, that can rarely progress to cirrhosis.

- During prolonged periods of fasting, patient develops hypoglycemia or elevated levels of ketone bodies in the blood (ketosis). Ketones are molecules produced during lipolysis, which occurs when stored sugars are not available.
- GSD-9 can affect skeletal muscle tissue in children and adults. Patient experiences fatigue, skeletal muscle weakness, skeletal muscle pain, and cramps during strenuous exercise.

Laboratory Diagnosis

GSD-9 can cause breakdown of skeletal muscle resulting in release of myoglobin and its excretion in the urine. Myoglobinuria can cause the **urine** to be red or brown. In a small number of patients with GSD type 9, both liver and skeletal muscles are affected, however skeletal muscle problems are usually mild.

Glycogen Storage Disease (GSD Type 10)

Glycogen storage disease type 10 is an autosomal recessive lysosomal storage disorder of glycogen metabolism. Disease is caused by mutation in the **PGAM2 gene** encoding skeletal muscle phosphoglycerate mutase enzyme.

- GSD-10 primarily affects skeletal muscles. Patient presents with muscle cramps following strenuous physical activity, recurrent episodes of myoglobinuria as a result of breakdown of skeletal muscle.
- Untreated cases of myoglobinuria can result in renal failure. In some cases of GSD type 10, microscopic tube-shaped structures called tubular aggregates are demonstrated in skeletal muscle fibers. It is not clear how tubular aggregates are associated with the signs and symptoms of the disorder.

GALACTOSEMIA

Galactosemia is an autosomal recessive disorder of carbohydrate metabolism that affects the body's ability to convert galactose (a sugar contained in milk, including human mother's milk) to glucose.

- The cause of classic galactosemia is deficiency of galactose-1-phosphate uridyltransferase, with resultant accumulation of galactose-1-phosphate in many tissues.
- Patient suffering from galactosemia presents with failure to thrive, infantile cataracts, mental retardation, and progressive hepatocellular failure leading to cirrhosis and death. Most of these changes can be prevented by early removal of galactose from the diet.
- Galactosemia due to galactokinase deficiency is much less frequent than classic galactosemia. The disorder is often marked only by **infantile cataracts**.

MUCOPOLYSACCHARIDE STORAGE DISEASES (MUCOPOLYSACCHARIDOSIS TYPE 2)

Mucopolysaccharide storage (MPS) diseases are autosomal recessive disorders and caused by disturbances of the normal breakdown of carbohydrate complex known as mucopolysaccharides. Examples of mucopolysaccharide storage diseases are Hurler syndrome, Hunter syndrome, Sanfilippo syndrome, Maroteaux-Lamy disease, Sly disease and Morquio disease.

- MPS diseases have certain common characteristics of osteoarthritis, which include deformities of the bones and weight-bearing large joints that interfere with mobility.
- All MPS diseases except Sanfilippo disease interfere with growth development, causing short stature.

Hurler Syndrome (MPS Type 1 Disease)

Hurler syndrome, the most severe form of mucopolysaccharidosis is an autosomal recessive lysosomal storage disorder. Disorder is caused by mutations in the IDUA gene located on 4p16.3, that encodes **α**-L-iduronidase enzyme resulting in accumulation of mucopolysaccharides in lysosomes, such as dermatan sulphate and heparan sulphate in the heart, brain, liver and other organs. Affected patient inherits one mutated copy of the gene from each parent, who is referred to a carrier.

Clinical Features

Patient of Hurler syndrome presents with skeletal abnormalities (abnormal bones in the spine, claw hands, stubby fingers, joint stiffness, dwarfism, growth retardation), progressive mental retardation, cognitive impairment, intellectual disability getting worse overtime, respiratory problems, cardiac valve deformities, characteristic facies, corneal clouding, deafness, hepatosplenomegaly and reduced life expectancy.

Laboratory Diagnosis and Treatment

A urine analysis is only one of first steps in diagnosing Hurler syndrome (mucopolysaccharidosis I). Definite diagnosis requires a test to measure α-L-iduronidase enzyme activity levels in the blood or skin cells. Mucopolysaccharidosis I is a progressive, debilitating, and often life-threatening disease. In severe cases, death occurs by the age of ten years. Some patients may have a normal life span with enzyme replacement therapy.

Sanfilippo Syndrome (MPS Type 3 Disease)

Sanfilippo syndrome also known as mucopolysaccharidosis type 3 (MPS type 3 disease) is an autosomal recessive lysosomal storage disorder in which large sugar molecules called glycosaminoglycans build up and accumulated in lysosomes in various tissues especially brain and spinal cord.

Clinical Features

Patient suffering from Sanfilippo syndrome presents with growth retardation, intellectual disability overtime, abnormal bones in spine, claw hand, delay speech, headache, respiratory tract infections, deafness, cardiac valves problems, joint disease, chronic diarrhea and sleeping problems.

Laboratory Diagnosis

Urine analysis reveals excretion of large amounts of mucopolysaccharide (heparan sulfate). Blood culture is done to grow microorganisms and their sensitivity to therapeutic agents.

Morquio Syndrome (MPS Type 4 Disease)

Morquio syndrome also known as mucopolysaccharidosis type 4 (MPS type 4 disease) is an autosomal recessive lysosomal storage disorder in which body cannot process certain types of sugar molecules called glycosaminoglycans resulting in accumulation in lysosomes in bones, joints, cartilage, skin and other tissues.

- The disorder can lead to a number of potential complications. MPS type 4 equally affects males and females.
- Patient suffering from Morquio syndrome presents with short stature with very short trunk, bell-shaped chest with ribs flared at the bottom, abnormal bone and spine development including scoliosis.

Scheie Syndrome (MPS Type V Disease)

Scheie syndrome is an autosomal recessive lysosomal storage disorder caused by deficiency of iduronidase enzyme resulting in building of glycosaminoglycans in the body. Disorder is the mild-subtype of MPS type 1 disease and the most severe type of MPS type 1 disease. Symptoms appear by the age of five years.

Clinical Features

Patient presents with mild coarsening of the facial features (large head, mouth, tongue and thick lips), hydrocephalus, large vocal cords, upper respiratory tract infections, nasal secretion, neurosensorial hearing loss, hepatomegaly, stiff joints, mild skeletal changes and carpal tunnel syndrome, umbilical hernia and inguinal hernia.

Laboratory Diagnosis

Urine analysis reveals high levels of glycosaminoglycans (GAGs). Aortic valve involvement may be present. A definite diagnosis requires molecular testing to measure enzyme activity in the blood or skin cells. Enzyme replacement therapy with iduronidase enzyme and surgery may be essential.

Maroteaux-Lamy Disease (MPS Type 6 Disease)

Maroteaux-Lamy disease is an autosomal recessive trait lysosomal storage disorder caused by ARSB gene (5q13-q14) encoding N-acetylgalactosamine-4-sulfatase (arylsulfatase B).

- Under physiologic state, N-acetylgalactosamine-4-sulfatase (arylsulfatase B) participates in breakdown of large sugar molecules called glycosaminoglycans (GAGs). GAGs are usually linked to proteins to form proteoglycans and include chondroid 4-sulfate, chondroid 6-sulfate, heparan sulphate, dermatan sulfate, keratan sulfate and hyaluronic acid.
- Deficiency of N-acetylgalactosamine-4-sulfatase (arylsulfatase B) results in accumulation of glycosaminoglycans in lysosomes of connective tissue and organs.

Clinical Features

Patient suffering from Maroteaux-Lamy disease presents with somatic features such as skeletal abnormality, including short stature and joint deformities but not by mental retardation. Other clinical findings include hepatosplenomegaly, decreased pulmonary function, cardiac valve deformities, reduced pulmonary function, otitis media, hearing loss, sinusitis, cloudy cornea, carpal tunnel syndrome, sleep apnea and umbilical or inguinal hernia.

Laboratory Diagnosis

A diagnosis of Maroteaux-Lamy disease is based on clinical features, family history, increased excretion of dermatan sulfate in urine, blood sample for enzyme of arylsulfatase, skin biopsy for arylsulfatase B and molecular testing for ARSB gene mutation.

Sly Syndrome (MPS Type 7 Disease)

Sly syndrome, also known as mucopolysaccharidosis type 7 (MPS type 7 disease), is an autosomal recessive lysosomal storage disorder caused by deficiency of the β-glucuronidase enzyme. Normally, β-glucuronidase enzyme is responsible for degradation of large sugar molecules called glycosaminoglycans. Deficiency of β-glucuronidase enzyme results in accumulation of glycosaminoglycans in skeletal, joints, brain, spinal cord, heart, spleen and liver.

Clinical Features

The most severe cases of Sly syndrome (MPS 7) are characterized by hydrops fetalis as a result of excessive accumulation of fluid in the body before birth, which can result in stillborn or death after birth. Neonatal jaundice or yellow discoloration of skin may occur. In MPS 7 disease, children present with short stature, macrocephaly, short neck, unusual short trunk and multiple bone deformities such as prominent breast bone (pectum carinatum), flared ribs, frequent hip dislocations, 'frozen' joints (contractures), club foot, and/or an inward curve of the knees and outward bowing of the ankles (genu valgum).

GAUCHER'S DISEASE

Gaucher's disease is most common autosomal recessive lysosomal storage disorder in European (Ashkenazi) Jewish lineage. Disorder is caused by a deficiency of the lysosomal glucocerebrosidase enzyme (lysosomal acid β-glucosidase), which leads to an accumulation of its substrate, glucosylceramide (membrane

glycosphingolipids) derived from the catabolism of senescent leukocytes, in the lysosomes of macrophages.

Diagnostic Hallmark

The hallmark of Gaucher's disease is the presence of '**Gaucher's cells**' with a distinctive fibrillary cigarette paper-like cytoplasmic appearance and eccentric nuclei, which are lipid-laden macrophages present in the red pulp of the spleen, liver sinusoids (Kupffer cells), lymph nodes, lungs (alveolar macrophages), and bone marrow. Bone marrow aspiration reveals numerous Gaucher's cells, but specific enzymes (chitotriosidase and angiotensin-converting enzyme markers of macrophage proliferation) assay is required to confirm the diagnosis. Mutations in the GBA1 gene can be demonstrated in these cases.

Clinical Features

Clinical features of Gaucher's disease are variable depending on three distinct variants of Gaucher's disease types 1, 2 and 3 based on the presence or absence of neurological complications. Gaucher's disease type 1 lacks neurological complications. Neurological complications are present in Gaucher's disease in types 2 and type 3. Comparison of three variants (types 1, 2 and 3) of Gaucher's disease is given in Table 5.52.

- **Type 1 or adult Gaucher's disease:** Gaucher's disease type 1 accounts for >90% of cases in Europe and USA. Patient presents with marked hepatosplenomegaly, bone pain and fracture due to erosion of femoral head and other long bones. Mild anemia and thrombocytopenia (easy bruising) are also present. Patient may live a normal life span. Gaucher's cells are demonstrated in the bone marrow, liver, spleen, and lymph nodes.
- **Type 2 or infantile Gaucher's disease:** Gaucher's disease type 2 occurs in newborns and infants present with neurological complications such as involuntary skeletal muscle spasm, difficulty is swallowing and loss of previously motor skills with fatal outcome before 1 year of age.
- **Type 3 or juvenile Gaucher's disease:** Gaucher's disease type 3 occurs during first decade of life and presents with neurological complications such as mental retardation, inability to coordinate voluntary movements and skeletal muscle spasms of the extremities or entire body; and visceral involvement. It is less severe than type 2 Gaucher's disease.

Morphology: Gaucher's Disease

Light Microscopy

- Gaucher's cells are demonstrated spleen, liver, bone marrow, CNS, tonsil, thymus, Peyer's patches, and lungs.
- Cytoplasm is fibrillary and crumpled (tissue paper-like).
- Histologic examination of liver biopsy shows Gaucher's cells with a distinctive cigarette paper-like cytoplasmic appearance and eccentric nuclei, which are lipid-laden macrophages present liver sinusoids (Kupffer cells). Histology of Gaucher's disease is shown in Fig. 5.43.

Treatment

The patients with Gaucher disease are treated by administration of enzyme replacement therapy by

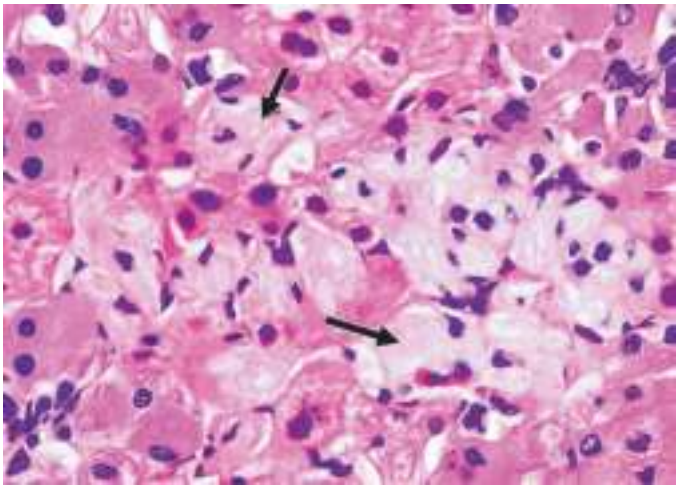


Fig. 5.43: Histology of Gaucher's disease. Gaucher's disease shows Gaucher's cells containing cigarette paper-like cytoplasmic appearance and eccentric nuclei, which are lipid-laden macrophages (arrows) (400X).

Table 5.52 Comparison of three variants (types 1, 2 and 3) of Gaucher's disease

Parameters	Gaucher's Disease Type 1	Gaucher's Disease Type 2	Gaucher's Disease Type 3
Age group	Adults (>90% in Europe and USA)	Infants	Early childhood
Frequency	>90% most common	Uncommon	Uncommon
Organs involved	Liver, spleen, bone marrow, lymph nodes	Liver, spleen, bone marrow and central nervous system	Central nervous system and viscera
Clinical features	Splenomegaly, hepatomegaly, cytopenia, fracture of femoral head	Failure to thrive and neurological impairment	Myoclonic convulsions
Prognosis	Normal life span	Fatal by one year of age	Less severe disorder

intravenous route (imiglucerase, velaglucerase, or taliglucerase). Orally administered inhibitors (miglustat of eliglustat) of glucosylceramide biosynthesis can be used.

NIEMANN-PICK DISEASE

Niemann-Pick disease is an autosomal recessive lysosomal storage disorder, which occurs due to SMPD1 gene mutation encoding protein resulting in accumulation of sphingomyelin in the lysosomes of phagocytes especially in type A and type B Niemann-Pick disease.

Diagnostic Criteria

Diagnostic hallmark of Niemann-Pick disease is presence of foamy histiocytes known as 'Niemann-Pick cells' containing sphingomyelin in liver, spleen, lymph nodes, skin, bone marrow, tonsil, GIT, and lungs.

- On light microscopy, Niemann-Pick cells are large cells 25–100 µm in size with eccentric nuclei and foamy cytoplasm. Foam cells show positivity with oil red O and Sudan black B.
- Electron microscopy of skin, rectal neurons, liver, or brain may demonstrate polymorphous cytoplasmic bodies. Niemann-Pick disorder is shown in Fig. 5.44.

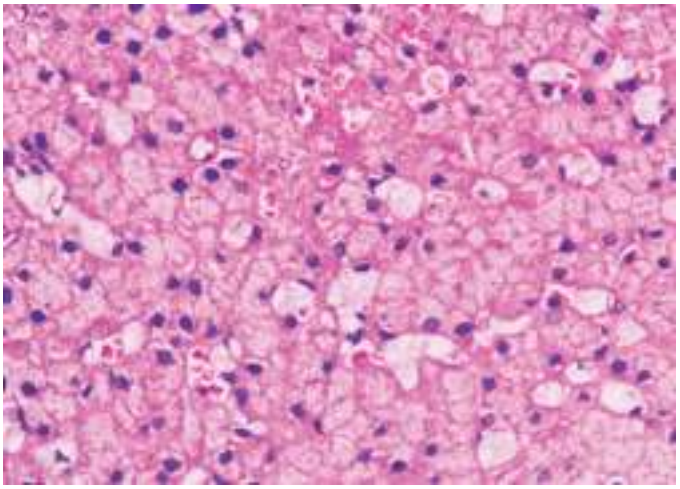


Fig. 5.44: Niemann-Pick disorder. Kupffer cells of liver show foamy histiocytes' containing sphingomyelin (400X).

Niemann-Pick Disease Variants

There are three variants of Niemann-Pick disease: types A, B and C. Comparison of three types (A, B, C) of Niemann-Pick disease is given in Table 5.53.

- **Niemann-Pick disease (type A):** It is a severe disease affecting infants within six months of life. Infant has extensive involvement of nervous system, liver and spleen. Patient presents with hepatosplenomegaly, lymphadenopathy, anemia, fever, a cherry red spot inside the macula of eye similar to that of Tay-Sachs disease, difficulty in feeding and neurologic manifestations with fatal outcome by three years of age.
- **Niemann-Pick disease (type B):** It is less severe disease, which does not involve central nervous system and organs. Patient presents with hepatomegaly, splenomegaly, pulmonary insufficiency and central nervous system manifestations with relatively better prognosis. Patient survives till adulthood.
- **Niemann-Pick disease (type C):** It occurs due to mutations in NCP1 and NCP2 genes, which encode cholesterol transport protein leading to accumulation of cholesterol within phagocytes of organs. Patient presents with hepatomegaly, splenomegaly, pulmonary insufficiency and central nervous system manifestations with relatively better prognosis. Patient survives up to adulthood.

Table 5.53 Comparison of three types (A, B, C) of Niemann-Pick disease

Parameters	Type A Niemann-Pick Disease (Classic Infantile Type)	Type B Niemann-Pick Disease (Visceral Type)	Type C Niemann-Pick Disease (Visceral Type)
Age group	Infants (severe disease)	Adults (less severe)	10 and 25 years
Basic defect	SMPD1 gene mutation encoding acid sphingomyelinase (ASM) protein resulting in accumulation of sphingomyelin in the lysosomes of phagocytes	SMPD1 gene mutation encoding acid sphingomyelinase (ASM) protein resulting in accumulation of sphingomyelin in the lysosomes of phagocytes	NPC1 or NCP2 gene mutations resulting in accumulation of sphingomyelin in the lysosomes of phagocytes
Clinical features	Hepatomegaly, splenomegaly, anemia, fever, profound neurologic manifestations and cherry red spots inside eye, difficulty in feeding	Hepatomegaly, splenomegaly, pulmonary insufficiency and central nervous system manifestations	Hepatomegaly, splenomegaly, central nervous system manifestations
Prognosis	Fatal outcome by three years of age (rarely survival beyond three years of age)	Survival in adulthood	Sometimes fatal outcome

- The diagnosis of Niemann-Pick disease type C (visceral type) should be considered in persons presenting with clinical manifestations: (a) fetal ascites or neonatal liver disease accompanied by prolonged jaundice and pulmonary infiltrates, (b) infantile hypotonia without evidence of progression for months to years, (c) vertical supranuclear gaze palsy followed by progressive ataxia, dystonia, dysarthria and seizures, (d) psychiatric presentations mimicking depression or schizophrenia, and (e) hepatomegaly or splenomegaly particularly, in early childhood.
- Molecular genetic testing is necessary to confirm the diagnosis in all persons with suspected Niemann-Pick disease.

ASPARTYLGLUCOSAMINURIA

Aspartylglucosaminuria (AGU) is an autosomal recessive lysosomal storage disorder. AGU is caused by mutation in AGU gene located on chromosome 4q34.3 encoding abnormal N-aspartylglucosaminidase enzyme, that cleaves N-acetylglycosamine-asparagine bond found into many glycopeptides and N-glycosylated proteins, and causes excessive accumulation of glycoasparagine in the body tissues and its excretion in urine. Low activity of N-aspartylglucosaminidase enzyme is demonstrated in lymphocytes, fibroblasts and trophoblasts. AGU is exceptionally found **outside of Finland**.

Clinical Features

Clinical manifestations of aspartylglucosaminuria appear several months after birth involving central nervous system and skeletal system. Clinical signs of AGU include slowly developing mental retardation, beginning of clumsiness (poor coordination of movements), delayed speech, mild gradual facial dysmorphism (coarsening), hyperkinesis, slight kyphoscoliosis and macroglossia. Hepatosplenomegaly is rare and reported in patients outside Finland.

BATTEN DISEASE

Batten disease is an autosomal recessive lysosomal storage disorder, that begins in childhood between five and ten years of age. Disorder is characterized by the accumulation of lipopigment in the brain as well as other tissues that lack nerve cells resulting in death of neurons in the brain, retina and central nervous system.

- Patient develops rapidly progressive neurological disturbances (e.g. deterioration of both intellect and neurological functions) and vision failure (optic atrophy). The life expectancy is between eight to ten years of age.
- No definite treatment is available to cure Batten disease. The seizures can be controlled with anti-seizures drugs.

GM2 GANGLIOSIDOSIS

GM2 gangliosidosis disease is a rare autosomal recessive lysosomal storage disorder that includes two disorders (e.g. Tay-Sachs disease and Sandhoff disease). It is caused by inherited deficiency in hexosaminidase A, B, and AB. The disorder progressively destroys nerve cells in the brain and spinal cord.

GM2 Gangliosidosis Type 1 (Tay-Sachs Disease)

GM2 gangliosidosis type 1 (Tay-Sachs disease) is a rare autosomal recessive trait lysosomal storage disorder seen in Ashkenazi (central European origin) Jewish descent.

- Tay-Sachs disease is caused by mutation in the HEXA gene located on chromosome 15 encoding the α-N-hexosaminidase enzyme resulting in accumulation of GM2 ganglioside in the neurons of brain and spinal cord, that progressively destroys neurons in brain and spinal cord.
- On average, half of the offsprings are expected to be heterozygotes and silent carriers of the gene mutation. Tay-Sachs disease is also known as amaurotic familial idiocy, which exists in two forms: infantile and the late-onset Tay-Sachs disease.

Clinical Features

Patient suffering from infantile type of Tay-Sachs disease presents with severe mental and motor deterioration, feeding problems, generalized weakness and an exaggerated startle reflex (sudden shock) in response to sudden loud noises, central nervous system degeneration, loss of motor skills (turning over, sitting and crawling), cherry red spot in the ocular macula and blindness between three to six months of age. Children die before 4 years of age. Late-onset of Tay-Sachs disease may become apparent anytime from adolescence through the mid of third decade, that progresses more slowly than the infantile Tay-Sachs disease.

GM2 Gangliosidosis Type 2 (Sandhoff Disease)

GM2 gangliosidosis type 2 (Sandhoff disease) is an autosomal recessive lysosomal storage disorder caused by mutation in gene encoding abnormal β-subunit of the hexosaminidase B enzyme resulting in accumulation of GM2 gangliosides in the lysosomes of neurons of brain and spinal cord and hence progressive loss of neurological function. Disorder occurs in two forms: infantile and juvenile/adult Sandhoff disease.

Clinical Features

Patient suffering from GM2 gangliosidosis type 2 (infantile Sandhoff disease) presents with clinical manifestations between the age of three to six months such as feeding problems, general weakness, an exaggerated startle reflex (sudden shock) in response to sudden loud noises, development of cherry red spots in the back of the eyes, splenomegaly, cardiac murmurs, progressive motor weakness and seizures. Juvenile/adult forms of Sandhoff disease are more variable in the age of onset and severity of symptoms.

METACHROMATIC LEUKODYSTROPHY

Metachromatic leukodystrophy (MLD) is an autosomal recessive lysosomal storage disorder characterized by accumulation of fats called sulfatides in myelin resulting in destruction of the myelin sheath surrounding the nerves in both central nervous system and the peripheral nervous system.

- When biopsy is examined under light microscope, sulfatide accumulation in cells appear as granules that are colored differently than other cellular material (metachromatic).
- There are three types of MLD based on the clinical manifestations: late-infantile, juvenile and adult forms. Patient initially presents with vague signs and symptoms followed by developmental delay, unsteadiness when walking, marked spasticity, seizures and profound mental retardation.

SCHINDLER DISEASE

Schindler disease is an autosomal recessive lysosomal storage disorder caused by mutation in the NAGA gene located in chromosome 22q13.2 encoding abnormal enzyme α-N-acetylgalactosaminidase **A and B** in lysosomes resulting in deficient activity of enzyme. There are two forms of Schindler disease: **type 1** infantile and **type 2** adult forms.

- Schindler disease type 1 appears during infancy until approximately one year of age and presents with loss of previously acquired skills that require coordination of physical and mental activities.
- Schindler disease type 2 affects adults and presents with development of wart-like discoloration on the skin, relative coarsening of facial features and mild intellectual impairment. The dilation of small lymphatic channels may lead to swelling.

MUCOLIPIDOSIS

Mucolipidosis is a group of inherited metabolic disorders that affect the body's ability to perform the normal turnover of various materials within cells. In this disorder, abnormal amounts of carbohydrate and lipids accumulate in the cells resulting in symptoms that range from mild learning disabilities to severe intellectual impairment, vision problems and skeletal abnormalities. Mucolipidosis occurs in types 1, 2, 3 and 4.

Mucolipidosis Type 1

Mucolipidosis type 1 is an autosomal recessive lysosomal storage disorder caused by a deficiency of the neuraminidase enzyme and accumulation of sialidated glycopeptides and oligosaccharides. Excessive swelling throughout the body is observed at birth. Newborn has coarse facial features such as flat nasal bridge, puffy eyelids, enlargement of gums, macroglossia, hypotonia, failure to thrive, skeletal malformations such as hip dislocation, sudden involuntary muscle contractions (called myoclonus), cherry red spots (macules) in their eyes, impaired intellect and respiratory infections with fatal outcome before the age of one year.

Mucolipidosis Type 2

Mucolipidosis type 2 is a slowly progressive autosomal recessive lysosomal storage disorder, that may begin during infancy or later. This disorder is characterized by growth retardation, visual disturbances, is mild coarse features, mild mental retardation, skeletal abnormalities (abnormalities of skull and face), facial dysmorphism, stiff skin, developmental delay and cardiomegaly.

Mucolipidosis Type 3

Mucolipidosis type 3 is a slowly progressive autosomal recessive lysosomal storage disorder, that affects many parts of the body. Signs and symptoms of disease typically appear around three years of age. Patient presents with short stature, cardiac valve abnormalities, mild clouding of cornea, stiffness of the hands and shoulders with later development of carpal tunnel syndrome, deterioration of hip joints and scoliosis.

Mucolipidosis Type 4

Mucolipidosis type 4 is an autosomal recessive lysosomal storage disorder, caused by mutation in ML4 gene mapped on chromosome 19q13. It is characterized by severe neurological manifestations (delayed psychomotor development) and ophthalmological abnormalities (corneal opacities and strabismus).

CYSTINOSIS

Cystinosis is an autosomal recessive lysosomal storage disorder caused by mutation in the CTNS gene located on chromosome 17p13, which results in an abnormal accumulation of the amine acid cystine within lysosomes due to lack of a cystine-specific transporter protein. Infantile cystinosis is most common severe

form of disease. Cystine accumulation occurs in cells throughout the body and causes progressive damage to multiple organs such as kidneys, eyes, skeletal muscles, pancreas, and brain. The clinical signs appear between three and six months of age.

Clinical Features

Patient suffering from cystinosis presents with polyuria, polydipsia, anorexia, vomiting, constipation and marked growth retardation secondary to impairment of proximal reabsorption capacity with severe fluid–electrolyte alterations (termed Fanconi syndrome).

- Skeletal bone deformities are observed secondary to hypophosphatemia rickets. Ocular changes due to cystine deposits in the cornea and conjunctiva result in photophobia after three years of age.
- Cystine deposition in various organs (pancreas, gonads, liver, spleen, skeletal muscles, cerebrum, cerebellum and kidneys) progressively lead to insulin-dependent diabetes mellitus, hypothyroidism, hypogonadism, male infertility, hepatosplenomegaly, skeletal muscle weakness, hypotonia, oropharyngeal dysfunction and end stage renal failure before the age of ten years.

AMINO ACID METABOLISM DISORDERS

Amino acids are 'building blocks' that join together to form protein. Amino acid metabolism disorders include phenylketonuria, alkaptonuria including ochronosis and maple syrup urine disease.

Phenylketonuria (PKT)

Normally, phenylalanine hydroxylase coverts phenylalanine to tyrosine in the liver. Gene mutation encoding abnormal **phenylalanine hydroxylase** results in failure of conversion of phenylalanine to tyrosine in the liver.

- **Clinical features:** High serum concentrations of phenylalanine are neurotoxic and enter cerebrospinal fluid resulting in progressive cerebral demyelination, mental deterioration and seizures by the age of one year.
  - Phenylalanine is catabolized into phenyl pyruvic acid and phenylacetic acid and excreted in urine in large amounts. Other manifestations include decreased pigmentation of hair, eyes, skin and musty body odor from phenylacetic acid in urine and sweat.
- **Screening test:** The concentration of phenylalanine in affected infants is usually normal at birth and increases rapidly during the first days of life. False-negative results are common immediately after birth. Blood sample for phenylketonuria is usually taken from the infant's heel within 2–3 days after birth.
- **Treatment:** Successful treatment is a phenylalanine-free diet. If affected mother is not controlled, the infant is at high risk of development of congenital heart disease, growth retardation, microcephaly, and mental retardation due to phenylketonuria.

Alkaptonuria

Due to deficiency of homogentisic oxidase enzyme causes incomplete metabolism of phenylalanine and tyrosine resulting in accumulation and urinary excretion of homogentisic acid (black pigment). Clinically, this disorder manifests alkaptonuria and ochronosis. The term alkaptonuria refers to urinary excretion of unmetabolized homogentisic acid imparting a dark color to urine on standing.

Pathology Pearls: Ochronosis

- The term ochronosis refers to pigment deposition in multiple tissues, most prominently in cartilage and connective tissue. Homogentisic acid (dark pigment) is deposited in the articular cartilage of vertebral column and later hips, knees and shoulder joints. Cartilage of nose, ears, larynx and bronchi is also affected.
- Larger joints may be distended by effusions, which is usually noninflammatory and synovium is hypertrophied.
- Cardiac valves, prostate, and sweat glands may also be involved.
- Light microscopy of the lesion shows granular pigment in chondrocytes and matrix is diffusely stained. Synovium shows evidence of acute or chronic inflammation.

Maple Syrup Urine Disease

Maple syrup urine disease is a rare inborn error of metabolism. It means the body is unable to process certain amino acids (the 'building blocks' of protein) causing a harmful build up of substances in the blood and urine. Normally, body breaks down protein foods such as meat and fish into amino acids. Defects in proteins forming branched-chain keto acid dehydrogenase (keto acid decarboxylase) complex results in maple syrup urine disease. Urine contains high levels of keto acids of leucine, isoleucine, and valine giving maple syrup odor.

- Newborns are treated with protein-modified diets. Patient presents with retardation of physical and mental health along with feeding problems.
- Untreated newborn may develop mental and physical disabilities with fatal outcome.

X-LINKED RECESSIVE DISORDERS

Genes for these traits are located only on the X chromosome (not on the Y chromosome). X-linked alleles always show up in males whether dominant or recessive because males have only one X chromosome. Selected

Table 5.54 Selected X-linked recessive disorders

Hematological Disorders	
▪ Glucose-6-phosphatase (G6PD) deficiency ▪ Hemophilia A (deficiency of factor VIII) ▪ Hemophilia B (deficiency of factor IX)	
Immunodeficiency Disorders	
▪ X-linked Bruton agammaglobulinemia ▪ Chronic granulomatous disease ▪ Wiskott-Aldrich syndrome	
Storage Disorders	
▪ Fabry's disease	▪ Hunter's disease
Skeletal Muscle Disorders	
▪ Duchenne's muscular dystrophy (absolute dystrophin gene mutation)	▪ Becker's muscular dystrophy (relative dystrophin gene mutation)
Metabolic Disorders	
▪ Diabetes insipidus	▪ Lesch-Nyhan syndrome
Other Disorders	
▪ Red-green color blindness ▪ Fragile X syndrome ▪ X-linked ichthyosis	▪ Menkes 'kinki hair' syndrome (inability to transport copper into the cells)

X-linked recessive disorders are given in Table 5.54 and selected X-linked recessive disorders due to enzyme deficiency are given in Table 5.55.

CLASSIC HEMOPHILIA

Classic hemophilia is common X-linked recessive disorder. X and Y chromosomes are called sex chromosomes. Mutation in factor VIII gene located on the tip long arm of X chromosome encodes deficient clotting factor VIII, that does not produce sufficient thrombin in the intrinsic pathway of the coagulation cascade resulting in bleeding.

Molecular Genetic Alterations

The gene for hemophilia is located on the tip of the long arm of X chromosome.

- Females inherit two X chromosomes, one from their mother and one from their father (XX).
- Males inherit an X chromosome from their mother and a Y chromosome from their father (XY). It means that if a son inherits an X chromosome from a carrier mother, will suffer from hemophilia, meaning thereby father cannot transmit hemophilia to his sons.
- Because daughter has two X chromosomes, even if she inherits hemophilia gene from the mother and healthy X chromosome from her father, does not suffer from hemophilia.
- A daughter who inherits an X chromosome that contains the gene for hemophilia is called a carrier. She can transmit the hemophilia gene onto her children. Hemophilia can occur in daughters, but is rare.
- Heterozygous females are carriers, which transmit the disease to the male progeny. The family pedigree of Queen Victoria shows a number of hemophilic descendants as she was a carrier of the disease. In females, the other normal X chromosome corrects the abnormality, but females can be asymptomatic carriers. In males, the disease is expressed because there is no normal X chromosome to correct the abnormality.

Clinical Features

Classification of hemophilia A according to plasma procoagulant factor VIII levels is as follows: (a) severe hemophilia A with plasma factor VIII levels less than 1% of normal levels (<0.01 IU/ml), (b) moderate hemophilia A with plasma factor VIII levels between 1 and 5% of normal levels (0.01–0.05 IU/ml), and (c) mild hemophilia A with more than 5% but less than 40% of normal (<0.05 to 0.40 IU/ml). Classification of hemophilia A according to plasma procoagulant factor VIII levels is given in Table 5.56. Clinical manifestations of hemophilia are shown in Fig. 5.45.

- **Severe hemophilia A:** Person presents with bleeding following tissue injury and spontaneous bleeding episodes into the joints and skeletal muscles. Recurrent hemarthroses can lead to progressive crippling deformities. Bleeding in gastrocnemius muscle results in 'equine gait'.

Table 5.55 Selected X-linked recessive disorders due to enzyme deficiency

Disorder	Enzyme Deficiency	Accumulation
Hunter's syndrome	L-Iduronosulphate sulphatase	Heparan sulfate, dermatan sulfate
Fabry's disease	Alpha-galactosidase A	Ceramide trihexoside
G6PD deficiency	Glucose-6-phosphate dehydrogenase	Red blood cells hemolysis without any accumulation
Classic hemophilia (factor VIII deficiency)	Factor VIII	Without any accumulation
Lesch-Nyhan syndrome	Hypoxanthine-guanine phosphoribosyltransferase (HGPRT)	Uric acid
Duchenne muscular dystrophy	Dystrophin	Without any accumulation

Table 5.56 Classification of hemophilia A according to plasma procoagulant factor VIII levels

Severity of Hemophilia A	Plasma Factor VIII Levels Percentage	Frequency
Severe hemophilia A	Less than 1% of normal (<0.01 IU/ml)	60%
Moderate hemophilia A	1–5% of normal (0.01–0.05/ml)	15%
Mild hemophilia A	More than 5% but less than 40% of normal (>0.05 to <0.40 IU/ml)	25%

Classification of hemophilia A according to plasma procoagulant factor VIII levels is also applicable to factor IX deficiency (Christmas disease)

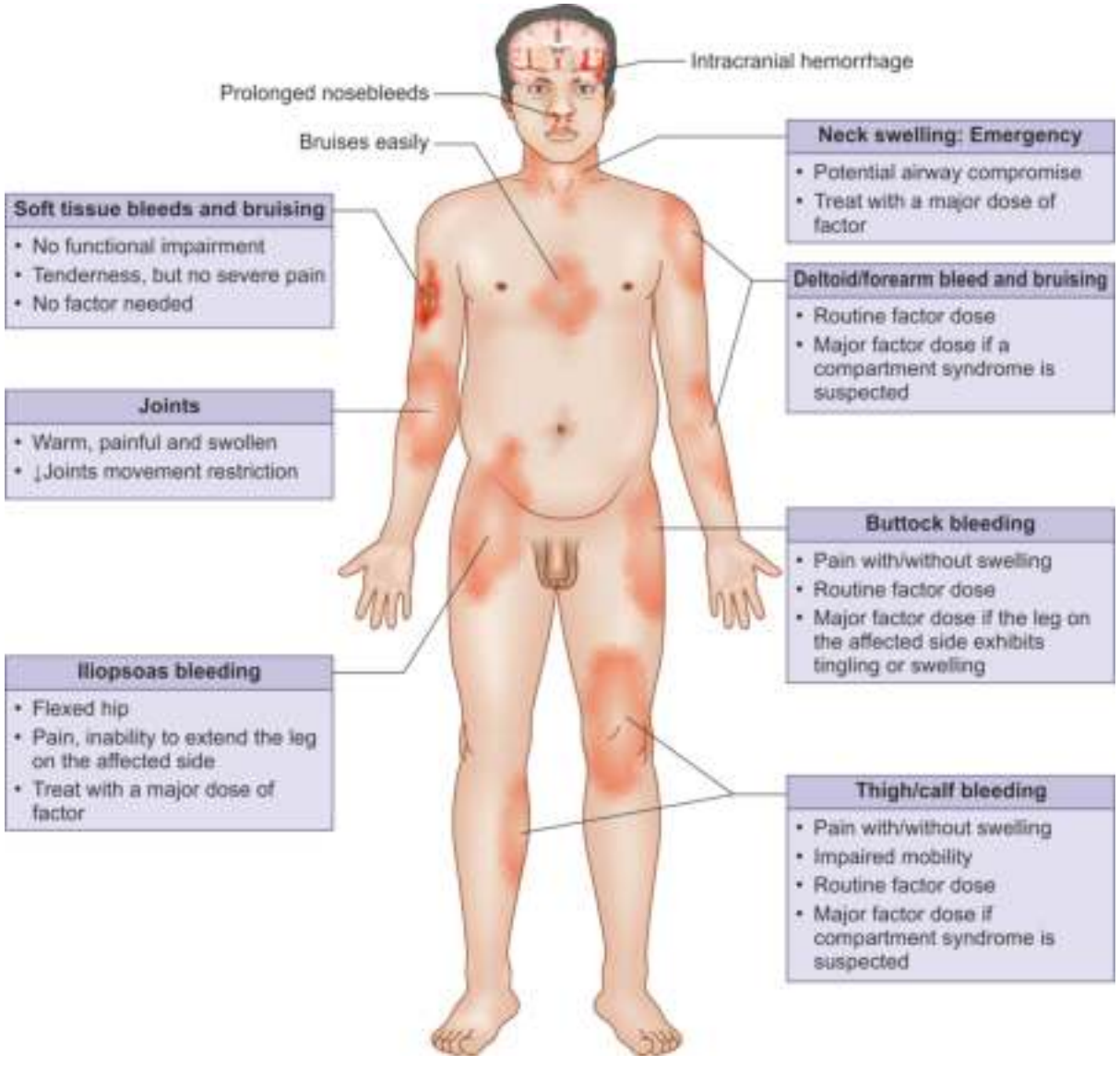


Fig. 5.45: Clinical manifestations of hemophilia.

- **Moderate hemophilia A:** Patient presents with bleeding episodes following injuries. Bleeding that occurs without obvious cause is called spontaneous bleeding episodes.
- **Mild hemophilia A:** Patient presents with bleeding only after severe injury, trauma or surgical procedures.

In many cases, mild hemophilia A is not diagnosed until an injury, surgery or tooth extraction results in prolonged bleeding. The first bleeding episode may not occur until adulthood. Women with mild hemophilia often experience menorrhagia, heavy menstrual periods, and can postpartum hemorrhage.

Laboratory Diagnosis

Hemophilia A is diagnosed primarily by reviewing a patient's family history as it is an inherited disorder. Physical examination and blood tests help in determination of the extent and severity of the disorder. Persons with known family history of the disorder can have tests done during pregnancy to determine if the fetus has inherited the disorder. Hemophilia cases are generally diagnosed between 0 and 2 years after birth with the mean age for diagnosis being nine months.

Treatment

Hemophilia A patient with bleeding manifestations is treated by infusion of concentrated FVIII clotting factor in fresh frozen plasma.

- The Medical and Scientific Advisory Council (MASAC) of the National Hemophilia Foundation encourages the use of recombinant FVIII product prepared from human-derived donor plasma by DNA technology.
- DDAVP (desmopressin acetate) is the synthetic version of vasopressin, a natural antidiuretic hormone that helps to stop bleeding. DDAVP can be administered in patients with mild hemophilia for bleeding in mucous membranes of the mouth and nose, joints and skeletal muscle before and after surgical procedures.
- Antifibrinolytic agents (e.g. aminocaproic acid and tranexamic acid) prevent the breakdown of blood clots in hemophilic patients, hence, it is recommended before dental procedures and to treat bleeding in nose and mouth.

DUCHENNE MUSCULAR DYSTROPHY

Duchenne muscular dystrophy is X-linked genetic disorder is the most common and severe variety of the disease due to deletion of one or many exons in the dystrophin gene (**DMD gene**) located on the short arm of the X chromosome (Xp21).

- Normally, dystrophin links the subsarcolemmal cytoskeleton to the exterior of the cell through a transmembrane complex of proteins. Boys with disorder cannot produce dystrophin at all.
- Dystrophin deficient skeletal muscles of pelvic and shoulder girdles predispose to death of myocytes during contraction. Serum levels of creatine kinase are markedly increased.

Clinical Features

Children fail to walk by 18 months of age due to weakness in the proximal muscles of the extremities, progressing to muscle necrosis, wasting, muscle contracture with fatal outcome in their teens. They develop pneumonia due to weakness of respiratory muscles. More than 90% of afflicted boys are wheelchair bound by the age of 10 years and bedridden by age 15 years.

Complications

The most common causes of death are complications of respiratory insufficiency caused by muscular weakness or cardiac arrhythmia due to myocardial involvement.

BECKER MUSCULAR DYSTROPHY

Becker muscular dystrophy is an X-linked recessive disorder caused by mutations in dystrophin gene, which encodes abnormal protein dystrophin in the muscle cell membrane. It is less common and less severe disorder than Duchenne muscular dystrophy.

- In Duchenne muscular dystrophy, dystrophin gene mutation results in the severe deficiency of protein dystrophin (<5%) in the muscle cell membrane.
- But in Becker muscular dystrophy, the mutation results in production of abnormal dystrophin or insufficient dystrophin protein in the muscle cell membrane.

Clinical Features

Becker muscular dystrophy is characterized by slowly progressive muscle weakness of the legs and pelvis in boys. Patient experiences difficulty in sports, climbing stairs and lifting heavy loads. Patient has calf muscles that look bigger than normal, even though these are weaker.

HUNTER SYNDROME (MPS TYPE 2 DISEASE)

Hunter syndrome is X-linked recessive disease and also known as mucopolysaccharidosis type 2 (MPS type 2 disease) in which deficiency of iduronate-2-sulfatase results in accumulation of large sugar molecules called glycosaminoglycans such as heparan sulfate and dermatan sulfate in the lysosomes in various tissues. Disease is transmitted from one generation to the next in a specific way. Hunter syndrome is less severe disease than Hurler syndrome.

Clinical Features

Patient suffering from Hunter syndrome presents with joint stiffness (immobile joints), skeletal muscle weakness, difficulty in walking, numbness, weakness and tingling sensations in hands; hepatosplenomegaly, upper respiratory tract infections, breathing problems at night, hearing loss, mild mental retardation, optic nerve edema, uveal effusion, retinal degeneration, visual field defects and cardiac lesions.

LESCH-NYHAN SYNDROME

Lesch-Nyhan syndrome is X-linked recessive disorder caused by deficiency of hypoxanthine-guanine phosphoribosyl transferase (hGPrT), resulting in impaired purine metabolism and excess production of uric acid. Patient presents with gout, mental retardation, choreoathetosis (abnormal involuntary muscle movements), spasticity, self-mutilation, and aggressive behavior.

GLYCOGEN STORAGE DISEASE (GSD) TYPE 8

Glycogen storage disease type 8 is X-linked recessive hepatic glycogen disorder resulting from lack of expression of phosphorylase-β-kinase activity. This is a regulatory enzyme in the activation cascade of glycogenolysis. GSD type 8 is mildest form of glycogenoses and characterized by hepatomegaly, increased glycogen in liver, growth retardation and decreased leukocyte phosphorylase, elevation of glutamate-pyruvate transaminase and glutamate oxaloacetate transaminase, hypercholesterolemia and hyperglyceridemia. Liver shrinkage occurs in response to glucagon.

FABRY DISEASE

Fabry disease is X-linked lysosomal storage disorder and also known as 'angiokeratoma corporis diffusum universale'. It occurs due to mutation in GLA gene encoding abnormal α-galactosidase A enzyme in lysosomes resulting in accumulation of ceramide trihexoside in body tissues such as skin, eyes, gastrointestinal system, kidney, heart and nervous system. Clinical manifestations are apparent until second or third decade of life.

Clinical Features

Patient suffering from Fabry disease presents episodes of fever, severe pain and burning sensations in hands and feet, decrease in sweat production, discomfort in warm temperatures and reddish to dark blue colored flat or raised skin rashes (angiokeratoma) in the lower trunk and area between the hips and knees; and cardiovascular and cerebrovascular involvement. Fabry disease is diagnosed by measuring the amount of α-galactosidase A enzyme activity. Patient dies of renal failure in early adult life.

X-LINKED DOMINANT DISORDERS

X-linked dominant inheritance refers to genetic disorders associated with mutations in one gene on X chromosome. A single copy of gene mutation is sufficient to cause disorder in males as well as females. If the trait is dominant, one of the parents must have the trait. X-linked dominant disorder can be passed down through families. In X-linked recessive traits, males are much more affected than females. X-linked dominant disorders are given in Table 5.57.

Table 5.57 X-linked dominant disorders

X-linked dominant hypophosphatemic rickets
Rett syndrome (95% of cases due to sporadic mutations)
Alport syndrome (most cases)
Incontinentia pigmenti
Giuffre-Tsukahara syndrome
Goltz syndrome
X-linked dominant protoporphyria
Fragile X-linked dominant syndrome

X-LINKED DOMINANT HYPOPHOSPHATEMIC RICKETS

X-linked dominant hypophosphatemic rickets can have several patterns of inheritance. Disorder results from mutation in the PHEX gene located on X chromosome. X-linked dominant hypophosphatemic rickets is characterized by inability of the kidneys to activate vitamin D and keep phosphate out of the urine and in the blood stream. Disease differs from most cases of rickets in that vitamin D supplementation does not cure the disease. Patient develops bone deformity, short stature, genu varum (bowing of legs), bone pains and severe dental problems.

RETT SYNDROME

Rett syndrome is an X-linked dominant disorder caused by mutation in MECP2 gene located on X chromosome in 95% of cases among females.

- After a short period of initial normal development, patient experiences neurological problems such as loss of speech and purposeful hand use, stereotypic hand movements and gait abnormalities.
- Additional clinical features include deceleration of head growth, seizures, autistic features and breathing abnormalities. Disease can also affect male infants. Most of them die before birth or early in infancy.

ALPORT SYNDROME

Alport syndrome is an inherited X-linked dominant disorder caused by mutation in **COL4A5 collagen gene** located on X chromosome, which codes for abnormal collagen protein. Collagen is important to the normal structure and function of the kidneys. Changes in collagen also results in ears and eyes problems. Disease

is characterized by progressive kidney disease (hematuria, proteinuria), hearing loss and eye abnormalities.

INCONTINENTIA PIGMENTI

Incontinentia pigmenti (IP) is a rare X-linked dominant genetic disorder, which occurs more common in females than males. Disease affects skin, teeth, nails and central nervous system. Patient presents with skin lesions blistering rash followed by wart-like lesions and brown hyperpigmented patches, and later hypopigmentation on arms and legs; small teeth, vision loss, intellectual disability and seizures.

GIUFFRÈ-TSUKAHARA SYNDROME

Giuffrè-Tsukahara syndrome, also known as radioulnar synostosis-microcephaly-scoliosis syndrome, is an X-linked dominant disorder. Disease is characterized by radioulnar synostosis with microcephaly, scoliosis, short stature, clinodactyly of the fifth fingers, partial webbing and intellectual disability.

GOLTZ SYNDROME

Goltz syndrome, also known as facial dermal hypoplasia, is an X-linked dominant multisystem disorder, that involves skin, skeletal system (hands, feet), face, eyes, cardiovascular system, central nervous system, urinary system and gastrointestinal system. In Goltz syndrome, ectodermal dysplasia affects normal development of hair, skin, nails and glands. Affected infant has thin skin, areas of missing skin, soft, yellow pink fat nodules in skin, and pigmentary changes.

X-LINKED DOMINANT PROTOPORPHYRIA

X-linked dominant protoporphyria is caused by mutation in ALAS2 gene located on X chromosome. Males usually develop the severe form of the disorder, while females remain asymptomatic.

- **Clinical features:** Patient presents with cutaneous photosensitivity since infancy that results in tingling, burning pain, and itching without blisters within minutes of exposure to sunlight, which persist for hours or days. Photosensitivity is lifelong. The photosensitivity and subsequent pain can be reduced by administration of α-melanocyte stimulating hormone analog.
- **Laboratory diagnosis:** The diagnosis of X-linked dominant protoporphyria is confirmed in a male with markedly elevated free erythrocyte protoporphyrin and zinc-chelated erythrocyte protoporphyrin by identification of hemizygous pathogenic gain of function in **ALAS2 gene** on molecular testing. Same molecular testing is performed to establish diagnosis in heterozygous female.

FRAGILE X SYNDROME

Fragile X syndrome has an X-linked dominant inheritance, which is caused by an expansion of the CGG triplet repeat with the FMR1 (fragile X mental retardation 1) gene located on the X chromosome.

- Disease is expressed in both sexes and characterized by moderate intellectual disability in affected males and mild intellectual disability in affected females.
- Males can develop large head, long face, prominent forehead and chin, ears, loose joints and large testes after puberty. Behavioral abnormalities including autistic behavior are common.

MITOCHONDRIAL DNA MUTATIONS ASSOCIATED DISORDERS

Mitochondrial inheritance is mediated through maternal lines (cytoplasmic mitochondrial genes), as mitochondria in the embryo are derived from **ovum**. Mitochondrial genes code for enzymes involved in oxidative phosphorylation.

- Only female parent transmits the trait to her children. Transmission of abnormal mitochondrial genes by female parent affects enzymes involved in oxidative phosphorylation. If an affected male has children, his progeny is unaffected.
- A mixture of genetically normal and abnormal mitochondria in tissues is termed 'heteroplasmy'. Disorders associated with mitochondrial DNA mutations are given in Table 5.58.

CHRONIC PROGRESSIVE EXTERNAL OPHTHALMOPLEGIA

Chronic progressive external ophthalmoplegia (CPEO) is a mitochondrial disorder that mainly involves extraocular of the eyes. Patient presents with slowly progressive symmetrical bilateral limitation of eye movements, eyebrows and ptosis. Mitochondrial DNA mutations are being recognized as the etiology of this disorder.

MITOCHONDRIAL DEAFNESS

Mitochondrial deafness is caused by pathogenic variants in mitochondrial DNA (mtDNA) and transmitted by maternal inheritance. The mother of a proband (usually) has the mtDNA pathogenic variant and may or may not have hearing loss.

KEARNS-SAYRE SYNDROME

Kearns-Sayre syndrome is a rare inborn error of metabolism and characterized by progressive external

Table 5.58 Disorders associated with mitochondrial DNA mutations

Mitochondrial Disorders	System Involved	Clinical Manifestations
Chronic progressive external ophthalmoplegia	Eye	Progressive weakness of extraocular muscles
Mitochondrial deafness	Ear	Progressive sensorineural deafness often associated with aminoglycoside antibiotics
Kearns-Sayre syndrome	▪ Eyes ▪ Heart	▪ Progressive weakness of extraocular muscles of early onset, retinal pigmentation ▪ Heart block
Leber hereditary optic neuropathy	Eyes	Painless, subacute visual loss, with central blind spots (scotomata) and abnormal color vision
Leigh disease	▪ Nervous system ▪ Eyes	▪ Proximal muscle weakness, sensory neuropathy, developmental delay, ataxia, seizures, dementia ▪ Visual pigment degeneration
MELAS (mitochondrial encephalopathy, lactic acidosis and stroke-like episodes)	Nervous system	Mitochondrial encephalomyopathy (cerebral structural changes), lactic acidosis, and stroke-like syndrome, other clinical and laboratory abnormalities; may manifest as diabetes mellitus
MERRF (myoclonic epilepsy with ragged-red fibers) syndrome	Nervous system and muscles	Myoclonic epilepsy, ragged-red fibers in muscle, ataxia, sensorineural muscle weakness and fatigue

ophthalmoplegia (impairment of eye movements and ptosis), pigmentary retinitis and an onset before the age of 20 years.

- Common additional features of Kearns-Sayre syndrome include deafness, cerebral ataxia, muscle weakness, kidney problems, dementia, and cardiac conductance defects.
- When muscle cells of affected persons are stained and viewed under a light microscope, which usually appear abnormal. The abnormal muscle cells contain an excess of structures called mitochondria, which are known as ragged-red muscle fibers.

LEBER'S HEREDITARY OPTIC NEUROPATHY

Leber's hereditary optic neuropathy (LHON) is a mitochondrial inherited disorder optic nerve disease caused by mutation in genetic code of the mitochondria, which are small subunits that reside within the cell. Disease is transmitted from mother to predominantly male offspring. Patient resents with sudden, painless central vision loss caused by degeneration of retinal ganglion cells and their exons during adult life.

LEIGH DISEASE

Leigh syndrome is a severe neurodegenerative disorder that usually becomes apparent in the first year of life. Disorder follows a mitochondrial inheritance pattern, also called maternal mitochondrial inheritance.

- Initially, infant has poor sucking ability, loss of head control and motor skills, loss of appetite, vomiting, and seizures.
- As the disease progresses, symptoms may include weakness, lack of muscle tone, spasticity, movement disorders, cerebellar ataxia and peripheral neuropathy.
- Complications can lead to impaired functions of respiration, heart and kidney. Death occurs within two to three years due to respiratory failure.

MITOCHONDRIAL ENCEPHALOPATHY, LACTIC ACIDOSIS AND STROKE-LIKE EPISODES

Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) is the most common maternally inherited mitochondrial disorder. More than 80% of MELAS cases have a common mutation A→G in the mitochondrial tRNA at 3243 of mitochondrial DNA.

- Neuroimaging reveals transient nature of the stroke-like episode abnormalities. The cardinal laboratory abnormalities include elevated serum lactate during the acute episodes and respiratory enzyme defects in skeletal muscle.
- Skeletal muscle biopsy also helps confirm the diagnosis by identification of abnormal proliferation of mitochondria. Current treatment options for MELAS are largely supportive.

MYOCLONIC EPILEPSY WITH RAGGED-RED FIBERS (MERRF) SYNDROME

Myoclonic epilepsy with ragged-red fibers (MERRF) syndrome is mitochondrial inheritance disorder, also called maternal mitochondrial inheritance.

- MERRF syndrome is a multisystem disorder involving skeletal muscle and nervous system. Patient presents with generalized epilepsy, ataxia, skeletal muscle weakness and dementia during childhood and adolescence.
- Diagnosis of MERRF syndrome is based on clinical features and skeletal muscle biopsy finding of ragged-red fibers. In over 80% of cases, MERRF syndrome occurs due to mutations in the mitochondrial gene called MT-TK.

BALANCED POLYMORPHISM

Genetic polymorphism refers to the occurrence of two or more clearly different phenotypes exist in the same population of species.

- Balanced polymorphism occurs in a population when two alleles are maintained in stable equilibrium. Heterozygous person (with one allele) survives longer than homozyous person (with two alleles).
- This variability allows an organism to adapt rapidly in the face of changing environmental conditions. Summary of the genetic disorder and infectious diseases pairings is given in Table 5.59.

GENETIC DISORDERS CONFERRING RESISTANCE TO INFECTIOUS DISEASES

Some genetic disorders can confer resistance to specific infectious diseases. These genotypes are maintained in the population regularly exposed to certain highly virulent infectious agents.

- Understanding, how human genetic disorders influence infectious disease susceptibility offers the opportunity for new insights into pathogenesis, genetic mechanisms of resistance, developing novel potential drug targets, risk stratification, response to therapy, vaccination and improving current therapies.
- Genetic disorder—infectious disease pairings include: (a) hemoglobinopathies—resistance to malaria, (b) cystic fibrosis—resistance to cholera, (c) Tay-Sachs disease—resistance to tuberculosis, (d) phenylketonuria low-risk of miscarriages, (e) Niemann-Pick type C1 disease—resistance to flaviviruses, (f) myasthenia gravis—resistance to rabies and (g) congenital disorder of glycosylation 2B and resistance to viral infections. Out of these first four (a, b, c, d pairs) of the resistance-associated pairings appear to be a result of selection pressures in geographic regions in which the specific pathogen is endemic.
- Additional disorders are also being discussed here, which include enzymopathies—infectious disease (G6PD deficiency—resistance to malaria, pyruvate kinase deficiency—resistance to malaria and hemosiderosis) and hemosiderosis—hypersusceptibility to typhoid fever.

HEMOGLOBINOPATHIES AND RESISTANCE TO MALARIA

Normal persons (HbA) are more susceptible to malaria. The genetic resistance to malaria infection, particularly *Plasmodium falciparum* is associated with hemoglobinopathies. Plasmodium has a life cycle that includes alternating hosts: (a) sexual cycle in the female Anopheles mosquito insect vector and, (b) human cycle that includes a liver stage and an erythrocytic stage. Malaria causes cycles of fever and chills.

Sickle Cell Anemia and Resistance to Malaria

The best-defined hemoglobinopathy showing protection against malarial parasite is sickle cell disease, which illustrates balanced polymorphism because carriers are resistant to malarial parasite (*Plasmodium falciparum*). Malaria causes cycles of fever and chills.

Table 5.59 Summary of the genetic disorder and infectious diseases pairings

Genetic Disorder	Genetic Basis of Disorder	Protection Against Infectious Disease
Sickle cell heterozygotes (HbA/HbS)	Mutations encoding hemoglobin or enzymes vital to RBCs metabolism	Malarial parasite and pneumococcal pneumonia
Cystic fibrosis	Mutations in gene encoding a chloride channel (CFTR)	Cholera
Phenylketonuria	Mutations in the gene encoding phenylalanine hydroxylase	Miscarriage in pregnant women
Congenital disorder of N-linked glycosylation and resistance to viral infections	Glycosidase 1 deficiency	Viral infections

- Sickle cell anemia is an autosomal recessive disorder caused by a point mutation in the β-chain of hemoglobin located on chromosome 11 causing the hydrophilic amino acid glutamic acid to be replaced within hydrophobic amino acid valine at the sixth position, that results into sickle-shaped red blood cells.
- Sickle-shaped red blood cells are fragile and clog the small blood vessels thus causing damage to the organs, severe anemia, joint pain, splenomegaly, recurrent infections and heart failure.
- Heterozygous persons (HbS/HbA) with one normal allele and one allele of the disease are relatively resistant to *Plasmodium falciparum* malaria.
- Homozygous persons (HbS/HbS) with copy of two alleles have greater number of red blood cells containing abnormal hemoglobin (HbS).
- About 10–20% population in Africa carries at least one sickle cell allele. Therefore, sickle cell trait shows a case of balanced polymorphism where sickle cell heterozygotes have advantage over sickle cell homozygotes in a population.

Thalassemia and Resistance to Malaria

Thalassemias are disorders involving alterations in the globin chains that make up the hemoglobin molecule.

- The disease is classified as α- or β-thalassemia depending on whether α- or β-hemoglobin chain harbors a mutation.
- Four genes, two from each parent, are required to produce enough α- or β-chains: α- or β-thalassemia trait occurs if one or two of the four genes are missing or altered.
- Researchers observed that β-thalassemia provides a high degree of protection against malaria, whereas α-thalassemia cases are more susceptible to malaria.

ENZYMOPATHIES AND RESISTANCE TO MALARIA

Both G6PD and pyruvate kinase deficiency are linked to resistance to malarial parasite. These enzymopathies have been recognized as important drug targets against *Plasmodium falciparum*.

Glucose-6-Phosphate Dehydrogenase Enzyme Deficiency

G6PD deficiency is an X-linked disorder that leads to increased oxidative stress in red blood cells due to food (fava beans), drugs (e.g. antimalarial or sulphonamide therapeutic agents) and various chemical agents. The disorder most commonly affects persons of Asian, African, Mediterranean of Middle Eastern descent.

- Patient presents with anemia, tiredness, jaundice and splenomegaly. The heterozygous who possesses G6PD deficiency has some protection against malaria. Red blood cells with low G6PD activity offer a hostile environment to malarial parasite growth and thus an advantage to G6PD carriers. The invasion of malarial parasite exacerbates G6PD deficiency disease, making red blood cells highly susceptible to phagocytosis. Infected G6PD deficient red blood cells are morphologically different compared to noninfected ones and are thus hypersusceptible to phagocytosis.
- In this instance, selection working both positively and negatively clearly represents a manifestation of the balance implied by the term balanced polymorphism.
- In addition to G6PD deficiency, other examples of balanced polymorphism include heterozygotes persons with β-thalassemia and ovalocytosis, which provide some protection against malaria.

Pyruvate Kinase Enzyme Deficiency

Pyruvate kinase enzyme is responsible for the final step of glycolysis in which phosphoenolpyruvate is converted to pyruvate with the production of ATP.

- Deficiency of pyruvate kinase leads to altered membrane rigidity of red blood cells, thus preventing entry of malarial parasite.
- Moreover, pyruvate kinase deficiency significantly reduces the intracellular concentration of glucose, a vital source of energy for the intracellular life of cycles of malarial parasite.

RED BLOOD CELL-ASSOCIATED ANTIGENIC POLYMORPHISM AND RESISTANCE TO MALARIA

Duffy blood group antigens serve as receptor for *Plasmodium vivax* invasion into red blood cells, which are determined by polymorphisms of the Duffy antigen receptor for chemokines (DARC), which a chemokine receptor as well as the receptor for *Plasmodium* species. A point mutation in the Duffy gene leads to lack of expression of the encoded receptor and thus negates attachment of *Plasmodium vivax* infection on the surface of red blood cells and thus provides protection against **malaria**.

CYSTIC FIBROSIS AND RESISTANCE TO CHOLERA

Cystic fibrosis is an autosomal recessive disorder caused by a mutation in the both copies of the cystic fibrosis transmembrane conductance regulator (CFTR) gene in the person resulting in defective chloride channel.

- The disorder most commonly affects Caucasian population. The body produces thick and sticky mucus that can clog the lungs, gastrointestinal tract and pancreatic duct system resulting in impaired functions.

- Heterozygous individuals with mutation if CFTR gene and less functioning chloride channels demonstrate balanced polymorphism and provide protection against the fatal diarrheal disease 'cholera'.
- Cholera is caused by toxin secreted by the bacterium *Vibrio cholerae* in the small intestine. The toxin acts through intracellular guanine nucleotide binding protein, which causes the release of cAMP, which in turn induces chloride ion movement through chloride channel into the lumen of the gastrointestinal tract leading to osmosis, loss of sodium ion and water into the lumen of gastrointestinal tract resulting in watery diarrhea.

TAY-SACHS DISEASE AND RESISTANCE TO TUBERCULOSIS

Tay-Sachs disease is an autosomal recessive disorder caused by mutation in **HEXA gene** located on chromosome 15 that codes for abnormal hexosaminidase A enzyme leading to accumulation of GM2 ganglioside in nerve cells in brain and spinal cord thus causes damage to the nerve cells resulting in deterioration of mental and physical abilities with fatal outcome by the age of four and five years.

- The disorder is more prevalent in Eastern European people of Jewish descent (Ashkenazi Jews).
- Symptoms of Tay-Sachs disease include deafness, progressive blindness, decreased muscle strength, paralysis, muscle stiffness, delayed mile stones, failure to thrive and red spots on the macula (an oval-shaped region near the center of the retina in the eyes).
- Heterozygous persons are protected against *Mycobacterium tuberculosis*, thus showing balanced polymorphism.

PHENYLKETONURIA AND LOWER RISK FOR MISCARRIAGES

Phenylketonuria (PKU) is a rare inherited inborn error of metabolism disorder that results in building of phenylalanine amino acid in the body due to non-functional hepatic enzyme, phenylalanine hydroxylase.

- Patient presents with intellectual disability, seizures, behavioral problems, mental disorders, musty smell and lighter skin.
- Researchers observed that heterozygotes women with phenylketonuria had a much lower risk of miscarriages due to protective effect of a gene against some factor causing fetal death, when compared to wild-type homozygous women.
- Phenylketonuria is an example of balanced polymorphism. Researchers also suggested that decrease in fetal mortality is mediated by the higher concentration of phenylalanine in the heterozygous pregnant women's blood.
- Phenylketonuria is most prevalent in Scotland, where the consistent damp climate promotes growth of *Aspergillus* species on grains and beans.
- *Aspergillus* species produce mycotoxin known as ochratoxin A, which is nephrotoxic, hepatotoxic and teratogenic.
- In pregnant women, ochratoxin A can cross placenta and cause spontaneous miscarriages. The ochratoxin A being an N-acetyl derivative of phenylalanine is a competitive inhibitor of phenylalanine. Inhibition of phenylalanine hydroxylase and other enzymes that use phenylalanine as a substrate is based on this structural homology.
- High frequency of the phenylketonuria gene present in women may help to protect against sudden miscarriages, thus ensuring normal pregnancy.

NIEMANN-PICK TYPE C DISEASE AND RESISTANCE TO FLAVIVIRUSES SUCH AS EBOLA AND MARBURG VIRUSES

Niemann-Pick type C (NPC) disease is an autosomal recessive lipid storage disorder caused by mutations in NPC1 gene in 95% of cases and NPC2 gene in 5% of cases.

- The disorder is characterized by accumulation of cholesterol within lysosomes due to shortage of NPC1 protein that normally transports cholesterol out of the lysosomes.
- Patients with classic childhood onset—Niemann-Pick type C usually appears normal in first two years of life and later presents with gradual-onset of neurologic symptoms such as ataxia, seizures, myoclonic jerks and loss of previously learned speech. Niemann-Pick type C demonstrates resistance to flaviviruses such as Ebola virus and Marburg virus.

MYASTHENIA GRAVIS AND RESISTANCE TO RABIES VIRUS

Myasthenia gravis is a neuromuscular autoimmune disorder that involves the destruction of acetylcholine receptors by autoantibodies, and inhibit neuronal transmission at the neuromuscular junction by accelerated degradation of acetylcholine receptors, blocking acetyl-binding sites, and degradation and simplification of synaptic folds.

- Loss of acetylcholine receptors at neuromuscular junction leads to pronounced muscle weakness and fatigue involving ocular muscles or multiple groups of muscles. Patient may present with muscles

weakness and drooping of eyes (ptosis), double vision, dysphonia, dysphagia, dysarthria, proximal limb weakness and life-threatening respiratory failure. Patients have low-risk of rabies infection.

- Rabies is a neurotropic rhabdovirus that causes viral encephalitis in warm-blooded mammals. The disease is transmitted through the bite of a rabid animal via direct contact with saliva through broken skin or mucous membranes in the eyes, nose or mouth; or nervous system tissue of an infected animal.
  - Rabies may enter the central nervous system through several routes.
  - The virus enters through the neuromuscular junctions of both the intrafusal and extrafusal muscles.
  - Virus binds to nicotinic acetylcholine receptors and gains entrance into the skeletal muscles via receptor-mediated endocytosis.
  - Following initial infection, rabies virus enters the peripheral nervous system where it undergoes replication in the dorsal root ganglion and then travels to the central nervous system through retrograde transport mechanism.
- Myasthenia gravis exerts its effects on musculature by either occupying or attacking acetylcholine receptors. It indicates that myasthenia gravis patients show diminished likelihood of rabies infection due to delay in the spread of the rabies virus in peripheral nervous system, immune clearance and additional medical intervention.
- Further research on the different mechanisms behind nicotinic acetylcholine receptors degradation in myasthenia gravis patients may offer new insights into new medications to better prevent rabies virus pathogenicity.

CONGENITAL DISORDER OF N-LINKED GLYCOSYLATION (GLYCOSIDASE 1 DEFICIENCY) AND RESISTANCE TO VIRAL INFECTIONS

Congenital disorder of N-linked glycosylation is caused by defects in the synthesis and processing of the asparagine-linked oligosaccharides of glycoproteins.

- Patient suffering from congenital disorder of N-linked glycosylation (CDG) presents with severe developmental delay, muscular hypotonia, recurrent edema, seizures, retrognathia, high arched palate and overlapping fingers.
- Congenital disorder of N-linked glycosylation has been linked to resistance to glycosylation-dependent viral infections (e.g. HIV-1, hepatitis C, dengue virus, herpes simplex 2 and influenzae) resulting from hinderane of viral entry and replication in cells.

SICKLE CELL ANEMIA AND SUSCEPTIBILITY TO PNEUMOCOCCAL INFECTIONS

While sickle cell anemia confers resistance to malaria, and also renders the patient to susceptible to invasive bacterial infections such as *Streptococcus pneumoniae*, *Haemophilus influenzae* type B and *Salmonella* non-*typhi*. In sickle cell anemia, the respiratory endothelium becomes hyperactive and overexpresses the receptor for platelet activating factor (PAF), which is a docking site for *Streptococcus pneumoniae*. Thus, overexpression of platelet-activating factor (PAF) receptor provides ample sites for the infection by bacteria.

HEMOSIDEROSIS AND SUSCEPTIBILITY TO TYPHOID FEVER

Hemosiderosis is a term used for excessive accumulation of iron such as hemosiderin in the tissues/organs. The lungs and kidneys are often sites of hemosiderosis. *Salmonella enterica* pathogen causes systemic infection designated typhoid fever.

- Hemosiderosis increases the susceptibility to Salmonella infection. The siderophore receptor protein (**SRP**) is important for the growth and survival of Salmonella. SRP is required for the activation of iron-dependent metabolic and virulence proteins as part of Salmonella physiology.
- The anti-Salmonella SRP vaccine blocks the iron transport through the SRP. The iron depletion ultimately results in the elemination of Salmonella pathogen.

POLYGENIC AND MULTIFACTORIAL DISORDERS

Polygenic disorders are more common than monogenic disorders. Polygenetic disorders are caused by abnormalities of two or more genes regulating their protein products. Environmental factors modulate polygenic disorders. Common polygenic disorders include ischemic heart disease, diabetes mellitus type 2, essential hypertension, gout, schizophrenia, bipolar disorder, hip dysplasia and neural tube defects.

DIABETES MELLITUS TYPE 2

Diabetes mellitus type 2 is a polygenic disorder, which has complex defects in both insulin secretion and its action resulting in metabolic derangements.

- The pathophysiology of diabetes mellitus type 2 is the result of a combination of polygene defects and environmental factors, characterized by

hyperglycemia as a result of peripheral insulin resistance (most common), impaired regulation of hepatic glucose production and declining β cell function, eventually leading to β cell failure in pancreas.

- Patient presents with polyuria, polydipsia, polyphagia, unexplained weight loss, sudden vision changes, tingling sensations in the hands and feet, tiredness and dry skin. Uncontrolled blood sugar in these patients leads to numerous complications such as cardiovascular disorder, neuropathy, nephropathy, retinopathy, foot damage, hearing impairment and Alzheimer disease.

ESSENTIAL HYPERTENSION

Essential hypertension is a multifactorial disease involving interactions among polygene, environmental, demographic, vascular and neuroendocrine factors. No single-genetic variant has emerged from linkage or association analyses as constantly related to blood pressure level in every sample in population. Essential hypertension can lead to morbidity and mortality. The discovery of genes, known as quantitative trait loci (QTLs), contributing to blood pressure control is considered the most direct means of achieving this objective.

NEURAL TUBE DEFECTS

Neural tube defects occur in the brain (anencephaly) or spine or spinal cord (spina bifida) in the newborns. These happen in the first month of pregnancy, often before a woman even knows that she is pregnant.

- Neural tube defects are caused by a combination of multiple genes and environmental factors such as folic acid deficiency, maternal insulin dependent diabetes mellitus and maternal use of certain anticonvulsant medications. Incidence of neural tube defects is 1 or 2 per 1000 of pregnancies.
- Ultrasonography can detect neural tube defects in fetus around 18–20 weeks pregnancy in 95% of cases.
- Patient develops paralysis, urinary and bowel control problems, blindness, deafness, intellectual disability, lack of consciousness and even fatal outcome.
- There is no permanent cure of neural tube defects. Treatment is focused to relieve pain and prevent further damage. Babies who have **spina bifida** may require **surgery** to correct the defect.

DEVELOPMENTAL DYSPLASIA OF THE HIP

Developmental dysplasia of the hip (DDH) is a disorder where the ball and socket; **joint** of the **hips** does not function properly in babies and young children. It is also called congenital dislocation of the hip.

- In a normal hip joint, the ball at the upper end of femur fits firmly into the socket, which is the part of the pelvis bone. DDH is caused by a combination of genetic, environmental and mechanical risk factors.
- Mechanical risk factors for DDH include high birth weight of the newborn, breech presentation, oligohydramnios, primiparity, intrauterine malposition, swaddling and laxity of ligaments.
- DDH is not associated with Marfan syndrome and Ehlers-Danlos syndrome, in which laxity of ligaments occur.
- Patient develops pain, unstable hip joint, limping when walking and unequal leg length.
- Mild DDH is managed by conservative treatment such as relieving pain, weight loss, lifestyle modification, joint injections and physiotherapy. Developmental dysplasia of the hip can be treated by periacetabular osteotomy procedure, that involves a series of cuts to the bone to reorient the acetabulum over the femoral head in order to restore a more normal anatomy.

GENETIC POLYMORPHISM

Genetic polymorphism is a difference in DNA sequence among persons, groups, or populations. Example of genetic polymorphism is blood groups. It is amazing to know that around 99.99% of the individual's genome among individuals are alike, and only 0.1% of genome differs in chromosomes.

- Genetic and environmental factors make human phenotype variations. When the genome DNA sequences on equivalent chromosomes of any two individuals are compared, there is substantial variation in the sequence at many points throughout the genome.
- Presently, the geneticists use the term 'genetic polymorphism' to describe functionally silent differences in DNA sequences that make each human genome unique among persons.
- In order to understand the phenomenon of genetic polymorphism, an emphasis has been done on the structures and functions of nucleotides, genes and nucleic acids including their relationship with polymorphism.
- **Genetic polymorphism** can be caused due to permanent transmissible change in the DNA sequence.

Table 5.60 Comparison of mutation and genetic polymorphism

Parameters	Gene Mutation	Genetic Polymorphism
Gene	Gene directly leads to disorder	Gene confers an increased risk, but does not directly cause disorder
Inheritance	Mendelian pattern of inheritance	Inheritance pattern not clear
Population affected	Rare	Common in population

Mutations can involve somatic and germline; and the length of the nucleotide sequences in genes and chromosomes.

- Genetic variation is of three types: single nucleotide polymorphism (SNP), copy number variations (CNVs) and repeat length polymorphism. Various types of genetic polymorphisms include; single nucleotide polymorphisms (SNPs), small scale insertions/deletions, polymorphic repetitive elements, microsatellite variation and haplotype. Comparison of mutation and genetic polymorphism is given in Table 5.60.

SINGLE NUCLEOTIDE POLYMORPHISM

Single nucleotide polymorphisms (SNPs) represent as variations in DNA sequences and can have a major impact on how human beings respond to the disease, pathogens, chemical agents, drugs and other therapies. Genetic polymorphism is studied by PCR-based diagnostic techniques involving DNA markers.

- Two persons possess exactly same DNA sequence in 99.99% of their DNA. Therefore, two persons vary in the remaining 01% of DNA. Single nucleotide polymorphism (SNP) is the most common type of genetic polymorphism among people.
- Each SNP represents an alteration in single DNA block called nucleotide (adenine, thymine, cytosine, or guanine) in every 1000 base pairs in the genome sequence in at least 1% of the population.
- Human genome comprises more than 6 million SNPs. Most commonly, SNP variations are found in the DNA between the genes in the regions of exons and introns. Less than 1% of SNPs occur in the coding regions. SNPs can be detected by polymerase chain reaction (PCR) and TaqMan assay protocol.

COPY NUMBER VARIATIONS

Human genome contains 5–24 million nucleotide base pair of copy number variations (CNVs), which represent large number of contiguous stretches of DNA from 1000 to millions nucleotide base pairs. About 50% persons have CNVs in the coding regions. CNVs range in size from one kilobase to several megabases that differ among individuals due to insertion, deletion, invasion, duplication or complex recombination, which are the basis of phenotype diversity. Recent evidence shows that the gene copy number can be elevated in cancer cells.

REPETITIVE DNA SEQUENCE CAUSING POLYMORPHISM

The term "repetitive DNA sequences" refers to homologous DNA fragments that are present in the genome. DNA sequences that are repeated in the genome do not code for protein. Repetitive DNA sequence can be divided into two classes: the tandem repetitive sequences (known as satellite DNA) and the interspersed repeats along the single stretch. The term satellite is used to describe DNA sequences that comprise short head-to-tail tandem repeats incorporating specific motifs. Satellite DNA is of two types: microsatellites and minisatellites. Satellite DNA types are given in Table 5.61.

Table 5.61 Satellite DNA types

Microsatellite DNA	Minisatellite DNA
Short tandem repeats that consist of 1–9 nucleotide base pairs monomer repeating sequences	Short tandem repeats that consist of 10–100 nucleotide base pairs monomer repeating sequences
Rich with adenine (A) and thymine (T) nucleotide base pairs	Rich with guanine (G) and cytosine (C) nucleotide base pairs
Also known as simple sequence repeats (SSR) or short tandem repeats (STR)	Also known as variable number tandem repeats (VNTR)

DISORDERS OF SEXUAL DIFFERENTIATION

Disorders of sexual development (DSD) occur when a baby is born with both male and female reproductive organs, atypical chromosomes or atypical anatomical appearances to their genitals.

- Presence of significant overlap in genital anatomy makes it difficult differentiate between sex of the baby. If newborn has disorders of sexual development, the sex chromosomes may still be male (XY) or female (XX). At the same time, newborn's reproductive organs and genitals may be of opposite sex.
- Physical examination provides important information about the degree of virilization of the external genitalia and the presence or absence of palpable gonads. Exact cause of disorder of sexual development is not known. In DSD, the tissue that eventually turns into testes or ovaries is present early in fetal development.
- Development of sexual organs is influenced by chromosomes, hormones and environmental factors.
  - Congenital adrenal hyperplasia due to 21-hydroxylase deficiency is the most common cause of DSD resulting in virilization of a child with 46, XX. In the past, disorders of sexual differentiation were given names such as intersex or hermaphroditism.
  - Different types of DSD in children include: 46, XX karyotype testicular disorder of sexual differentiation, female with ambiguous or male genitals (46, XX DSD), 46, XY female DSD, mixed genitals and sex organs (46, XX ovotesticular), sex chromosome DSD, and Mayer-Rokitansky-Küster-Hauser syndrome.

46, XX KARYOTYPE TESTICULAR DISORDER OF SEXUAL DEVELOPMENT

46, XX karyotype testicular disorder of sexual development (DSD) is characterized by external genitalia ranging from normal to atypical with testosterone deficiency. Incidence is 1 in 15000 of live births. The disorder is caused by translocation of a small Y chromosome fragment including SRY (sex determining region of Y chromosome) gene to X chromosome or another chromosome. In SRY gene negative cases, copy of recurrent NR5A1, copy number variations, involving regulatory genes (SOX3, SOX9) and common recurrent NR5A1 have been reported.

- **Clinical features:** SRY positive cases (80–90%) develop normal male to atypical external genitalia, undescended testes with absent Müllerian structures and infertility, and after puberty with short stature, normal pubic hair, normal penis size, small testes, gynecomastia and azoospermia-related sterility. Undescended testes and hypospadias are also reported in literature.
  - On the other hand, SRY negative newborn cases (10–20%) present with penoscrotal hypospadias and undescended testes.
  - Long-term complications due to male hypogonadism include: low libido, erectile dysfunction of penis, decreased secondary characteristics, osteopenia and depression.
- Diagnosis of 46, XX karyotype testicular disorder of sexual development (DSD) is based on clinical signs and cytogenetic or molecular testing (SNP) array confirming an XX genome. SRY gene mutation is detected by fluorescence *in situ* (FISH) or polymerase chain reaction (PCR).
- Genetic counseling is essential for the affected persons and their families. Recurrence risk depends on the type of genetic alterations found. SRY positive cases are generally not inherited. Inheritance pattern in SRY negative cases depends on the genetic cause.
- Testosterone replacement therapy is given to correct hormonal imbalance. Reduction mammoplasty may be considered in some cases. The affected persons are sterile.

46, XX KARYOTYPE FEMALE WITH NORMAL OVARIES AND UTERUS BUT MALE OR AMBIGUOUS GENITALIA

A newborn with this disorder of sexual development (DSD) has 46, XX the female chromosomes (XX karyotype) with normal ovaries and uterus, but male or ambiguous external genitalia.

- Patient has enlarged clitoris that resembles like a penis, with closed lower section of vagina.
  - Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency is the most common cause of DSD resulting in virilization of a child with 46, XX.
  - Newborn with 21-hydroxylase deficiency fails to synthesize cortisol and aldosterone, hence body produces more androgens in female baby.
- Exposure to excessive androgens during fetal life results in male or ambiguous external genitalia. Newborn with 21-hydroxylase deficiency can cause life-threatening salt losing nephropathy.

46, XY DISORDER OF SEXUAL DIFFERENTIATION

A normal male has one X chromosome and one Y chromosome in each cell (XY, karyotype).

- In XY, female disorder of sexual development, some female children have similar chromosomal pattern, i.e. one X chromosome and one Y chromosome in each cell (XY, karyotype), but their external genitals may not clearly indicate male or female.
- Some male infants have underdeveloped testes or absence of testes with reduced or no sperm production; and penoscrotal hypospadias.
- Female infants have underdeveloped female reproductive system or absence of female reproductive system (uterus and fallopian tubes).
- Androgen insensitivity syndrome (AIS) is one of the causes of this disorder, in which body is not sensitive to androgens. Individuals with 46, XY DSD are treated by surgery and hormonal replacement therapy.
- These patients are at great risk of development for gonadal tumors and benefit from regular surveillance or surgery to remove abnormally developed gonads.

MIXED GENITALIA AND SEX ORGANS (46, XX OVOTESTICULAR DISORDER OF SEXUAL DIFFERENTIATION)

Synonyms of 46, XX ovotesticular disorder of sexual differentiation are true gonadal intersex or true hermaphroditism.

- Infant is born with the internal reproductive organs (gonads) of both sexes (ovaries and testes).
  - Gonads can be any combination of ovary, testes or combined ovary and testes (ovotestes).
  - The external genitalia are usually ambiguous but can range from normal male to normal female. Infant has abnormal vagina and hypoplastic uterus.
- Infant has undescended testes (cryptorchidism) and hypospadias in penis. Upon reaching puberty, feminization, breast development and menstruation may occur.
- Most affected persons are infertile but spermatogenesis or ovulation is possible. Tumors of testes or ovaries have been reported but are very rare.
- Children with this type of DSD have female chromosomal pattern (XX). Exact cause of this disorder is not known; however, some cases have been linked to genetic material normally found on the Y chromosome that is misplaced and translocated on the X chromosome or another chromosome.
- Small number of cases have genetic variations of other genes such as deletions in DMRT1 gene, mutation in SRY gene, mutation in MAP3K1 gene, mutation in NR5A1 gene, mutation in RSPO1 gene and duplication of SOX9 gene.

SEX CHROMOSOME DISORDER OF SEXUAL DIFFERENTIATION [CHILDREN MAY HAVE EITHER ONE X CHROMOSOME (X0) OR AN EXTRA CHROMOSOME (XXY)]

Some children have neither male (XY) nor female (XX) chromosomes. Instead, these children may have either one X chromosome (X0) or an extra chromosome (XXY). Reproductive organs are normally formed in these children as seen in normal individuals, which may not go through normal sexual development at puberty. Children with female sex organs may not start having menstrual periods, and may have small breasts.

MAYER-ROKITANSKY-KÜSTER-HAUSER (MRKH) SYNDROME

Müllerian agenesis, also known as Mayer-Rokitansky-Küster-Hauser syndrome or vaginal agenesis, is a congenital malformation characterized by a failure to development of Müllerian duct, resulting in absence of uterus and variable degrees of vaginal hypoplasia of its upper portion.

- However, ovaries and vulva are still present. Females still develop breasts and pubic hair at puberty. Exact cause of this disorder is not known. Females have normal XX chromosomal pattern.
- The first sign of Mayer-Rokitansky-Küster-Hauser syndrome is that a girl does not have menstrual periods. Sexual intercourse may be difficult because the vagina is shorter than normal. It is sometimes possible to obtain ova from these females to make a surrogate pregnant.

PSEUDOHERMAPHRODITE

Pseudohermaphrodite has gonads of only one sex, but the appearance of the external genitalia does not correspond to the gonads present.

MALE PSEUDOHERMAPHRODITE

Male pseudohermaphrodite is congenital disorder also known as testicular feminization syndrome, which occurs due to a congenital deficiency of the androgen receptor.

- The cause may be tissue resistance to androgens (testicular feminization), defects in testosterone synthesis, or hormones administered to the mother during pregnancy.
- Patient has cryptorchid testes, but the external genital organs appear feminine or ambiguously female, with signs of virilization. The condition has also been linked to chromosomal anomalies, such as 46, XY/45, X mosaicism.

FEMALE PSEUDOHERMAPHRODITE

In female pseudohermaphrodite, the gonads are always ovaries, but the external genitalia are not clearly female. Disorder is most often caused by increased androgenic hormones from congenital adrenal hyperplasia, an androgen-secreting adrenal or ovarian tumor in the mother, or hormones administered to the mother during pregnancy. Patient shows 46, XX genotypically normal female.

GENOME IMPRINTING DISORDERS

Each person inherits two copies of every autosomal gene, one copy of gene from mother and one copy of gene from father. Both copies of autosomal genes are functional for the majority of these genes.

- However, in a small subset, one copy of the gene is 'turned off' (silenced) through DNA methylation in a parent-of-origin dependent manner. The repressed allele is methylated, while the active allele is unmethylated.
- These genes are termed 'imprinted genes' because one copy of the gene was epigenetically marked or imprinted in either the sperm or ovum. Thus, the allelic expression of an imprinted gene depends upon whether it resided in a male or female of the previous generation. Imprinted expression can also vary between tissues, developmental stages and species.
- Genome imprinting is a normal process, when genome imprinting combined with genome mutations can result in diseases.
- Genomic imprinting disorders are a group of congenital diseases characterized by overlapping clinical features affecting growth, development and metabolism; and common molecular disturbances affecting genomic imprinted chromosomal regions and genes.
- Many of inherited diseases and human development violates Mendelian law of inheritance, this way of inheriting is studied by epigenetics. Prader-Willi syndrome and Angelman syndrome have same cytogenetic deletion (15) (q11q13) resulting in differing phenotypes in progeny depending on whether the cytogenetic deletions were transmitted by the mother or the father.

PRADER-WILLI SYNDROME

Prader-Willi syndrome (PWS) is a disorder caused by the loss of expression of the paternal active genes in a particular region of chromosome 15 between 15q11 and 15q15. Incidence is 1 in 12,000 to 15,000 per live births.

- Prader-Willi syndrome can cause a wide range of symptoms, and affect the child's physical, psychological and behavioral development.
- Patient presents with hypogonadism, hypotonia, mental retardation, short stature, small hands and feet, strabismus, behavior problems, and uncontrolled appetite leading to obesity and diabetes mellitus.
- Men who suffer from Prader-Willi syndrome may have small sex organs, deep voice and loss of facial hair. These patients are not able to have children.

ANGELMAN SYNDROME

Angelman syndrome is a genetic disorder caused due to UBE3A gene mutation located on maternal chromosome 15 or by paternal uniparental disomy.

- Uniparental disomy occurs when two copies of a chromosome or part of chromosome are inherited from one parent and nothing comes from the other parent.
- These cytogenetic changes occur as random events during the formation of sperm or ovum or in early development.
- This disorder was once known as 'happy puppet syndrome' because of the child's sunny outlook and jerky movements. Incidence is 1 in 12,000 to 20,000 per live births.
- Normally, people inherit one copy of the gene from each parent, and both copies of the gene become active in many areas of the body.
- Angelman syndrome occurs when only one copy of the UBE3A gene in certain areas of the brain. Patient presents with delayed development, intellectual disability, severe impairment of speech, inappropriate laughter, ataxia, epilepsy and small head size.

DIAGNOSTIC APPROACH OF GENETIC DISORDERS

Genetic disorders are the leading causes of morbidity and mortality in human beings. The prevalence of genetic disorders varies widely in different clusters depending on their organization, ethnic origin and socioeconomical factors.

- Genetic disorders are caused by mutations in single-gene (monogenic), polygenic (combination of genes); chromosomal (whole or part) abnormalities and multifactorial diseases.
- Genetic disorders are diagnosed by history, physical examination, pedigree construction, genetic testing such as cytogenetic testing, biochemical testing and molecular testing.
- Cytogenetic and molecular techniques are being widely used to detect alterations in genes and chromosomes. Barr bodies and Y chromatin are also analyzed.

HISTORY AND PHYSICAL EXAMINATION

Diagnostic approach of genetic disorders requires a comprehensive clinical examination composed of three major elements: (a) physical examination, (b) detailed medical family history; and (c) clinical and laboratory testing reports, if appropriate and available.

- **Physical examination:** Distinctive facial features can suggest the diagnosis of a genetic disorder. Physical examination includes measurement of head circumference, distance between the eyes and length of the arms and legs. Neurological examination and fundoscopy of eyes should be performed.
- **Personal medical history:** Detailed information about a patient's health, often going back to birth, can provide clues to a genetic diagnosis. A personal medical history includes past health problems, hospitalizations, surgeries, allergies, medications, and the results of any medical or genetic testing that has already been performed.
- **Family medical history:** Because genetic disorders often run-in families, information about the health of family members can be a critical information for diagnosing these disorders. The geneticist must ask about health conditions in a patient's parents, siblings, children, and possibly more distant relatives. This information can give clues about the diagnosis and inheritance pattern of a genetic disorder in a family.
- **Laboratory tests, including genetic testing:** Imaging studies including X-rays, computerized tomography (CT) scans and magnetic resonance imaging (MRI) are done to analyze structures inside the body. Molecular, chromosomal, and biochemical genetic testing are performed to diagnose genetic disorders. Other laboratory tests that measure the levels of certain substances in blood and urine can also help suggest a diagnosis.

PEDIGREE CONSTRUCTION

A pedigree is a diagram that shows the history of a trait as it is passed from one generation to the next. Study of pedigree construction helps in determining the mode of inheritance of genetic disorder, i.e. autosomal domonant, autosomal recessive or sex-linked. It is useful to identify carriers of genetic disorders and genetic counseling. Schematic representation of family pedigree is shown in Fig. 5.46A to C.

Pathology Pearls: Inheritance Pattern Analysis by Pedigree Construction

- **Autosomal dominant inheritance:** In autosomal dominant inheritance, one parent carries a gene and other parent is normal. Only one copy of the abnormal gene needs to be inherited resulting to expression in both homozygous or heterozygous state in 50% of cases. Children of both sex are affected.
- **Autosomal recessive inheritance:** In autosomal recessive inheritance, both the parents carrying abnormal gene are inherited for the disorder resulting to **homozygous state** affected children of both sex. In **heterozygous state**, person is a silent career.
- **X-linked recessive inheritance:** In X-linked recessive inheritance, one of the X chromosomes carrying mutant gene in female is transmitted to male resulting to hemophilia. Because affected males do not have normal X chromosome to correct the abnormality, hence, suffer from hemophilia. Heterozygous female is an asymptomatic carrier.
- **X-linked dominant inheritance:** X-linked dominant inheritance is a rare variant of X-linked inheritance. Hemizygous males as well as heterozygous females phenotypically manifest the disorder.
- **Mitochondrial inheritance:** Mitochondrial inheritance is mediated through maternal lines (cytoplasmic mitochondrial genes), as mitochondria in the embryo are derived from ovum.
  - Mitochondrial genes code for enzymes of oxidative phosphorylation. Only female parent transmits the trait to all of their children.
  - Transmission of abnormal mitochondrial genes by female parent affects enzymes of oxidative phosphorylation. If an affected male has children, his progeny is unaffected.
  - A mixture of genetically normal and abnormal mitochondria in tissues is termed '**heteroplasmy**'.

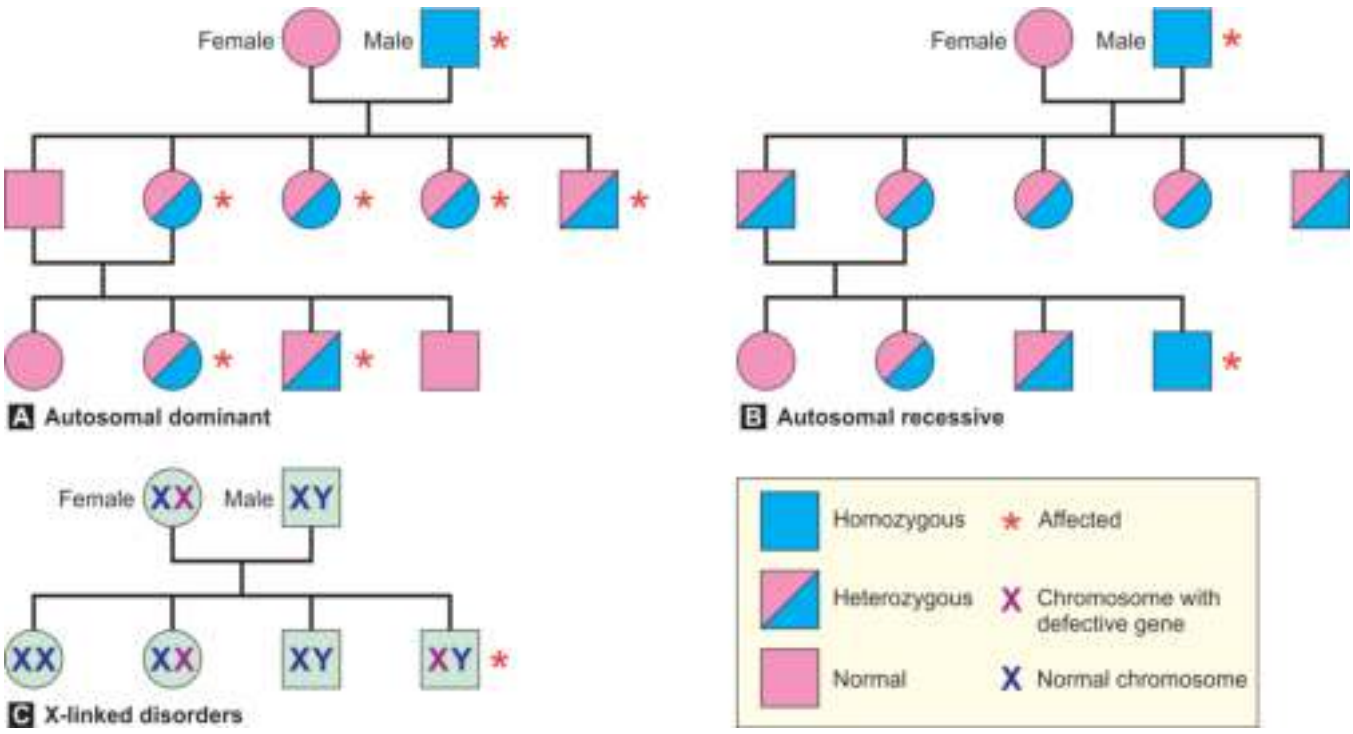


Fig. 5.46: Schematic representation of family pedigree. Family pedigrees are used to determine patterns of inheritance: (A) autosomal dominant, (B) autosomal recessive, (C) X-linked disorders and individual genotypes.

Table 5.62 Uses of genetic tests in clinical practice

Newborn screening
Carrier testing
Prenatal testing
Pre-implantation testing
Diagnostic testing
Prognostic testing
Predictive/predispositional testing

GENETIC TESTING

Genetic testing plays a vital role in determining the risk of developing certain genetic disorders as well as screening and sometimes medical treatment. Different types of genetic testing can be performed for many different purposes. Uses of genetic tests in clinical practice are given in Table 5.62.

Pathology Pearls: Genetic Testing uses

- **Prenatal testing:** Prenatal testing is performed to detect changes in a fetus's genes or chromosomes, which helps the couples to be aware of increased risk of having a baby with a genetic disorder. Down syndrome (trisomy 21) and Edward syndrome (trisomy 18) patients are often screened. A tissue sample for genetic testing can be obtained through amniocentesis or chorionic villus sampling.
- **Newborn screening:** Newborn screening is the most type common of genetic testing. Early detection of genetic disorders can lead to interventions to prevent the onset of symptoms or minimize the severity of the disorder. This type of genetic testing is important because if results show there is a genetic disorder such as congenital hypothyroidism, sickle cell disease or phenylketonuria (PKU) care and treatment can start right way.
- **Pre-implantation testing:** Pre-implantation testing is performed when attempt is made to conceive a child through *in vitro* fertilization. The embryos are screened for genetic abnormalities. Embryos without abnormalities are implanted in the uterus in hopes of achieving pregnancy.
- **Carrier genetic testing:** Carrier genetic testing can be performed in individuals, who have a history of genetic disorder and to help the couples about risk of genetic disorder passing to their children such as cystic fibrosis, sickle cell disease or Tay-Sachs disease.
- **Genetic testing of couple:** Genetic testing of couple can provide information about a couple's chance of having a child with a specific genetic disorder.
- **Diagnostic genetic testing:** Diagnostic genetic testing is performed to confirm a diagnosis in a symptomatic person such as cystic fibrosis or Huntington's disease or used to monitor prognosis of a disease or response to treatment.
- **Pharmacogenetic testing:** Pharmacogenetic testing performed to determine what medication and dosage will be most effective and beneficial for the patient.

- **Predictive or predispositional testing:** Predictive or predispositional testing can detect persons at risk of getting a disease prior to the onset of symptoms, which is useful if a person has a family history of a specific genetic disorder and an intervention is available to prevent the onset of disease or minimize severity of the disorder. Predictive testing can detect gene mutations that increase a person's risk of developing conditions with a genetic basis of certain type of cancer. Early detection of genetic disorders can lead to interventions to prevent the onset of symptoms.

TYPES OF GENETIC TESTING

Several different methods are currently performed in genetic testing laboratories. The type of genetic test depends on the type of abnormality being analyzed. In general, three major types of genetic testing are available, which include: cytogenetic (chromosomal), biochemical and molecular methods.

CYTOGENETIC TESTING

Cytogenetic testing involves the examination of chromosomes to identify structural abnormalities under microscope.

- Chromosomes of a dividing human cell can be analyzed clearly in white blood cells, specifically T cells, which are easily collected from blood. Cells from other tissues such as bone marrow, amniotic fluid, and placenta can also be cultured for cytogenetic analysis.
- Following several days of cell culture, chromosomes are fixed, spread on microscope slides, and stained. The staining methods for routine analysis allow each of the chromosomes to be individually identified.
- The distinct bands of each chromosome revealed by staining allow for analysis of the chromosomal structure.

Karyotyping

Karyotyping is a technique to examine pairing and ordering of chromosomes in genome, thus providing a genome-wide photographs of an individual chromosome. It is performed in a sample of cells such as amniotic fluid (amniocentesis), blood (lymphocytes), bone marrow biopsy and placental tissue. Schematic representation of amniocentesis is shown in Fig. 5.47.

- The sample is put into a special dish or tube and allowed to grow in the laboratory for several days. Karyotypes are prepared from mitotic cells that have

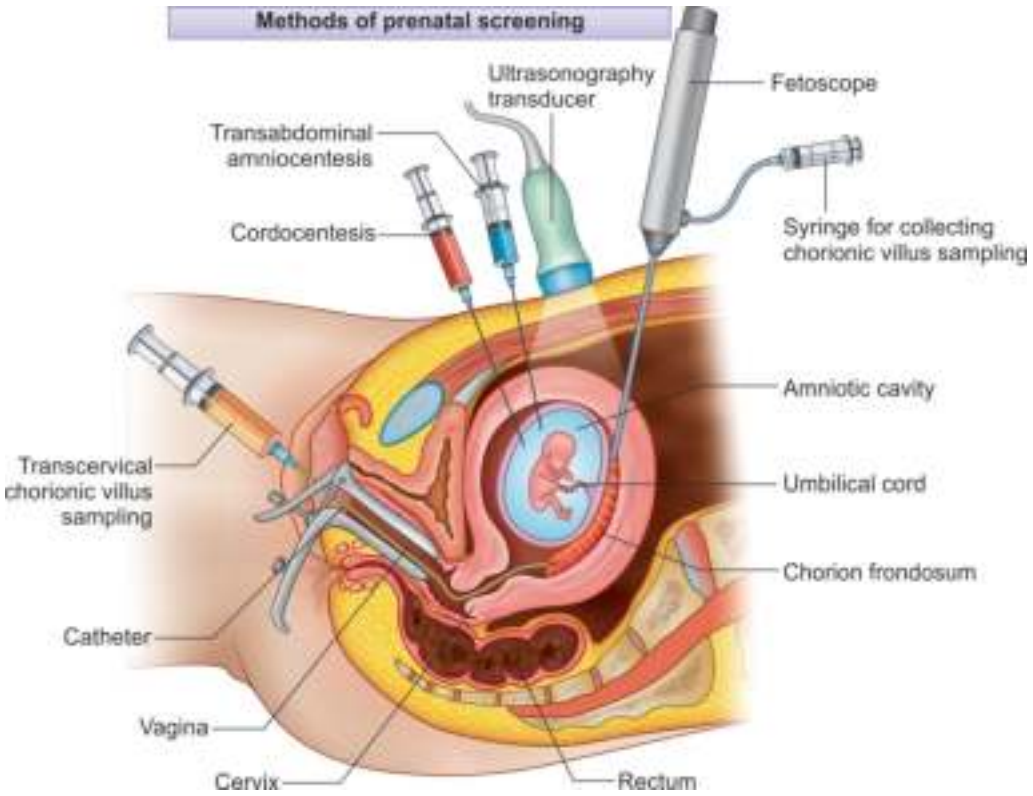


Fig. 5.47: Schematic representation of amniocentesis. In amniocentesis, a syringe is used to collect amniotic fluid. Ultrasound imaging is used to guide the tip of the syringe to prevent damage to the placenta and fetus. Fetal cells in the collected amniotic fluid can then be chemically tested or used to produce a karyotype of the developing baby.

Table 5.63 Chromosomal banding techniques

Technique	Staining Employed
G-banding technique	Giemsa stain to analyze condensed chromosome
R-banding technique	Reverse Giemsa stain after incubation in hot phosphate buffer
C-banding technique	Analysis of centromeres involves acid treatment of metaphase chromosomes, followed by incubation in alkali (e.g. barium hydroxide) and then staining with Giemsa dye
Q-banding technique	Quinacrine fluorescence stain to obtain bright and dull regions produced along the chromosome

been arrested in the metaphase or prometaphase of the cell cycle by colchicine, when chromosomes assume their most condensed conformations.

- Cells are spread on the slides, stained with Giemsa stain, examined under microscope, and photographed that reveal characteristic structural features for individual chromosome such as size, shape, number, arrangement of chromosomes and location of centromere in the cell sample.
- Chromosomes contain thousands of genes that are stored in the DNA, the basic genetic material. According to Denver classification based on the length, chromosomes are classified into seven groups: A to G. Grouping and characteristics of chromosomes are given in Table 5.63.

Pathology Pearls: Clinical Significance of Karyotyping

Karyotyping can be performed to detect for prenatal or postnatal diagnosis of many genetic disorders such as premature ovarian failure and Philadelphia chromosome in chronic myelogenous leukemia (CML).

- **Prenatal diagnosis:** Sonography, first trimester screening, quad screening, amniocentesis, chorionic villi biopsy sampling, and percutaneous umbilical cord blood sampling (PUBS) are important procedures that allow prenatal diagnosis. Karyotype analysis of chromosomes is usually performed either for prenatal congenital abnormalities.
- **Postnatal diagnosis:** In postnatal diagnosis, cells are derived from blood lymphocytes, or from biopsy of skin to obtain fibroblasts. Karyotype analysis of chromosomes is usually performed for postnatal diagnosis of pediatric disorders such as mental or growth retardation, failure of sexual development at puberty; obstetric disorders such as infertility, recurrent abortions, pregnancy in older women; and solid tumors including leukemias.

Pathology Pearls: Chromosome Banding Techniques

Chromosome banding techniques are either based on staining chromosome with a dye. The most common methods of dye-based chromosome techniques are G (Giemsa) banding, R (reverse) banding, C (centromere) banding and Q (quinacrine) banding. Chromosomal banding techniques are given in Table 5.63.

- **G (Giemsa) banding:** G-banding is a technique used in cytogenetics to produce a visible karyotype by staining condensed chromosomes and showing dark and light regions banding patterns along chromosome.
- **R (reverse) banding:** R-banding is a technique used in cytogenetics to produce the reverse of the G-band stain on chromosomes. R-(reverse) banding is obtained by incubating the slides in hot phosphate buffer, then a subsequent treatment with Giemsa stain.
- **C (centromere) banding:** C-banding (synonym: constitutive heterochromatin or centromere banding) has been used to analyze the centromeres. This technique involves acid treatment of metaphase chromosomes, followed by incubation in alkali (e.g. barium hydroxide) and then staining with Giemsa dye.
- **Q (Quinacrine) banding:** Q-banding is the first chromosome banding pattern reported. The resulting pattern is similar to the G (Giemsa) banding pattern in a way that the bright and dull regions produced along the chromosome correspond to the dark and light regions in G (Giemsa) banding patterns respectively.

Y Chromosome

Smears are prepared from oral mucosa to analyze Y chromosome. Long arm of Y chromosome exhibits intense fluorescence with quinacrine dye. Under ultraviolet light, Y chromatin appears as a bright dot. Multiple numerical aberration of sex chromosome can be demonstrated by immunofluorescence study.

Pathology Pearls: Lyon Hypothesis

- In females, during embryogenesis, inactivation of one of two X chromosomes derived from maternal or paternal is known as Lyon hypothesis.
- Inactivation of one of two X chromosomes is transmitted to all somatic cells except germ cells. Ovaries will always possess active chromosomes.
- Inactive X chromosome in the somatic cells becomes condensed in the nucleus is called nuclear sexing.

Barr Bodies (Sex Chromatin)

Barr bodies are also known as sex chromatin, which appear as clumps of chromatin in the interphase nuclei of all somatic cells in females. According to the Lyon hypothesis, each Barr body represents inactivated X chromosome.

- **One Barr body** is present in normal female cells (XX). Barr bodies are absent in males (XY). The number of Barr bodies is always one less than the number of X chromosomes.
- Barr body is absent in Turner syndrome with 45, XO karyotyping. One Barr body is present in Klinefelter syndrome with 47, XXY karyotyping.
- Triple X syndrome with 47, XXX karyotyping demonstrates two Barr bodies. Three Barr bodies are present in poly-X female with 48, XXXX karyotyping.
- Assessment of Barr bodies was considered as diagnostic tool in past. It has now been supplanted by more definitive and sophisticated analytic procedures. Smears stained by scrapping of oral mucosa or circulating neutrophils demonstrates 'Barr bodies', which appear as drumstick appendage attached to one of the nuclear lobes. A minimum of 30% cells positive for sex chromatin indicates female genetic constitution.

BIOCHEMICAL GENETIC TESTING

Biochemical genetic testing is the study of different proteins in the body that may be unusual in some way. These studies can be performed to analyze enzyme defect or mutation in gene from the samples obtained such as blood, urine, amniotic fluid, cerebrospinal fluid, or tissue.

- Normally, different types of proteins are required in biochemical reactions in the cells. Several classes of proteins such as enzymes, transporters, structural proteins, hormones and receptors perform multiple functions. Inborn errors of metabolism are inherited disorders in which the body cannot properly turn food into energy.
- Genetic disorders are usually caused by defects in specific proteins (enzymes) that participate in breakdown of food constituents. Newborn screening is done around 24–48 hours after birth.
- Since proteins are less stable than DNA and can degrade quickly, the sample must be collected, stored properly and transmitted promptly according to the laboratory's specifications. Types of protein changes resulting in altered function are given in Table 5.64.

Table 5.64 Types of protein changes resulting in altered function

No protein made
Too much or too little protein made
Misfolded protein made
Altered active site or another critical region
Incorrectly localized protein (buildpu of protein)
Incorrectly assembled protein

MOLECULAR GENETIC TESTING

Molecular genetic testing (DNA testing) is done to study single-gene or short lengths of DNA to identify variations or mutations that lead to a genetic disorder. Diagnostic molecular methods for evaluation of gene expression are given in Table 5.65.

Table 5.65 Diagnostic molecular methods for evaluation of gene expression

Method	Principle	Requirement and Comments
Immunohistochemistry	Recognizes variably molecule-specific epitopes with antibody	▪ Frozen or formalin fixed paraffin-embedded tissue section ▪ Specific monoclonal antibody
SDS-PAGE and Western blot	Sorting of proteins by molecular weight masses, electrotransfer onto membrane and probing of the membrane with antibody	▪ Fresh or frozen possibly alcohol fixed ▪ Specific antibody required ▪ Technique confirms the presence of immune-reactive protein of the expected molecular weight ▪ Tissue topography lost
mRNA *in situ* hybridization	Technique demonstrated specific transcripts on tissue sections	▪ Fresh, frozen possibly formalin fixed tissue ▪ Specific probe required
RT-PCR (reverse transcriptase-polymerase chain reaction)	Reverse transcription to cDNA, followed by PCR amplification, preferably quantitatively	▪ Fresh or frozen tissue ▪ Thermal cycler, or quantitative PCR system) light cycler/ real-time PCR ▪ No topography correlation
cDNA microarray analysis	Hybridization of tumor cDNA onto the filters or slides and simultaneously screens potentially thousands of transcripts	▪ Specially prepared filters/slides with probes, hybridization, and filter reader are required ▪ No topography correlation

- Direct DNA analysis is applicable when the gene sequence of interest is known. DNA is extracted from the cells obtained from blood, bone marrow, amniotic fluid (amniocentesis), buccal mucosa and analyzed.
- Some genetic diseases can be caused by numerous different mutations, making molecular testing challenging. For example, more than 1000 gene mutations in the cystic fibrosis conductance regulator (CFTR) gene can cause cystic fibrosis. It is not possible to examine the entire sequence of the CFTR gene routinely to identify the causative gene mutation because the CFTR gene is quite large.
- Majority of cystic fibrosis cases are caused by about 30 mutations; hence these cystic fibrosis cases are tested before more comprehensive molecular genetic testing is performed.

MOLECULAR GENETIC TESTING: METHODOLOGY

Cytogenetics is the study of structure and number of chromosomes using a microscope, whereas molecular cytogenetics is the study of chromosomes and DNA at the molecular level using DNA technology.

- As the number of genetic tests has expanded rapidly over the last decade, so have the different types of genetic testing methodologies used.
- The type of genetic test employed depends on the type of abnormality being measured. In general, three categories of genetic testing—cytogenetic, biochemical, and molecular—are available to detect abnormalities in chromosome structure, protein function, and DNA sequence, respectively.
- Direct DNA analysis is applicable when the gene sequence of interest is known. For small DNA mutations, direct DNA testing is typically the most effective method, particularly if the function of the protein is unknown and a biochemical test cannot be developed.
- A DNA test can be performed on any tissue sample and requires very small amounts of sample. Several different molecular technologies, including direct sequencing, polymerase chain reaction-based assays, and hybridization, can be used to perform testing.
- Polymerase chain reaction (**PCR**) is a common procedure used to amplify targeted segments of DNA through repeated cycles of denaturation (heat-induced separation of double-stranded DNA), annealing (binding of specific primers of the target segment to parent DNA strand), and elongation (extension of the primer sequences to form a new copy of the target sequence). The amplified product can then be further tested.
- For some genetic disorders, many different mutations can occur in the same gene and result in the disease, making molecular testing challenging. However, if the majority of cases of a particular genetic disorder are caused by a few mutations, this group of mutations is first tested before more comprehensive testing such as sequencing is performed.
- Comparative genome hybridization (**CGH**) or chromosomal microarray analysis (**CMA**) is a molecular cytogenetic method for analyzing gains or losses in DNA that are not detectable with routine chromosome analysis. The CGH method is based on the proportion of fluorescently-labeled patient DNA to normal reference DNA, which can detect small deletions and duplications, but not structural chromosomal changes such as balanced reciprocal translocations or inversions or changes in chromosomal copy number.
- DNA microarray analysis, also referred to as gene, genome, or DNA chip analysis, is a tool for determining gene expression. Molecules of mRNA bind, or hybridize, specifically to a DNA template, typically a gene or portion of a gene, from which it originated. When an array contains many DNA microarray templates, the expression level of hundreds to thousands of genes from an individual patient sample can be measured using a computer to detect the amount of mRNA bound to each site on the array.
- Protein microarray analysis is used to quantify the amount of protein present in **biological samples**. Similar to chromosome and DNA microarray analysis, the hybridization of labeled target proteins in a patient sample is measured against a reference sample. Also referred to as a biomarker, the presence, absence, increase, or decrease of a particular protein can be an indicator of disease in a person. For example, analysis of the cerebrospinal fluid of a patient for amyloid β or τ proteins may be used to diagnose Alzheimer's disease.

FLUORESCENCE *IN SITU* HYBRIDIZATION

Fluorescence *in situ* hybridization (FISH) is a molecular cytogenetic technique that uses fluorescent probes and that binds to genes on chromosomes visualized by fluorescence microscopy. FISH technique is used for rapid diagnosis of chromosomal abnormalities such as trisomies, monosomies, microdeletions, translocations.

- FISH technique is used to identify chromosomal abnormalities such as insertions, deletions (e.g. DiGeorge syndrome del22), translocations (9 and 22 in chronic myeloid leukemia) and copy number aberrations.
- The basic elements of FISH technique include a DNA probe and target sequence. Before hybridization, these DNA probes are labelled directly (right panel) or indirectly (left panel).
  - For indirect labeling, DNA probes are labeled with modified nucleotides that contain a hapten, whereas direct labeling uses nucleotides that have been modified to contain a fluorophore, which are consequently detected by fluorescent antibodies and finally visualized under the fluorescence microscope. FISH technique is faster with directly labeled probes.
  - FISH technique with indirectly labeled probe offers the advantage of signal amplification by using several antibodies, and production of a signal that is brighter compared with background levels.
- FISH technique has gained recognition worldwide as a physical mapping technique and support for large-scale mapping the chromosomes related to the human genome project. Three FISH probes are used to detect the chromosomal alterations. Refer to FISH technique in Chapter 14: Molecular Diagnostic Techniques in Clinical Practice, Section II.

COMPARATIVE GENOME HYBRIDIZATION

Comparative genome hybridization (CGH) is a special FISH technique used to detect all genome imbalances (i.e. chromosomal copy number changes bypassing cell culturing) occurring in human beings.

- Fundamental of CGH is to compare total genome DNA of the given abnormal (patient) sample and total genome DNA of the normal (person) cells sample. Both genome DNA samples of patient and normal person are labeled with two different fluorescent dyes. The final mixture is added, hybridized and observed under fluorescence microscopy and the results are compared with green to red fluorescence ratio along chromosomal axis, which represents loss or gain of genetic material in the tumor at that specific locus.
- Drawback of conventional CGH is that it cannot detect the structural chromosomal aberrations without copy number changes such as mosaicism, balanced chromosomal translocations, inversions and the ring chromosomes. CGH can only detect gains or losses relative to the ploidy level. Refer to comparative genome hybridization (CGH) in Chapter 14: Cellular–Molecular Diagnostic Techniques in Clinical Practice, Section II.

QUALITATIVE FLUORESCENCE POLYMERASE CHAIN REACTION

Qualitative fluorescence polymerase chain reaction (QF-PCR) is a rapid molecular technique for detection of the chromosome copy number by amplification of repeat sequences at chromosome-specific polymorphic loci. QF-PCR is performed to prenatal diagnosis of aneuploidy in fetus in mid-pregnancy for cost effective and rapid diagnosis.

MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION

Multiplex ligation-dependent probe amplification (MLPA) method works under PCR-based technology, that utilizes up to 40–50 probes, each specific for a different DNA sequence (mainly exons of a specific gene of interest) to evaluate the relative copy number changes of each DNA sequence in a single PCR reaction.

- MLPA can also be used to analyze the subtelomeric regions of chromosomes or multiple regions of specific chromosomes to detect aneuploidy. Software programs are used for analysis.
- MLPA is performed to detect familial adenomatous polyposis, breast cancer (BRCA1 and BRCA2), ovarian cancer, Duchenne muscular dystrophy, hereditary neuropathy with liability to pressure palsies, spinal muscular atrophy and Charcot-Marie-Tooth disease.

WHOLE-EXOME SEQUENCING

Exome sequencing (synonym whole-exome sequencing, i.e. WES) is a genome technique used for sequencing all of the protein coding regions of genes in a genome (known as exome).

- The exome refers to the sequence encompassing all exons of protein coding genes in the human genome. Exome sequencing differs from whole genome sequencing. Whole genome sequencing involves sequencing all coding regions (exons), noncoding regions (intron), nuclear DNA and mitochondrial DNA.
- However, ALL protein coding genes are found in the exome. Whole exome sequencing (WES) has four basic steps: (a) library preparation, (b) cluster generation, (c) sequencing, and (d) data analysis and interpretation of the results.

BIOCHEMICAL GENETIC TESTING: METHODOLOGY

Biochemical genetic testing is the study of enzymes or proteins instead of a gene, that may be unusual in some way in the body. Many biochemical genetic diseases are known as 'inborn errors of metabolism' in children, because they are present at birth and disrupt a key metabolic pathway.

- Biochemical genetic testing measures enzyme or protein activity, level of metabolites and protein structure in samples such as blood, urine, cerebrospinal fluid and amniotic fluid. Because gene products are relatively unstable than DNA or RNA, that can degrade quickly, hence the sample must be collected, stored properly, and transported immediately according to the laboratory's specifications.
- Biochemical genetic testing can be done by various diagnostic tools such as high-performance liquid chromatography, gas chromatography/mass spectroscopy and tandem spectrometry metabolites.
- Biochemical genetic testing techniques estimate both qualitative and quantitative determination of metabolites. In addition, bioassays may employ fluorometric, radioisotopes or thin layer chromatography methods.

CHAPTER 6

Neoplasia

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LEARNING OBJECTIVES

NEOPLASIA: GENERAL CONSIDERATIONS

- Human deoxyribonucleic acid (DNA) and cancer
  - Deoxyribonucleic acid (DNA) packaging
  - Gene expression: DNA replication, transcription and translation
- Four classes of normal cellular regulatory genes
  - Proto-oncogenes, oncogenes and viral oncogenes
  - Tumor suppressor genes
  - Apoptosis regulatory genes
  - DNA repair genes
- Molecular/cytogenetic alterations in human cancers
  - Genomic instability linked to human cancers
  - Epigenetic alterations
  - Cancer stem cell properties
- Histogenesis of neoplasms
  - Intraepithelial neoplasia
  - Dysplasia in mesenchymal tissues
- Kinetics of tumor growth and angiogenesis
- Tumor invasion and metastasis
- Tumor metastatic cascade

NOMENCLATURE AND CLASSIFICATION OF TUMORS

- Tumor nomenclature
  - Histogenesis of tumors
- Tumor classification hierarchy
  - Benign epithelial tumors
  - Benign mesenchymal tumors
  - Borderline tumors
  - Malignant epithelial tumors
  - Malignant mesenchymal tumors
  - Locally malignant tumors
  - Mixed benign and malignant tumors
  - Germ cell tumors
  - Primitive neuroectodermal tumors (PNETs)
  - Neuroendocrine tumors
  - Hematolymphoid malignancies
  - Miscellaneous terminology related to neoplasms

FEATURES USED TO DISTINGUISH BENIGN TUMORS FROM MALIGNANT TUMORS

- Distinguishing features of benign and malignant tumors
  - Two basic cellular components in tumors
  - Degree of differentiation in tumors
  - Nucleocytoplasmic features in tumors
  - Growth rate in tumors
  - Cellularity and cell polarity in tumors
  - Mitotic figures in tumors
  - Tumor monoclonal properties: analysis techniques
  - Telomerase activity in tumors
  - Tumor angiogenesis
  - Tumor invasion and metastasis

NORMAL PROCESSES THAT REGULATE CELLS AND INHIBIT CARCINOGENESIS

- Cell cycle and cell division
  - Cell cycle and cell division: phases
  - Cell cycle checkpoints
  - Cell cycle regulators
- DNA repair protects cellular genomes from genotoxic stresses
  - DNA repair pathways
- Telomeres and senescence
- Apoptosis inhibits tumor growth

EPIDEMIOLOGY OF CANCER

- Global impact of cancer
  - Cancer epidemiology and prevention strategies
  - Immunodeficiency states in cancer epidemiology
  - Acquired premalignant diseases
  - Interactions between genetic and environmental factors linked to inherited cancer syndromes

PREFERENTIAL ROUTES OF METASTASIS OF TUMORS

- Local invasion: first step metastasis
- Lymphatic route of metastasis
- Hematogenous route of metastasis
  - Liver metastasis
  - Pulmonary metastasis
  - Bone metastasis
  - Brain metastasis
  - Adrenal gland metastasis
- Seeding of cancer stem cells and transcoelomic route of metastasis
  - Peritoneal cavity seeding
  - Pleural cavity seeding
- Perineural and leptomeningeal routes of metastasis
- Intraepithelial route of metastasis
- Iatrogenic malignant tumor implantation during surgery

MOLECULAR BASIS OF CANCER

- Chromosomal abnormalities and dysregulation of genes: mechanisms
  - Numerical alterations in chromosomes
  - Structural alterations in chromosomes
  - Genes dysregulation and carcinogenesis
- Cellular and molecular hallmarks of cancer
  - Growth signal autonomy (unrestricted cell proliferation)
  - Insensitivity to growth inhibitory (suppressor) signals (inactivation/biallelic loss of tumor suppressor genes)
  - Evasion of apoptosis (programmed cell death)
  - Enabling replicative potential (immortality)
  - Induction and sustained tumor angiogenesis
  - Activating tissue invasion and metastasis
  - Reprogramming energy metabolism (Warburg effect)
  - Evasion of immune destruction
  - Genomic instability (mutated phenotype)
  - Tumor-promoting inflammation
  - Phenotypic plasticity and disrupted differentiation
  - Nonmutational epigenetic reprogramming in cancer stem cells
  - Polymorphic microbiomes and cancer

CARCINOGENIC AGENTS: CELLULAR INTERACTION AND CARCINOGENESIS

- Chemical carcinogenesis
  - Multistep carcinogenesis: mechanisms
  - Chemical carcinogenic agents: classification
- Radiation-induced carcinogenesis
  - Ultraviolet radiation-induced carcinogenesis
  - Ionizing radiation-induced carcinogenesis

- Viral carcinogenesis
  - Oncogenic RNA viruses associated cancers
  - Oncogenic DNA viruses associated cancers
- Bacterial carcinogenesis
  - *Helicobacter pylori*-induced gastric carcinogenesis
  - *Borrelia burgdorferi*-induced breast carcinogenesis
  - *Campylobacter jejuni*-induced colorectal carcinogenesis
- Parasite-induced carcinogenesis
- Hormonal carcinogenesis
  - Role of hormones in breast carcinoma
  - Hormonal replacement therapy in pregnant women linked to clear cell carcinoma of vagina in their daughters
  - Role of hormones, obesity and leptin in endometrial carcinoma
  - Role of hormones in prostatic adenocarcinoma

ROLE OF ONCOGENES AND TUMOR SUPPRESSOR GENES IN CARCINOGENESIS

- Cell signaling
  - Growth factors
  - Cell surface receptors
  - GTP-binding proteins (G proteins)
  - Signal transduction proteins
  - Transcription factors
  - Chromatin remodeler proteins
  - Cell cycle regulatory proteins
  - Apoptosis regulatory proteins
  - DNA repair proteins maintaining genome integrity
- Proto-oncogenes, oncogenes and viral oncogenes
  - Proto-oncogenes function during physiologic and pathologic state
- Tumor suppressor genes
  - Tumor suppressor gatekeeper genes
  - Tumor suppressor caretaker genes
  - Tumor suppressor genes function as inhibitors of Wnt/β-catenin mitogenic signaling activator pathway
  - Tumor suppressor genes function to inhibit cell cycle progression
  - Tumor suppressor genes function to inhibit tumor angiogenesis
  - Tumor suppressor genes: genomic stability enabler (most important)
  - Tumor suppressor genes function DNA repair system
  - Tumor suppressor genes function by various mechanisms
- Epigenetics in cancer
- Tumor invasion and metastasis: molecular mechanisms
  - Epithelial–mesenchymal transition in development of malignant tumor and its clinical significance
  - Tumor microenvironment
  - Metastasis multi-step process

CLINICAL ONCOLOGY

- Local effects of tumors on the host
- Non-metastatic systemic effects of cancer on the host
  - Cancer-associated cachexia
  - Paraneoplastic syndromes
- Host defense against cancer
  - Cancer immunosurveillance and immunoediting
  - Cancer stem cells evasion of immune response
- Tumor markers: diagnostics in clinical practice
  - Molecular basis of tumor markers
  - Tumor markers: detection methods
  - Tumor markers: classification and uses
  - Recommendations for advising tumor marker tests
- Diagnostic approach of cancer
  - Clinical history
  - Past medical history
  - Lifestyle, social history and environmental factors
  - Systemic evaluation of the body systems
  - Blood tests
  - Urine analysis
  - Imaging techniques
  - Cytologic examination
  - Surgical pathology
- Treatment modalities for treating cancer
  - Surgical treatment
  - Radiation therapy
  - Hormonal therapy
  - Chemotherapy
  - Immunotherapy
- Prognosis of solid cancers
- Prevention modalities of cancers

MOLECULAR DIAGNOSTICS IN ONCOLOGY

- Purpose to analyze cancer biomarkers
- Specimen requirements for molecular diagnostics in cancer
- Molecular diagnostics uses in oncology
- Molecular techniques: methodologies
  - Liquid biopsy: cell-free DNA technology
  - Targeted mutation analysis methods
  - Whole genome sequencing analysis
  - Ribonucleic acid (RNA) biomarkers
  - Proteins as biomarkers
  - Chromosomes analysis (karyotyping test)

NEOPLASIA: GENERAL CONSIDERATIONS

Cancer is the most common cause of mortality across world. Knowledge of the non-neoplastic cell proliferation is helpful in understanding neoplasia. Some non-neoplastic cellular proliferations include hyperplasia (increase in number of cells in the tissue or organ), metaplasia (change from one type of differentiated tissue to another type, usually in response to an irritating stimulus), and **dysplasia** (disordered development of cells with nuclear features of malignancy, i.e. an alteration in their size, shape and organization). Anaplasia refers to lack of differentiation (almost always indicative of malignancy, that exhibits pleomorphism, numerous atypical mitoses, abnormal nuclear morphology and cellular disorganization, i.e. loss of polarity). Oncogenesis (neoplasia) is a process of development of benign or malignant tumor.

- **Neoplasia** is defined as autonomous, purposeless, excessive, progressive, and disorganized cell proliferation, and monoclonal growth with irreversible permanent genetic alteration in cells that persists in the same excessive manner after cessation of the initiating injurious stimuli, which has evoked the change (Ancient Greek, *neo*—new and *plasia*—creation). Neoplasia term can be applied to any tumor mass, benign or malignant. **Oncology** (Greek *onkos*—tumor + logia) is the study of neoplasms (tumors), including the etiology and pathogenesis.
- **Neoplasm (new growth)**, synonymous with tumor, is composed of cells that deviate from normal programme of cell division and differentiation. Depending on the biological behavior, tumors are classified as benign (slow-growing, non-invasive without metastasis), borderline low-malignant potential and malignant (rapid growth, invasive and metastasis), which may arise from epithelial tissue, mesenchymal tissue, neuroectodermal tissue, hematolymphoid tissue and germ cells.
  - The most common tumors originate from tissues of rapid turnover of cells, i.e. epithelium of mucous membranes, skin, breast and reproductive organs and hematolymphoid tissues, which are exposed to environmental mutagens. **Carcinoma** is a

malignant tumor of epithelial origin (skin, prostate, lung, colon). **Sarcoma** is a malignant tumor of mesenchymal origin (soft tissue and bone).
  - In clinical practice, some malignant tumors (e.g. basal cell carcinoma) may grow locally and invade normal tissues but never metastasize. Some malignant tumors produce distant metastases only after a very considerable time, while at the other end of spectrum, some malignant tumors metastasize very early to distant organs.
  - Familial cancer syndrome refers to an inherited susceptibility to malignancy, typically involving a mutation either in an oncogene (autosomal dominant inheritance) or tumor suppressor gene (autosomal recessive inheritance).
- Neoplasia results from mutations that '**turn on**' oncogenes and '**turn off**' tumor suppressor gene (inactivation/biallelic loss). Malignant tumors most often have both activation of oncogenes and inactivation/biallelic loss of tumor suppressor genes. Degree of vascularization is the important determinant of tumor growth potential. Cancer stem cells (CSCs) can bind to laminin and fibronectin in connective tissue, and secrete tissue degrading matrix metalloproteinases (MMPs), i.e. collagenases and proteases, and then invade surrounding tissues and disseminate to distant organ(s). Cancer stem cells may attain 'immortality' or the ability to keep dividing indefinitely.
- **Carcinogenesis** results from accumulation of complementary gene mutations in a stepwise manner overtime. During carcinogenesis, transformation of normal cell to cancer stem cell (CSC) must follow several steps involving disruption of key cellular processes: acquire self-sufficiency in growth signals, ignore growth inhibitory signals, evade apoptosis, acquire defects in DNA repair, acquire ability to undergo uncontrolled cell division (i.e. cell cycle regulation, cell cycle progression, apoptosis and senescence), clonal expansion, accumulate oncogenic mutations, promote angiogenesis for providing oxygen and blood supply to tumor, tumor heterogeneity, invade basement membrane and surrounding tissue, and metastasize to distant organ(s). Immune system fails to recognize and respond to eliminate cancer stem cells.
  - Most of malignant tumors are caused by combined interaction of genetic factors, lifestyle and environmental mutagens. Three major environmental factors, which induce tumorigenesis include chemical carcinogens, radiation and viruses. Gene mutations that contribute to the evolution of malignant phenotype are referred to driver mutations. Along with driver gene mutations, passenger mutations are present in genome of cancer stem cells, because they often occur during somatic cell division and have no functional consequences.
  - Most carcinomas are initiated by inactivation/biallelic loss of tumor suppressor genes. Malignant tumors most often have both activation of oncogenes and inactivation/biallelic loss of tumor suppressor genes. Mutation in single gene is not sufficient to cause cancer. Subsequent progression to malignant phenotype involves additional gene mutations such as gain-of-function ('dominant') mutations occur in proto-oncogenes and loss-of-function ('recessive') mutations due to inactivation/biallelic loss of tumor suppressor genes in all types of malignant tumors. The genetic alterations persist in the progeny of the initial cancer stem cell (CSC).
  - Malignant tumors occur due to uncontrolled proliferation of cancer stem cells (CSCs), which are monoclonal. For example, clonal plasma cells produce the same type of immunoglobulin including the same light chain. On contrary, inflammatory polyclonal plasma cells produce various immunoglobulins with heavy and light chains. Some of the cancer stem cells undergo further gene mutations, which are passed to their progeny, that is called '**clonal evolution**' or progression to malignant phenotype. Some of the subclones grow more rapidly and disseminate more readily to distant organ(s), while other subclones are so abnormal, that proliferation of subclones does not take place and undergo programmed cell death (apoptosis). Aneuploidy indicates irregular increase in the cellular DNA content.
- Typically, a long-time pass between the initiated event and onset of malignant disease by multistep carcinogenesis, i.e. initiation, promotion and progression. Cancer stem cells continue to grow and replicate each time undergoing successive changes and further undergo gene mutations. Eradication of the malignant tumor requires removal of the cancer stem cells, which possess protein product encoded by BMI1 gene that inhibits normally functional negative regulators of the cell cycle.
- Primary malignant tumor growth and available blood supply determine the cell cycle duration. Epithelial tissue cells have a shorter cell cycle duration than connective tissue cells, hence carcinomas derived from epithelial tissue grow more rapidly than do sarcomas derived from connective tissue. Malignant tumors require an available blood supply to provide nutrients and oxygen that promote tumor growth from

one-to-two (1–2 mm) millimeter in size to large-size malignant tumor. Cellular components of malignant tumor microenvironment secrete angiogenic factors, which stimulate tumor angiogenesis to meet the demand of the malignant tumor.

- Cancer stem cells also produce their own growth factors, which bind to numerous growth factor receptors present on the cell membranes of rapidly growing cancer stem cells. Increase in growth factor receptors, in conjunction with the changes in the cell membranes, further enhance uncontrolled proliferation of cancer stem cells.
- The immune surveillance system includes CD8+ cytotoxic T cells, natural killer cells (NK cells), and macrophages, which recognize tumor-associated neoantigens presented by major histocompatibility complex (MHC) class molecules on antigen-presenting cells (APCs). The equilibrium between signals from the tumor microenvironment and immune system shifts, which fails to eliminate cancer stem cells.
  - In this chapter, we will discuss the basic terms associated with tumors, features used to distinguish benign tumors from malignant tumors, epidemiology and etiology of tumors, effects of tumors including paraneoplastic syndromes, molecular carcinogenesis, proto-oncogenes and tumor suppressor genes, diagnostics, tumor markers and immunohistochemistry, and tumor grading and staging.

Pathology Pearls: Carcinogenic Agents Linked to Cancer

- Chemical carcinogens (asbestos, nickel, cadmium, vinyl chloride, benzene, tobacco, processed meat)
- Physical carcinogens (ultraviolet X-rays, ionizing radiation, i.e. atomic bomb explosion, and nuclear power plant accidents)
- Viral carcinogens (HPV, HBV, HCV, EBV, HTLV-1, herpesvirus 8)
- Microbial carcinogens (*Helicobacter pylori*)
- Fungus derived carcinogens (aflatoxin of *Aspergillus flavus* fungus)
- Parasitic carcinogens (*Schistosoma haematobium*, *Opisthorchis viverrini*, *Clonorchis sinensis*)
- Oncogenes (RAS, ABL, Myc, HER2/neu, cyclin D1, cyclin E, β-catenin gene mutations, chromosomal translocation, gene arrangements, nucleotide base pair aberrations, gene amplification and gene overexpression)
- Inactivation/biallelic loss of tumor suppressor genes (RB, TP53, APC, WT1, NF1, NF2, BRCA1 BRCA2 genes)
- Defective DNA repair system
- Chromosomal/genomic instability (xeroderma pigmentosum, ataxia-telangiectasia, Fanconi anemia, Bloom syndrome)

HUMAN DEOXYRIBONUCLEIC ACID (DNA) AND CANCER

Genetic changes that cause cancer can be either inherited or somatic (acquired) that arise from exposure to certain environmental mutagens (carcinogens), i.e. chemical agents, tobacco smoke, solar ultraviolet-rays, ionizing radiation and human papillomavirus (HPV). Genetic changes can also happen because of random mistakes in deoxyribonucleic acid (DNA) that occur during cell division and germline mutations. Genes are segments of DNA that carry instructions to make a specific protein or several proteins.

- At the simplest level, chromatin is a double-stranded helical structure of deoxyribonucleic acid (DNA) that winds around each other like a twisted ladder. DNA is made up of nitrogenous base pairs, and sugar phosphate backbone. Each DNA strand is made of three billion nucleotide base pairs: adenine (A), thymine (T), guanine (G) and cytosine (C).
- A linear sequence of deoxyribonucleotides is linked together by 3′–5′ phosphodiester linkages. The informational content of DNA resides in the sequence in which monomers—purines and pyrimidine deoxyribonucleotides are ordered. The polymer deoxyribonucleotides possess a polarity; one end has a 5′-hydroxyl or phosphate terminal, while other has a 3′-phosphate or hydroxyl terminal.
- Double helical deoxyribonucleic acid (DNA) molecule has two strands. Each DNA strand possesses a polarity, and antiparallel, i.e. one DNA strand runs in the 5′–3′ direction and the other DNA stand in the 3′–5′ direction. Sugar phosphate backbone joins together nucleotides in a DNA sequence. DNA nucleotide base pairs are linked by covalent bonds, formed between the deoxyribose sugar of one nucleotide base and the phosphate group of the subsequent nucleotide base. This arrangement makes and alternating chain of deoxyribose sugar and phosphate groups in the DNA polymer, a structure known as 'sugar-phosphate backbone'.
- Both strands of double helical deoxyribonucleic acid (DNA) molecule are stabilized by hydrogen bonds between nucleotide base pairs, i.e. two hydrogen bonds between adenine (A): thymine (T) nucleotide base pairs, while three hydrogen bonds between cytosine (C): guanine (G) nucleotide base pairs, and store the same biological information of DNA replication. In Watson and Crick's model, the two strands of DNA twist around each other to form a right-handed helix. The nucleotide base residues in double helix deoxyribonucleic acid (DNA) molecule form a spiral in clockwise direction.

- In double-stranded DNA molecule, genetic information is stored in the sequence of nucleotide base pairs on template DNA strand, that is copied during ribonucleic acid (RNA) synthesis. The opposite noncoding DNA strand constitutes more than 98% of DNA that lacks protein sequences.
- Major and minor grooves wind along the DNA molecule parallel to the phosphodiester backbones, in which proteins can interact with specifically exposed atoms of the nucleotide base pairs via specific hydrophobic and ionic interactions without disrupting the nucleotide base pairs of double-helical DNA molecule.
- Mitochondria have their own DNA, which is circular and does not utilize histones. Mitochondrial DNA is passed almost exclusively from the mother to offspring through the ovum. Mitochondrial DNA test talks about maternal ancestors of offspring.

DEOXYRIBONUCLEIC ACID (DNA) PACKAGING

Deoxyribonucleic acid (DNA) exists in the condensed to form chromosomes to fit into the nucleus. As DNA is replicated during S phase of the cell cycle, histone octamer core proteins are synthesized in the cytosol by ribosomes in parallel, and imported to the nucleus, where they are assembled into core particles and incorporated into the growing chromatin strand, as nucleosomes ('beads on string'). DNA methylation changes the expression of a DNA segment without changing the nucleotide base sequences. It is worth mentioning that mitochondria have their own DNA, which is circular and does not utilize histones. Human double-stranded deoxyribonucleic acid (DNA) molecule packaging is shown in Fig. 6.1.

Deoxyribonucleic Acid (DNA) Supercoiling

DNA supercoiling reduces the space and allows for much more DNA to be packaged.

- Negative supercoiling is the left-handed coiling of DNA in counterclockwise direction and also known as 'underwinding of DNA'.
- Positive supercoiling is the right-handed coiling of DNA; thus, winding occurs in the clockwise direction and also known as the 'overwinding of DNA'.
- Certain DNA topoisomerase enzymes are able to change supercoiled DNA topology to facilitate several essential functions such as DNA replication, transcription and recombination.

Histone Octamer Core Proteins

Since DNA is negatively charged in nature because of sugar phosphate backbone, it is wrapped around the positively charged histone octamer core proteins, and found in nuclei that package and order the DNA into the basic fundamental structural unit of chromatin called 'nucleosome'.

- Histone octamer core proteins contain two pairs of H2A, H2B, H3 and H4 proteins. Histone linker protein is highly basic and rich in **proline** and **lysine**. Histone linker protein H1 binds the nucleosome at the starting and ending sites of the DNA, thus locking the DNA into place and help in the formation of high order structure and thereby stabilizing the chromatin fiber. Phosphate group gives a negative charge. Lysine and arginine give histone octamers a positive charge. Histone octamer core proteins play an important role in the maintenance of gene expression.
- Recent studies revealed that deletion of the four histone octamer core proteins (e.g. H2A, H2B, H3 and H4) lack the N-terminal tail, that affect the histone–DNA interactions and decreased nucleosome stability. Histone octamer proteins work with nonhistone proteins to stabilize the structure of DNA.
- Histone octamer core proteins form nucleosomes that are basic units of chromatin. The presence of nonhistone proteins is essential for the packaging of chromatin at higher level, i.e. chromatin fibers and chromosomes essential for function of histone proteins. Histone octamer core proteins play an important role in the maintenance of gene expression. Nonhistone proteins play a key role in regulation of gene expression.
- Histone tails are flexible structures that flank both ends of the histone fold and protrude from the nucleosome. Post-translational modifications of histone tails modulate DNA accessibility within the nucleosome, which are essential for stable folding of oligonucleosome arrays into condensed chromatin fiber-to-fiber interactions, involved in higher-order chromatin architecture. Histone tails also play an important role in providing structural stability to nucleosomal core particle itself, gene transcription and expression in the nucleosome.
- Histone methylation mostly makes DNA inactive by changing the expression of DNA segment without changing the nucleotide base sequences. Histone methylation usually causes reversible transcriptional suppression, but can also cause activation on location of methyl groups. On contrary, histone acetylation relaxes DNA coiling, allowing for transcription and making DNA active.

Nucleosomes

Nucleosome is the fundamental unit of chromosome, which consists of 146 nucleotide base pairs of the DNA wrapped tightly around a positively-charged histone octamer, forming 'beads on a string' joined with linker DNA into coiled chromatin.

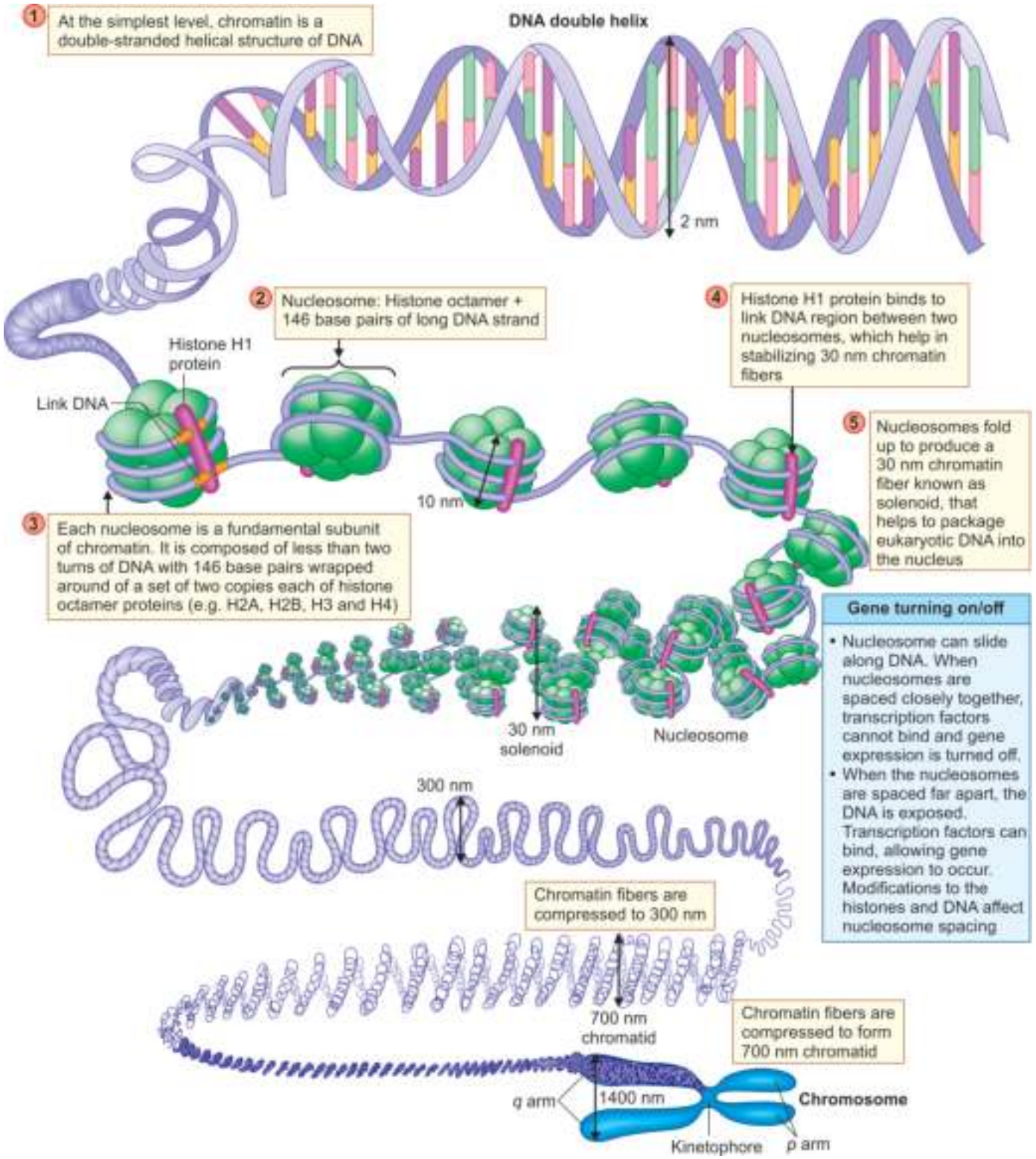


Fig. 6.1: Human double-stranded deoxyribonucleic acid (DNA) molecule packaging. DNA molecule is composed of two linear strands that run opposite to each other and twist together. DNA is wrapped around positively charged histone octamer. composed of amino acids (i.e. lysine and arginine), found in nuclei that package and order the DNA into structural units called nucleosome. The end result is compression of chromatin into chromosome. The nucleosome is fundamental unit of chromatin consists of 146 nucleotide base pairs of the DNA around a histone octamer and play a role in gene regulation. Switching gene 'turning on' or 'turning off' gene occurs due to nucleosomal spacing along DNA. Nucleosomes can slide along DNA. When nucleosomes are spaced closely together, the transcription factors cannot bind and gene expression is turned off. When the nucleosomes are widely spaced far apart, the DNA is exposed, and the transcription factors can bind, which cause allowing gene expression ('turning on') to occur. Modifications to the histones and DNA affect nucleosome spacing.

- Both histone proteins and nonhistone proteins are involved in the formation of the chromatin structure of DNA and providing structural support to the DNA. The basic difference between histone proteins and nonhistone proteins is in the structure they provide. Histone proteins become core protein molecules to form nucleosomes that are basic units of chromatin. The presence of nonhistone proteins is essential for the function of histone proteins.
- Nucleosomes play a key role in compaction of chromatin structure and gene regulation, which can slide along DNA. When nucleosomes are closely spaced together, transcription factors cannot bind and thus gene expression is 'turned off'. When the nucleosomes are spaced far apart, the DNA is exposed to transcription factors, which can bind to exposed DNA and thus gene expression is '**turned on**'. Modifications to the histone octamers and DNA affect nucleosome spacing.

Solenoid and Chromatin Condensation

Nucleosomes are packaged into a condensed thread-like chromatin fiber that helps to package eukaryotic DNA into the nucleus, sometimes described as 'beads on a string'. The end result is formation of chromatin fibers (30 nm), which are condensed into chromatid (700 nm), which is further compressed to chromosome (1400 nm), i.e. forming two strands of a replicated chromosome during mitotic cell division and visible under electron microscope. Chromatids are connected by a centromere called 'sister chromatids'. Nonhistone proteins are additional set of proteins required for packaging of chromatin at higher level, i.e. chromatin fibers and chromosomes.

Chromatin Fibers

The architecture of the chromatin dictates whether it permits or inhibits transcription and other DNA-template processes, i.e. DNA replication, DNA repair and recombination. Changes in the chromatin structure are central to transcriptional regulation. One main pathway to alter chromatin structure involves covalent modifications of the histone tails.

Chromosome

Each chromosome has two sister chromatids with two short arms (p arms), two long arms (q arms), and a centromere holding it together at the center. Human beings have 23 pairs of chromosomes (46 in total): one set comes from mother and one set comes from father. Of these 23 pairs, one pair is sex chromosomes so depending on whether one is male (XY) or female (XX). The other 22 pairs are autosomes (non-sex chromosomes), that look same for both males and females.

Deoxyribonucleic acid (DNA) making up each of our chromosomes contains thousands of genes. At the ends of each of chromosome is repetitive nucleotide TTAGG sequence of DNA containing 15000 nucleotide base pairs are called telomeres. Telomeres serve critical role of preserving genomic sequence by protecting the genome from degradation and inhibiting chromosomal fusion and recombination during DNA replication. Sister chromatids and centromere of chromosomes are also involved in chromosome organization within nucleus.

Heterochromatin and Euchromatin Regions in Chromosomes

Deoxyribonucleic acid (DNA) is tightly bound with histone octamer core proteins and condensed, that appears darker region on chromosome known as 'heterochromatin'. DNA is unfolded to form a beaded structure, that appears transcriptionally, accessible lighter region on the chromosome known as 'euchromatin'. Cell division occurs by two processes: mitosis and meiosis. The critical difference between mitosis and meiosis is that mitosis produces two genetically identical daughter cells, whereas meiosis produces four genetically different daughter cells.

Genes in Human Genome

Approximately, 20,000–25,000 genes located on 23 pairs of chromosomes are packed into the human genome. Gene is the unit of DNA made up of nitrogenous nucleotide base pairs, and sugar phosphate backbone, which carries the instructions for making specific set of protein(s). Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product, i.e. protein. Gene directs the production of an average three proteins with the assistance of enzymes and messenger molecules. Structure of human gene is shown in Fig. 6.2.

- **Gene structure:** Gene contains 5'–3' untranslated regions: (a) promoter region near to gene (start site transcription), (b) enhancer region far away (enhancer site of transcription which does not specify amino acids), (c) exons (coding sequences, which specify a sequence of amino acids), (d) introns (noncoding sequences), (e) transcriptional and (f) translational start and stop codon sites. Regulatory sequences play a role in determining when and where the protein is produced.
- **Gene 'turning on' and 'turning off':** Many proteins such as transcription factors and complex of mediator proteins 'turn on' the gene and help RNA polymerase to read the gene, which creates copies called pre-messenger RNAs (**pre-mRNAs**), which transmit the instructions for building the desired protein. The pre-messenger RNAs (pre-mRNAs) get cleaned

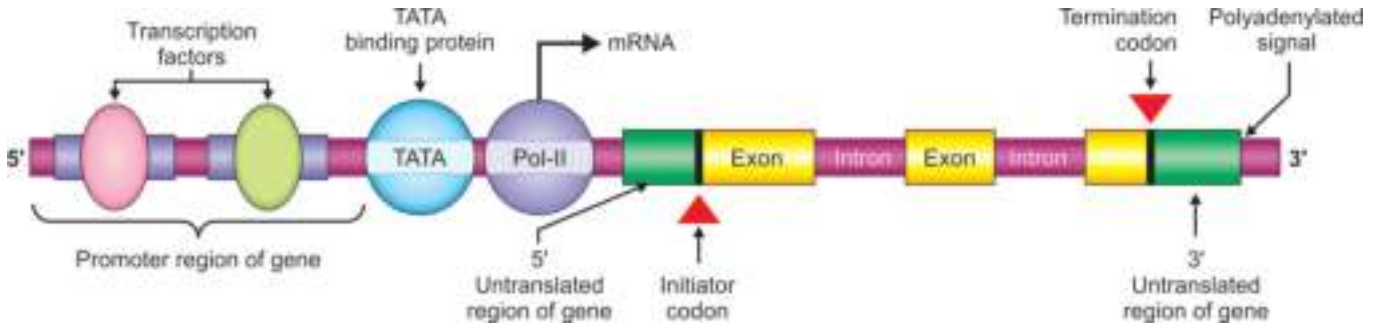


Fig. 6.2: Structure of human gene. Gene consists of 5'–3' untranslated regions, promoter, enhancer/silencer, coding sequences (exons, which specify a sequence of amino acids), noncoding sequences (introns, which do not specify amino acids) and regulatory sequences that play a role in determining when and where the protein is produced (transcriptional and translational start and stop codon sites). Transcription factors and mediator proteins complex 'turn on' the gene and help RNA polymerase to read the gene.

up a little and introns are excised, then mature mRNAs leave the nucleus via nuclear pore and enter the cytoplasm resulting in production of desired protein with the help of transfer RNAs (tRNAs) and ribosomal RNA (rRNA) unit.

GENE EXPRESSION: DNA REPLICATION, TRANSCRIPTION AND TRANSLATION

Histone octamer core proteins play an important role in the maintenance of gene expression. On contrary, nonhistone proteins play a key role in regulation of gene expression, which involves DNA replication, transcription and translation. The Central Dogma of Molecular Biology states that genetic information flows only in one direction from DNA→RNA→protein, i.e. polypeptide (chain of polypeptide). Gene expression involves three processes: DNA replication, transcription and translation. Gene expression involving DNA replication, transcription and translation is shown in Figs 6.3 and 6.4.

DNA Replication

DNA replication is the process by which the genome's double-stranded molecule of DNA is copied with its own complete identical helices of DNA in the cell before cell division through mitosis or meiosis. DNA must be replicated in order to ensure that each new daughter cell receives the correct number of chromosomes. Enzymes that participate in DNA replication process include DNA helicase, DNA primase, DNA polymerases, topoisomerase (DNA gyrase), exonuclease and DNA ligase.

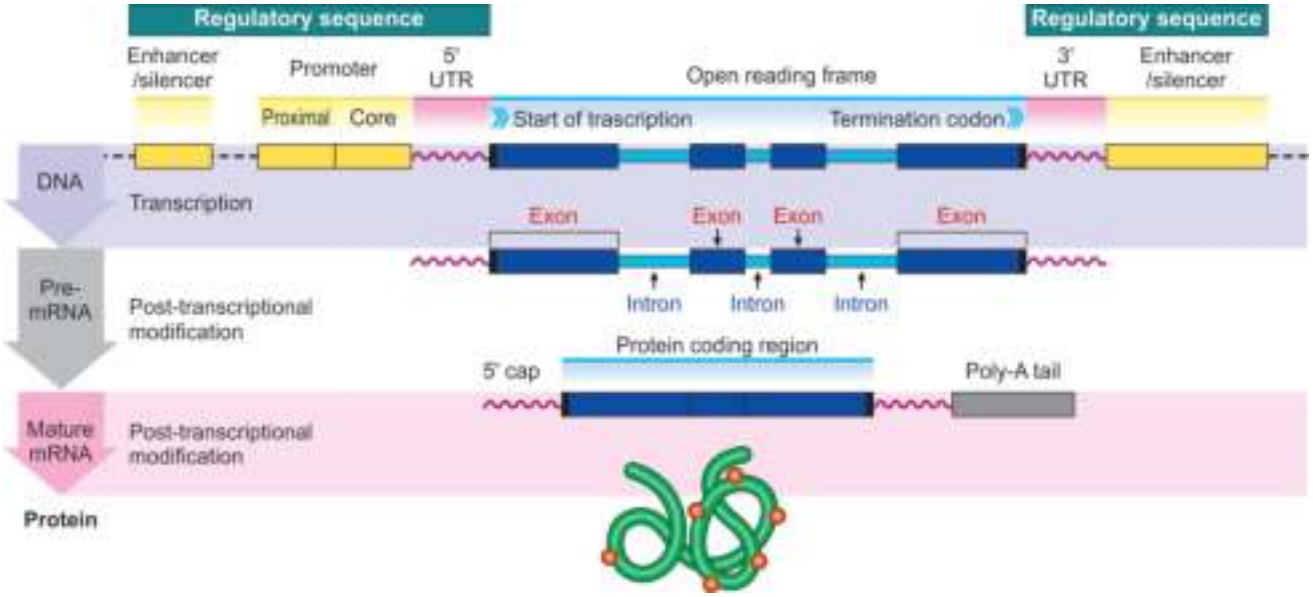
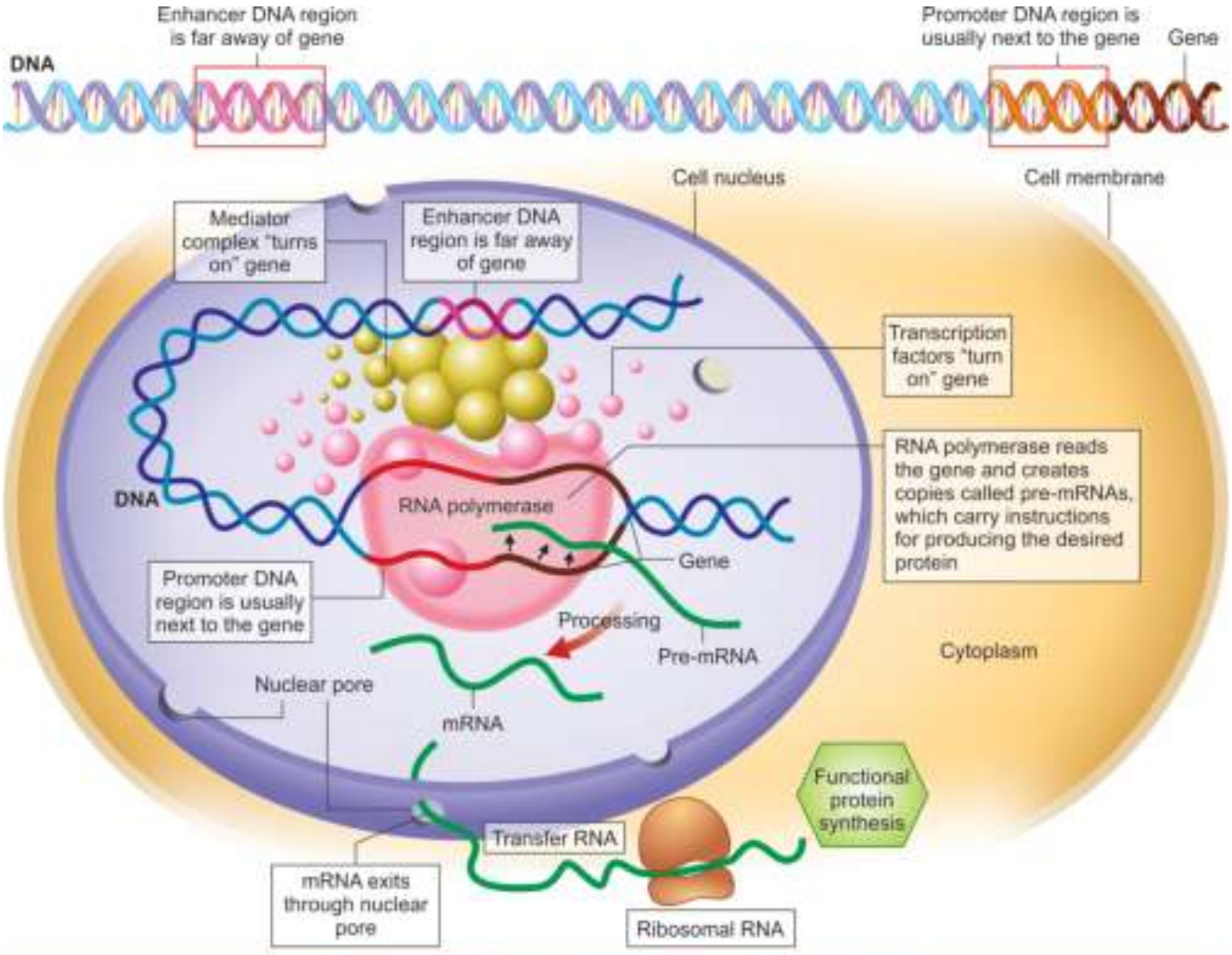
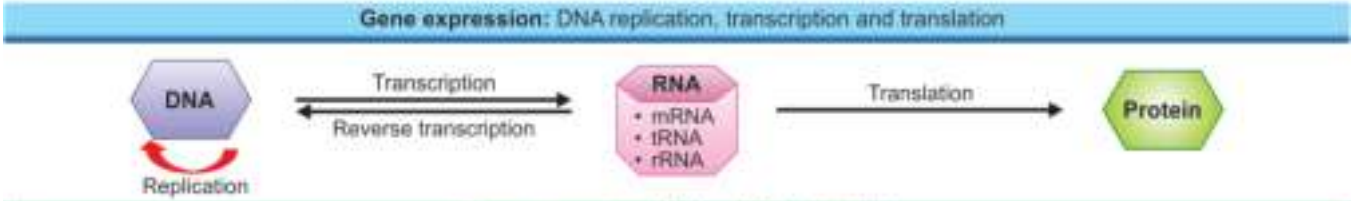


Fig. 6.3: Gene expression involving DNA replication, transcription and translation. Genetic information is transcribed from DNA into primary RNA transcript (pe-messenger RNA) that becomes a messenger RNA (mRNA) after processing, that can be translated into the proteins, noncoding regions (introns) must be removed and protein-coding regions (exons) joined by RNA splicing to produce mature messenger RNA products (mRNA, tRNA and rRNA).





Gene expression (DNA→RNA→Protein)	
• DNA replication	DNA→DNA copy occurs in nucleus.
• Transcription	DNA→mRNA occurs in nucleus, then mRNA moves on to ribosomes into the cytoplasm for translation.
• Translation	Protein synthesis occurs in cytoplasm (mRNA transcript is decoded into protein with the help of a ribosome and tRNA).
Human genome contains around 30,000 genes, each gene codes for one protein.	

Fig. 6.4: Gene expression involving DNA replication, transcription and translation. Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product such as protein. Transcription is the process of making messenger RNA (mRNA) from a DNA template by RNA polymerase. Transcription factor is a protein that binds to DNA and regulates gene expression by promoting or suppressing transcription. Transcription factor and mediator proteins complex 'turn on' the gene and help RNA polymerase to read the gene.

Transcription

Transcription is the process of making messenger RNA (mRNA) from a deoxyribonucleic acid (DNA) template strand by RNA polymerase. Transcription process yields a primary RNA transcript that gets a cap added to the 5′ end and undergoes polyadenylation and splicing to produce messenger RNA (mRNA).

- Transcription factor is a protein that binds to DNA and regulates gene expression by promoting or suppressing transcription. Transcription factor and

mediator proteins complex 'turn on gene' and help RNA polymerase to read the gene.

- Transcription is regulated by controlling the rate of gene expression by helping or hindering RNA polymerase binding to DNA. Increase in the rate of gene expression is termed upregulation, activation or promotion. Decrease in rate of gene expression is termed downregulation, repression or suppression. Coactivator is a protein that works with transcription factors to increase the rate of gene expression. Corepressor is protein that works with transcription factors to decrease the rate of gene expression.

Translation

Following transport of mRNA to the cytoplasm, mature messenger RNA (mRNA) is translated in the cytosol to specific amino acid sequence of polypeptide molecule onto ribosome. Each set of three nucleotide base pairs is translated into specific sequence of single amino acid in a polypeptide chain. Like DNA transcription, translation has three stages: initiation, elongation and termination.

- **Primary RNA transcript splicing and messenger RNA formation:** A primary transcript is the single-stranded ribonucleic acid (RNA) protein product synthesized by transcription of DNA, and processed to yield various mature RNA products such as messenger RNA (mRNA), transfer RNA (tRNAs) and ribosomal RNA (rRNA).
- **Protein synthesis:** Specifically, messenger RNA (mRNA) copies the information in a gene in DNA. The messenger RNA (mRNA) moves out of the cell nucleus and into the ribosomal machinery called ribosome located in cell's cytoplasm, and the information is used to link together small molecules called amino acids in the right order to make a specific protein (e.g. immunoglobulins, enzymes, messenger proteins, structural component proteins and transport/storage proteins). Depending on their size and the sequence of amino acids, proteins can fold or coil into certain shapes, which then perform nearly all cellular functions.

FOUR CLASSES OF NORMAL CELLULAR REGULATORY GENES

Four classes of normal cellular regulatory genes include growth-promoting proto-oncogenes, growth-inhibiting tumor suppressor genes, genes involved in programmed cell death (apoptosis) and DNA repair genes, which are the principal targets of mutations causing malignant phenotype. Broad categories of genes are given in Table 6.1. Molecular carcinogenesis is shown in Fig. 6.5.

Table 6.1 Broad categories of genes

Proto-oncogenes

- HER2/neu (ERBB2) proto-oncogene
- RAS family proto-oncogenes
- Myc proto-oncogene
- SRC family of receptor tyrosine kinase proto-oncogenes
- hTERT gene (encodes telomerase reverse transcriptase)
- BCL-2 (B cell lymphoma) proto-oncogene
- EGFR proto-oncogene
- CCND1 proto-oncogene (encodes cyclin D protein)

Tumor Suppressor Genes

- Tumor suppressor caretaker genes
  - BRCA1 and BRCA2 genes
  - DNA mismatch repair genes (MLH1, MSH2, MSH3, MSH6, PMS1, PMS2)
  - Fanconi anemia DNA repair genes (FANCA, FANCB, FANCC, FANCD-1, FANCD-2, FANCE, FANCF, and FANCG)
  - DNA nucleotide excision repair genes (XPA, XPB, XPC, XPD, XPE, XPF, XPG, and XPV)
- Tumor suppressor gatekeeper genes
  - TP53, RB1, APC, BRCA, and APC/β-catenin genes
- Tumor suppressor genes function to inhibit Wnt/β-catenin mitogenic signaling activator pathway
  - APC, NF1, NF2, PTCH, SMAD2, SMAD4, and PTEN genes
- Tumor suppressor genes function to inhibit cell cycle progression
  - CDKN2A, and RB genes
- Tumor suppressor genes function to inhibit angiogenesis
  - vHL (von Hippel-Lindau), SDHA, SDHB, SDHC, SDHD, and STK11 genes
- Tumor suppressor gene functions as genomic stability enabler (most important)
  - TP53 gene
- Tumor suppressor genes function by various mechanisms
  - WT1, MEN1/SMAD4/DPC4, ATM, CHEK2, TGF-β, and p16 genes

DNA repair genes function to repair damaged DNA

- DNA mismatch repair genes (MLH1, MSH2, MSH3, MSH6, PMS1, and PMS2)
- Fanconi anemia DNA repair gene (FANCA, FANCB, FANCC, FANCD-1, FANCD-2, FANCE, FANCF, and FANCG)
- DNA nucleotide excision repair genes (XPA, XPB, XPC, XPD, XPE, XPF, XPG, and XPV)

Apoptosis regulatory genes function to inhibit cell cycle and promote apoptosis

- TP53, BAX, and c-Myc pro-apoptotic genes
- BCL-2 anti-apoptotic gene

- **Proto-oncogenes** are normal genes coding for a number of proteins, which stimulate cell division during G0 to G1 phase of cell cycle. Tumor suppressor genes are normal genes, which inhibit cell division during G1 phase of cell cycle. Apoptosis regulatory

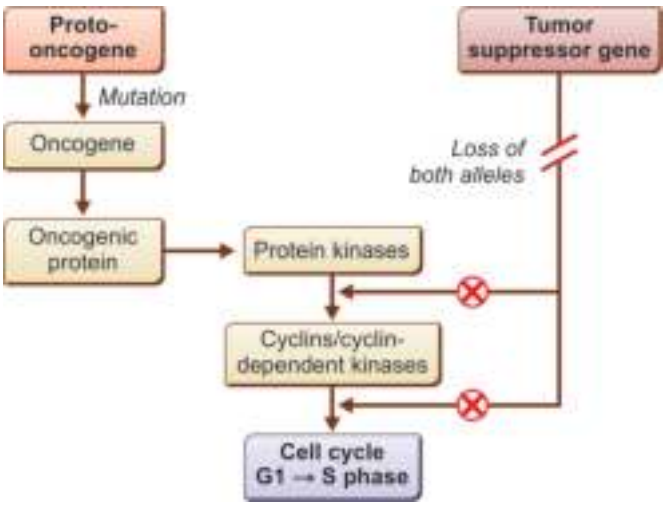


Fig. 6.5: Molecular carcinogenesis. Neoplasia can either results from mutations that 'turn on' the oncogenes or both alleles loss of tumor suppressor gene. Gain-of-function ('dominant') mutations occur in proto-oncogenes, which become activated and called oncogenes. Loss-of-function ('recessive') mutations in tumor suppressor gene leads to inactivation, or both alleles loss resulting in loss-of-function of the decoded protein.

genes eliminate damaged cells by activation of cysteine caspases and maintain tissue homeostasis, which include TP53 tumor suppressor gene, anti-apoptotic BCL-2 gene and the pro-apoptotic gene BAX. Normal wild type TP53 gene can limit cell proliferation after DNA damage by two mechanisms: (a) arresting cell division and (b) activating apoptosis. DNA mismatch repair is regulated by DNA repair genes, which is a highly conserved biological pathway that plays a key role in maintaining genome stability.

- Normal controls are defective in cancer stem cells, so that the balance between factors stimulating and inhibiting cell growth is permanently lost resulting in uncontrolled cell division. In the cancer stem cells, proto-oncogenes are permanently mutated to become oncogenes, which cause uncontrolled cell proliferation.
- Activity of the oncogenes is complex and is activated by many ways, i.e. gene amplification (Myc gene in neuroblastoma or increased mRNA transcription due to chromosomal translocation 8 and 14 in Burkitt lymphoma). Activated oncogenes can either produce excess of proteins or abnormal functional proteins. Only one copy a given oncogene needs to be activated to cause uncontrolled cell proliferation. On contrary, when tumor suppressor gene is mutated due to inactivation/biallelic loss that results in loss or reduction in its function, thus normal cell can progress to cancer stem cell (CSC), usually in combination with other genetic changes. Characteristics of oncogenes (mutated form of proto-oncogenes) and tumor suppressor gene mutation are given in Table 6.2.

PROTO-ONCOGENES, ONCOGENES AND VIRAL ONCOGENES

Proto-oncogenes

Proto-oncogenes are normal growth-promoting genes. Each proto-oncogene encodes specific growth factor, growth factor receptor and intracellular regulatory proteins responsible for providing positive signals leading to cell growth, cell division, apoptosis and maintenance of normal cell. In general, extracellular growth factor binds to growth factor receptor, which activates intracellular downstream signal transducers. The signaling pathway initiates DNA transcription of genes involved in cell growth, which involves binding of transcription factors to DNA regulatory proteins and recruiting chromatin remodelers to carry out gene transcription.

Oncogenes

Oncogenes are mutated forms of proto-oncogenes (normal cellular genes), which occur as a result of point mutation, gene amplification, balanced

Table 6.2 Characteristics of oncogenes (mutated form of proto-oncogenes) and tumor suppressor gene mutation

Characteristics	Oncogenes	Tumor Suppressor Gene Mutation
Number of gene mutation events required to contribute to tumorigenesis	One allele loss	Two alleles loss
Function of the mutant allele	Dominant (gain-of-function)	Recessive (loss-of-function)
Activity demonstrated in gene transfer assays	Yes	Yes
Associated with hereditary syndromes (inheritance of germline mutations)	Seldom (c-RET proto-oncogene)	Often
Somatic gene mutations contribute to cancer development	Yes	Yes
Tissue specificity of mutational event	Somatic mutation in specific tissue cell	In inherited cases, there is often a tissue preference

chromosome translocation (without genetic loss), deletion, overexpression, chromosomal rearrangement and insertional mutagenesis. Balanced chromosome translocations contribute to molecular carcinogenesis due to overexpression of oncogenes or generation of novel fusion-proteins with altered functions and generally involved in promoting unrestricted cell growth and cell division.

- Oncoproteins are the products of oncogenes, which are formed when a gene is transcribed and translated to RNA and manufacturing the proteins. Oncogene gets permanently 'turned on', which is known as 'gain-of-function', i.e. dominant gene mutation. This means that only one copy of proto-oncogene needs to be mutated in order to induce carcinogenesis. In general, most soft tissue sarcomas and blood cancers are initiated by activation of an oncogene.
- Genomic sequencing has demonstrated numerous small deletions and insertions, as well as chromothripsis (chromosomal shattering), in which a chromosome is shattered and then reassembled in a disorganized way.

Viral Oncogenes

Viral oncogenes are responsible for oncogenesis resulting from persistent virus infection when integrated into host genome. Virus-encoded oncoproteins that activate growth factor receptors can also induce human cancers.

TUMOR SUPPRESSOR GENES

A tumor suppressor gene encodes gene product involved in cell cycle arrest and prevents carcinogenesis. Tumor suppressor genes regulate diverse cellular processes, i.e. cell cycle checkpoint responses, detection and repair of DNA damage, protein ubiquitination and degradation, mitogenic signaling, cell specification, cell differentiation and migration and tumor angiogenesis.

- Caretaker tumor suppressor genes encode proteins that engage in maintaining the genome stability and DNA repair thus prevent cancer. Examples of caretaker tumor suppressor genes are BRCA1, BRCA2, DNA mismatch repair (MLH1, MSH2, MSH3, MSH6, PMS1, PMS2), Fanconi anemia DNA repair, and DNA nucleotide base pairs excision repair genes. In contrast, gatekeeper tumor suppressor genes regulate cell cycle.
- Gatekeeper tumor suppressor genes encode proteins, which either stimulate cell proliferation and differentiation or apoptosis. Loss-of-function of gatekeeper tumor suppressor genes allows enhanced cell proliferation and transmission of mutated genes. Mutation in tumor suppressor genes results in loss-of-function (recessive gene mutation). Examples of gatekeeper tumor suppressor gene include TP53, RB1, APC, BRCA, APC/β-catenin genes. TP53 gene has been called the 'guardian of genome'. TP53 gene mutations have been demonstrated in many human cancers. Germline mutation of TP53 gene is a feature of Li-Fraumeni syndrome. Dysregulation of tumor suppressor genes is linked to carcinogenesis.
- Mitogenic signaling tumor suppressor genes encode protein, that engage in inhibition of mitogenic signaling. Examples of mitogenic signaling inhibitor genes include APC, NF1, NF2, PTCH, SMAD2, SMAD4, and PTEN. Mitogenic signaling normally activates RAS, GTPase, that then activates the rest of the MAPK signaling pathways, ultimately expressing proteins that stimulate cell cycle progression. It is likely that most cancers have some mutation in the RAS/RAF/mitogen-activated protein kinase (MAPK) signaling pathway most commonly in RAS.
- Cell cycle progression tumor suppressor genes encode proteins that inhibit cell cycle progression. Examples of cell cycle progression tumor suppressor genes include CDKN2A and RB.
- Tumor angiogenesis and tumor suppressor genes encode proteins that inhibit tumor angiogenesis. Examples of tumor angiogenesis and tumor suppressor genes include vHL (von Hippel-Lindau), SDHA, SDHB, SDHC, SDHD, and STK11. Tumor angiogenesis is the formation of new blood vessels that tumors need to grow. Angiogenesis is caused by the release of angiogenic factors by the tumor and host cells near tumor microenvironment.
- Genomic stability enabler TP53 tumor suppressor gene encodes p53 protein, that acts as guardian of the genome. TP53 gene is the most frequently mutated in >50% of human cancers.
- Tumor suppressor genes function by various mechanisms include WT1, MEN1, SMAD4/DPC4, ATM, CHEK2, TGF-β, and p16.

Pathology Pearls: Li-Fraumeni Syndrome (TP53 Gene Mutation) Linked to Human Cancers

- TP53 tumor suppressor gene mapped on chromosome 17 is the guardian of the genome, which normally serves to arrest the cell cycle and induce apoptosis under condition of DNA damage. When TP53 gene undergoes mutation, apoptosis function is lost and cells with DNA damage will continue to divide and proliferate, possibly acquiring mutations which confer malignant behavior.
- Germline mutation in TP53 gene (aberrant p53) in Li-Fraumeni syndrome is linked to many cancers (i.e. breast carcinoma, osteosarcoma, soft tissue sarcomas, brain tumors, acute leukemia and adrenocortical carcinoma) including cancers of gastrointestinal tract, lung, kidney, thyroid gland, skin, ovary,

testes and prostate. It is important to note that not every person with mutation in TP53 gene will necessarily develop cancer, but the risks are substantially higher than the general population.

- Li-Fraumeni syndrome involves TP53 tumor suppressor gene, with the 'Knudson' two-hit hypothesis. A person inherits mutated one allele of TP53 gene (**first hit**), but still has another functional allele of TP53 gene. Loss of both alleles of TP53 gene (second hit) leads to cell growth control loss, allowing clonal expansion of neoplastic cell.
- Familial cancers often involve more than one organ in patients with Li-Fraumeni syndrome, which include breast carcinoma, osteosarcoma and soft tissue sarcomas. Female at birth, who has Li-Fraumeni syndrome has 100% chance of developing breast cancer.
- Affected persons with germline mutation in TP53 tumor suppressor gene can develop cancers in multiple organs. However, it must be noted that TP53 gene mutations are most commonly observed in sporadic cancers. A primary sporadic cancer may be treated by surgery, radiation and chemotherapy.
- Diagnosis of Li-Fraumeni syndrome depends on clinical manifestations and/or genetic testing for mutation in the TP53 gene. Molecular genetic testing is performed for early detection of cancer in these patients.

APOPTOSIS REGULATORY GENES

Apoptosis regulatory genes are involved in extrinsic death receptor pathway and intrinsic mitochondrial pathway. Apoptosis is programed cell death mechanism characterized by nuclear condensation, cell shrinkage, membrane blebbing, DNA fragmentation, formation and phagocytosis of apoptotic bodies. Caspases, a family of cysteine proteases, are the central regulators of apoptosis. For example, BCL-2 oncogene produces aberrant protein associated with the cell membrane that prevents programed cell death (apoptosis). Oncogenes may also synthesize oncoproteins that reduce apoptosis leading to survival of the cells. Dysregulation of apoptosis regulatory genes evades apoptosis and survival of cancer stem cells leading to malignant phenotype.

DNA REPAIR GENES

During DNA repair, DNA glycosylases remove the damaged nucleotide base pairs by cutting them out of the DNA strand through the cleavage of the covalent bonds between the nucleotide base pairs and the sugar phosphate backbone. The resulting gap is then filled by a specialized DNA repair polymerase and sealed by ligase. XPA is DNA repair protein that initiates recognizing damaged DNA and forming complexes with other proteins such as XPB and XPD involved in DNA repair process. XPB and XPD proteins act as helicases that unwind the damaged DNA. Dysregulation of DNA repair genes is linked to carcinogenesis.

MOLECULAR/CYTOGENETIC ALTERATIONS IN HUMAN CANCERS

Recently, human cancers may be viewed as a genetic disease with various chromosomal and nucleotide base pair aberrations, which include chromosomal translocations, gene mutations, gene deletions, gene amplifications, gene arrangements during the transformation of a normal cell to cancer stem cell (CSC).

- Most forms of localized DNA damage occur by various mechanisms: (a) single-stranded DNA breaks, (b) double-stranded DNA breaks, (c) DNA adducts, (d) nucleotide base pair insertions and deletions, and (e) mismatch of nucleotide base pairs.
- Single-stranded DNA break is repaired by nucleotide base pair excision mechanism. Double-stranded DNA break is repaired by non-homologous end-joining (NHEJ) or homologous recombination (HR). DNA adduct is repaired by nucleotide base pair excision.
- Failure to DNA repair system results in xeroderma pigmentosum, an autosomal recessive disorder linked to skin cancers. Mismatch nucleotide base pair is repaired by insertion of correct nucleotide base pair. Failure to DNA repair is linked to development of colorectal carcinoma, pancreatic carcinoma, breast carcinoma and ovarian carcinoma.

GENOMIC INSTABILITY LINKED TO HUMAN CANCERS

Genetics is the study of heredity (biological process) whereby parents pass certain genes onto their children. Every child inherits genes from both of his/her biological parents, and in turn, child expresses specific traits.

- Genes are present within the chromosomes, which carry instructions to make proteins involved in regulation of cellular functions especially how cells grow and divide. Mutation in gene can affect how it functions. Gene mutations can be present either acquired in somatic tissues or inherited in germ cells (egg or sperm).
- Most human cancers are caused by somatic (acquired) gene mutations in 75–80% cases, because there is no identified germline gene mutation involved. On contrary, inherited cancers account for 20–25% of cancers due to germline gene mutations. Many of the targeted therapies used to treat human cancers are designated to address changes in cell growth caused by particular gene mutations.
- Carcinogenesis is an evolutionary process whereby cells accumulate numerous permanent gene mutations in the nucleotide base pair sequences. Genomic instability and high rates of gene mutation cause

malignant tumor to acquire additional numerous mutations and chromosomal alterations during its evolution, most are termed 'passenger mutations' because they do not confer malignant phenotypes. Driver gene mutations cause transformation of normal cells to cancer stem cells (CSCs) and subsequent clonal expansion and progression to malignant tumor growth.

Pathology Pearls: Terminology of Gene Alleles and Mutations

- **Driver gene mutation:** Genetic alteration in driver gene that facilitates tumorigenesis and survival.
- **Passenger gene mutation:** Genetic alteration in passenger gene that is not essential for tumorigenesis.
- **Gene mutation with gain-of-function:** Gene mutation that increases a gene aberrant product's activity or results in a new function. Dominant gene mutations act to oppose normal gene function.
- **Gene mutation with loss-of-function:** Gene mutation that decreases the gene product's function. Loss-of-function gene mutation partially disrupts normal function.
- **Haploinsufficiency:** Haploinsufficiency occurs when one functional copy (allele) of a gene is inactivated or deleted and the remaining **wild-type copy (allele)** of the gene is not sufficient to produce the needed gene product to preserve normal function.
- **Gatekeeper tumor suppressor genes:** Gatekeeper tumor suppressor genes encode protein products that restrain cell growth and their loss-of-function allows enhanced cell proliferation and transmission of gene mutations.
- **Caretaker tumor suppressor genes:** Caretaker tumor suppressor genes encode protein products that maintain genomic stability involved in deoxyribose nucleic acid (DNA) repair.
- **Landscape genes:** Landscape gene mutations encode aberrant products that contribute to the abnormal neoplastic growth of cells by fostering a microenvironment conductive to unregulated cell proliferation.
- **Gradualism:** The gradual and stepwise accumulation of tumorigenic gene mutations occur overtime.
- **Chromothripsis:** The nearby simultaneous acquisition of multiple gene mutations in a tumor via catastrophic shattering and then reassembly of chromosomes.
- **Genetic modifiers:** Genetic modifiers are defined when the effects of one gene and modified by single or multiple genes.
- **Gene interactions:** The gene interactions can be defined as genetic enhancers or suppressors, dominant/recessive (interaction between alleles of the same gene), or epistatic/hypostatic (phenotype suppression).

Somatic (Acquired) Gene Mutations Linked to Cancers

Somatic (acquired) gene mutations occur in the particular parent cells after birth and cannot be transmitted to the offspring. Such persons lack family history of cancer or inherited changes in their DNA, that would increase risk for developing that cancer. Somatic gene mutations are much more common than germline mutations.

- **Causes of somatic gene mutations:** Somatic gene mutations can be induced by environmental mutagens (chemical agents, ultraviolet radiation, ionizing radiation and biologic agents) encountered throughout life in somatic cells leading to uncontrolled cell proliferation and development of cancers (e.g. breast, ovary, lung, colon, kidney, urinary bladder and prostate gland) in 90% of cases. Intercalating agents insert into the DNA molecule and cause single-nucleotide insertions and deletions. Oxidative reactions alter the chemical structures of nucleotide base pairs. Ionizing radiation alters nucleotide base pairs and breaks phosphodiester bonds. Ultraviolet radiation induces production of pyrimidine dimers, which cause distortion in the double helix DNA molecule resulting in inhibition of DNA replication and transcription.
  - Many of the mutated genes that contribute to development of cancer fall into three broad categories: oncogenes (RAS family of genes, HER2/neu, cyclin D1, cyclin E, β-catenin), tumor suppressor genes (BRCA1, BRCA2, TP53, RB), and DNA repair genes. Mutations in the growth promoting genes have usually autosomal dominant inheritance, because a mutation in a single copy of the proto-oncogene is usually sufficient to produce stimulatory effect. Dominant acting growth promoting genes that cause cancer are termed oncogenes.
  - Targeted therapies are now available for many gene mutations found in human cancers that can control the growth of cancer at least for a period of time. Few examples of genomic changes in cancer include: (a) EGFR mutations, ALK rearrangements, ROS1 rearrangements, MET and RET mutations in lung cancer, and (b) BRAF mutations in melanoma and some lung cancers.
- **Mechanisms of somatic gene mutations:** Gene mutations most often occurs by substitutions, in which a single nucleotide base is changed into different nucleotide base. Other gene mutations result in the loss (deletion) or addition (insertion) of one/more nucleotide base pairs. Nucleotide substitutions occur by two mechanisms: transition or transversion. In transposition, a purine nucleotide is replaced with a purine nucleotide, or a pyrimidine nucleotide is replaced with a pyrimidine nucleotide of the same class. In transversion, there is change in the class of the nucleotides, i.e. a purine nucleotide is replaced with a pyrimidine nucleotide, or pyrimidine nucleotide is replaced with a purine nucleotide.

- **Consequence of somatic gene mutations:** Gene mutation results in abnormal transcribed DNA sequence (mRNA), abnormal translated proteins and lethal phenotype due to mutated RNA and protein. Mutations range in size, which can affect anywhere from a single DNA nucleotide base pair to a large segment of a chromosome that includes multiple genes. Gene mutations alter the function of essential proteins, which adversely affect the health of individuals. Gene mutations occur by various mechanisms, which include nonsense, missense, insertion, deletion, duplication, frameshift mutations and trinucleotide repeat expansion.
- **Somatic gene mutations and cancer:** Cancer is an abnormal growth of cells, which tend to proliferate in an uncontrolled manner, that can disseminate via lymphatic route to draining lymph nodes, via hematogenous route to distant organ(s) and via transcoelomic route to peritoneum. During process of carcinogenesis, pre-malignant cells accumulate genetic mutations until a fully malignant phenotype develops. Although, cancer has a genetic basis, yet it is not necessarily hereditary. Most human cancers arise from somatic gene mutations instead of inherited (germline) gene mutations. Two main groups of genes are implicated in development of cancer: activation of oncogenes and inactivation/biallelic loss of tumor suppressor genes.

Germline (Inherited) Gene Mutations Linked to Cancers

Germline gene mutations are present at birth and exist in all cells of the body. It is not certain that all cases with germline gene mutations develop cancer. Family members develop site-specific cancers in more than one site. Germline gene mutations related human cancers may have either autosomal dominant inheritance with mutation in proto-oncogenes or autosomal recessive inheritance with mutation in tumor suppressor genes. Germline gene mutations in BRCA1/BRCA2 tumor suppressor genes increase the risk of breast cancer and ovarian cancer in women. Germline mutation in TP53 gene (aberrant p53) in **Li-Fraumeni syndrome** is linked to many cancers. Screening of family members is recommended at a younger by genetic testing on blood or saliva to detect germline gene mutations. Survivors of inherited cancer syndrome have limited treatment options except follow-up care.

Familial Cancer Syndromes

Familial cancer syndromes are inherited susceptibility to malignancy, typically involving a mutation in either an oncogene (autosomal dominant) or tumor suppressor gene (autosomal recessive). Most inherited cancer syndromes follow autosomal-dominant inheritance, in which the patient's first-degree relatives (parents, children and siblings) have a 50% risk of carrying the causative gene mutation themselves. Extensive clinical history of screening of early development of cancers is required in the management of these patients developing colorectal cancer, breast carcinoma, Wilms' tumor, or renal cell carcinoma. Molecular genetic testing is done to detect inherited cancers-causing gene in cancer stem cells as well as tissues of family members.

- **Heterozygous inheritance:** In these cases, one faulty allele has to be present for an individual to have a predisposition to cancer. Individuals with one normal allele and one faulty allele are known as heterozygous inheritance. The detrimental effects of recessive cancer gene are less common within population as compared to their dominant cancer gene counterparts. Familial adenomatous polyposis coli is an example of familial cancer syndrome.
- **Homozygous inheritance:** Homozygous inheritance of these recessive cancer genes does not cause cancer directly, but rather, the loss-of-functional protein that they encode results in genomic instability, which provides an ideal environment for oncogenic mutations and predisposes the affected person to cancer.

EPIGENETIC ALTERATIONS

Epigenetic alterations are commonly demonstrated in human cancers. Epigenetic alterations are genetic modifications that impact gene activity such as 'turning on'/'turning off' without changing the nucleotide base sequences of DNA building blocks. A common type of epigenetic alteration is called DNA methylation that involves the attachment of methyl groups to DNA building blocks. When methyl groups are present on a gene, that gene is turned off or silenced, and no protein is produced from that gene.

- Another common epigenetic change is 'histone octamer modification'. Histones are structural proteins in the cell nucleus. Deoxyribonucleic acid (DNA) wraps around histones, giving chromosomes and their shape. Histones can be modified by the addition or removal of chemical methyl groups or acetyl groups, which determine how tightly DNA is wrapped around histone octamer proteins and affect whether a gene can be 'turned on' or 'turned off'.
- Errors in the epigenetic process, such as modification of the wrong gene or failure to add a chemical group to a particular gene or histone octamer proteins, can lead to altered gene activity or inactivity. Malignant tumors often have hypomethylation of the cell genome (increases gene expression and overall, for

increased metabolic activity) with hypermethylation of the cell genome (silences tumor suppressor genes that control cell growth).

CANCER STEM CELL PROPERTIES

Cancer stem cells (CSCs) are relatively/absolutely autonomous, and monoclonal in nature, which undergo uncontrolled proliferation in disorganized manner, and become unresponsive to extracellular growth factors and regulatory mechanisms operating inside normal cells/tissues.

- Cancer stem cells proliferate, invade surrounding tissues, and disseminate via lymphatic route to lymph nodes, via hematogenous route to distant organ(s), and via transcoelomic route to peritoneum. Cancer stem cells synthesize angiogenic factors, and growth factors, which promote angiogenesis and tumor growth. Cancer stem cells have ability to escape immune surveillance resulting in development of clinical cancers.
- Proprieties of transformed cancer stem cells (CSCs) include: (a) loss of cell-to-cell contact (continue to grow over one other, piling up into dense aggregates), (b) altered cell morphology (rounded shape, refractile in phase contrast microscope), (c) reduced requirement for mitogenic growth factors by cancer stem cells, (d) increased transport of glucose in the CSCs, and (e) inability to halt cell proliferative response to deprivation of growth factors. Transformation of normal cell to cancer stem cell is associated with genetic instability, immortalization, aberrant growth control and tumorigenicity. General characteristics of transformed cancer stem cells are given in Table 6.3.

Pathology Pearls: Anchor-independent Cancer Stem Cell Growth on Culture Dish

- Each of cancer stem cell large colonies may contain several hundred cells in anchor-independent growth.
- Ability to grow without attachment to solid substrate (anchorage independence) is analyzed by suspending cells in semi-solid medium such as agarose or methylcellulose, to prevent their attachment to a solid substrate, specifically, the bottom of the Petri dish.
- The ability of cancer stem cells to proliferate while held in suspension, the phenotype of anchor independence is usually a good predictor of their ability to form tumors *in vivo*.

Table 6.3 General characteristics of transformed cancer stem cells

Genetic Characteristics
▪ Aneuploidy (abnormal number of chromosomes) ▪ Heteroploidy ▪ Increased spontaneous gene mutation ▪ Mutated proto-oncogenes (i.e. oncogenes get 'turned on' hence autosomal dominant inheritance) ▪ Inactivation or deletion of both copies of tumor suppressor genes (which get 'turned off', hence results in autosomal recessive inheritance disorder)
Structural Changes
▪ Cytoskeleton protein alterations in cancer stem cells ▪ Extracellular matrix (ECM) alterations ▪ Cell adhesion molecules (CAMs) alterations ▪ Cancer stem cell polarity disruption
Growth Characteristics
▪ Ability of cancer stem cells to proliferate indefinitely (cell immortalization) ▪ Loss of contact inhibition among cancer stem cells (ability to grow over another) ▪ Ability of cancer stem cells to grow without attachment to solid substrate (anchorage independence growth—good predictor of their ability to form tumors *in vivo*) ▪ Density limitation of growth reduction ▪ Reduced requirement of mitogenic growth factors of cancer stem cells (growth factor-independent) ▪ Shortening of doubling time of cancer stem cells population ▪ Increased transport of glucose in cancer stem cells ▪ High saturation of density (ability to accumulate large number of cancer stem cells in culture dish)
Neoplastic Characteristics
▪ Tumorigenesis ▪ Tumor angiogenesis ▪ Increased protease secretion by cancer stem cells, and causing degradation of extracellular matrix (ECM) ▪ Invasion of cancer stem cells in the surrounding tissue ▪ Metastasis of cancer stem cells to distant organ(s)

HISTOGENESIS OF NEOPLASMS

Histogenesis is a method of classifying neoplasms on the basis of the tissue cell of origin. Most human organs (except for nervous system) are composed of epithelial or connective tissue cells or both. Benign tumors refer to those tumors incapable of metastasis associated with good prognosis. Borderline tumors of low-malignant potential lack stromal invasion. Malignant tumors are capable of invasion and/or metastasis, often fatal if not treated effectively. Metastasis refers to spread of a malignant tumor from one site to another site via lymphatic, hematogenous and transcoelomic routes.

INTRAEPITHELIAL NEOPLASIA

Intraepithelial neoplasia represents an intermediate stage in the production of malignant tumor. All the cytomorphological features are present such as disordered squamous epithelial growth marked by

the loss of polarity and presence of nuclear hyperchromasia, but the cells have not evaded the surrounding tissues.

- Intraepithelial neoplasia is frequently observed in the cervix uteri at the junction of ectocervix and endocervix.
- The concept of progressive premalignant cell proliferation applies in other organs such as breast, stomach, colorectal region, esophagus, bronchus, prostate, urinary bladder, oral cavity and vulva.
- Low-grade intraepithelial neoplasia does not involve whole thickness of epithelium, which is reversible if the irritant is removed. On the other hand, severe dysplasia/carcinoma *in situ* refers to epithelial malignancies confined to the epithelium without invasion through the basement membrane. Comparison of dysplasia and anaplasia is given in Table 6.4.

Cervical Intraepithelial Neoplasia

Cervical intraepithelial neoplasia (CIN) is graded as CIN I (mild dysplasia, involving lower third of epithelium and usually reversible), CIN II (moderate dysplasia, involving up to middle third of epithelium and usually reversible) and CIN III (severe dysplasia, involving entire thickness of epithelium up to surface layer). Carcinoma *in situ* in cervical epithelium is synonymous with CIN III and involves severe dysplastic changes extending through the entire thickness of the epithelium up to surface layer but not invading the basement membrane, i.e. carcinoma *in situ*, which shares similar molecular and genetic alterations with squamous cell carcinoma. Increased expression of TP53 is demonstrated in moderate and severe dysplasia (carcinoma *in situ*).

- **Pathophysiology:** Squamous cell carcinoma of cervix arises from the ectocervix, and adenocarcinoma originates from endocervix. Epidemiologic risk factors for cervical carcinoma are early sexual activity, multiple sexual partners and cigarette smoking.
  - Human papillomavirus (HPV) 16, 18, 31 and 33 is frequently associated with cervical squamous cell carcinoma. HPV-DNA sequences are often integrated into the genome of dysplastic or malignant cervical epithelial cells.
  - HPV viral E6 protein binds and inactivates the gene product of TP53 tumor suppressor gene, and HPV viral E7 protein binds and inactivates the gene product of RB tumor suppressor gene, thus allowing the cervical epithelial cells to accumulate and causes DNA damage leading to cell cycle progression.
  - Extracellular HPV viral E7 protein affects endothelial cells by increasing production of IL-6 and IL-8, promoting progression to invasive cervical carcinoma. Development of cervical intraepithelial neoplasia (CIN I, CIN II, CIN III) and invasive cervical carcinoma is shown in Fig. 6.6.
- **Diagnostics:** Gold standard to evaluate of cervical intraepithelial neoplasia, i.e. dysplasia is still on routine histologic examination. Thus, there is a natural history from metaplasia-to-dysplasia-to-invasive carcinoma. This is best evidenced in development of the uterine cervix and respiratory tract neoplasms.

DYSPLASIA IN MESENCHYMAL TISSUES

Osteofibrous dysplasia of bone is non-inherited developmental disorder and characterized by fibrous stroma with trabeculae of immature woven bone reflecting defective bone maturation as a result of post-zygotic mutation in GNAS1 gene, which encodes G protein-coupled receptor activating adenyl cyclase resulting in excessive production of adenosine monophosphate that derives cellular proliferation.

- Osteofibrous dysplasia variants include monostatic (70–75%) and polyostotic (25–30%). Polyostotic fibrous dysplasia may be associated with McCune-Albright syndrome (precocious puberty and skin pigmentation) or Mazabraud syndrome (hyperplasia

Table 6.4 Comparison of dysplasia and anaplasia

Characteristics	Dysplasia	Anaplasia
Greek word	Greek word dysplasia means *dys* (bad) and *plasis* (formation)	Greek word anaplasia means *ana* (backward) and *plasis* (formation)
Definition	Dysplasia describes abnormal arrangement of cells, when mature cells partially lose their morphological features	Anaplasia describes cells that have lost the distinct characters which define them as particular tissue type
Pathology	Less advanced lesion	More advanced lesion
Examples	Cervical intraepithelial neoplasia (CIN), bronchial dysplasia, pituitary dysplasia, hip dysplasia, cemento-osseous dysplasia, dentin dysplasia, osteochondral dysplasia, osteofibrous dysplasia and myelodysplastic syndrome	Poorly differentiated carcinoma

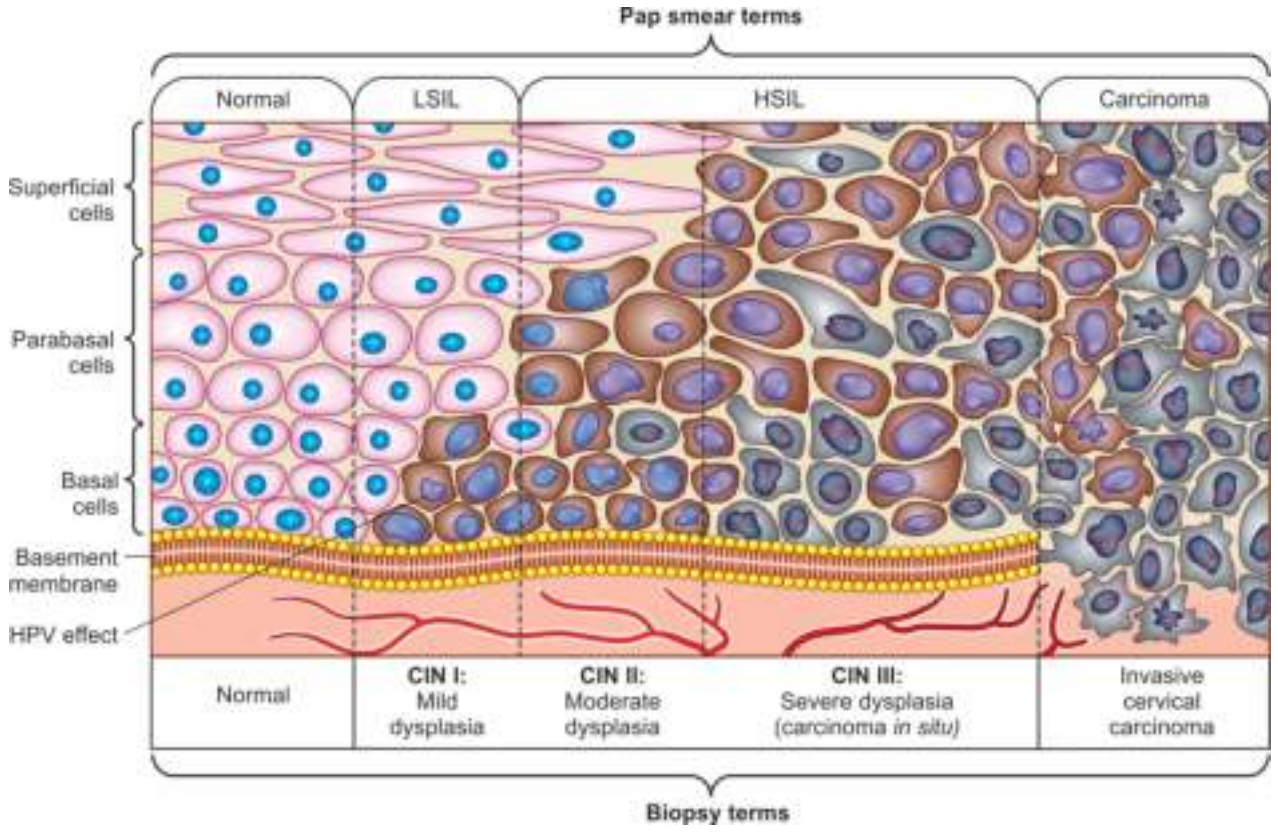


Fig. 6.6: Development of cervical intraepithelial neoplasia (CIN I, CIN II, CIN III) and invasive cervical carcinoma. Cervical intraepithelial neoplasia-I (CIN I) shows moderate dysplastic cervical epithelium in the lower one-third. Cervical intraepithelial neoplasia-II (CIN II) shows moderate dysplastic cervical epithelium in the lower two-thirds. Cervical intraepithelial neoplasia-III (CIN III) involves full thickness of cervical epithelium that does not invade the basement membrane and shows severe dysplastic cells. About 25% of untreated cases of cervical intraepithelial neoplasia may progress to cervical carcinoma. So, involved cervis must be excised with conization to prevent development of carcinoma. LSIL denotes low-grade intraepithelial lesion. HSIL denotes high-grade intraepithelial lesion.

of endocrinal glands and skin pigmentation). Radiograph shows well-circumscribed lesion with sclerotic rim of variable density giving 'ground glass appearance'.

- Osteofibrous dysplasia can undergo malignant phenotype in less than 1% of cases in polyostotic osteofibrous dysplasia or in patients with McCune-Albright syndrome.

KINETICS OF TUMOR GROWTH AND ANGIOGENESIS

Tumor growth depends on the activity of specific cell membrane receptors which regulate signaling pathways within the cell. Cell signal transduction involves the communication process where signals from outside the cell are transferred to the nucleus inside the cells.

- **Growth factors:** Several important growth factors have been identified, which bind to their cognate receptors, which include epidermal growth factor receptor (EGFR) family, platelet-derived growth factor receptor (PDGFR), BCR-ABL, KIT, vascular endothelial growth factor (VEGF), transforming growth factor (TGF), and fibroblast growth factor (FGF).
- **Receptor tyrosine kinases:** Receptor tyrosine kinases (RTKs) are a subgroup of growth factor receptors involved in the signal transduction process. RTKs play an important role in various cellular processes such as cell growth, proliferation, migration, differentiation and survival. Normally functioning growth factor receptors emit cytoplasmic signals in response to binding ligand. However, mutations in the genes encoding aberrant growth factor receptor molecules can cause subtle alterations in protein structure, such as amino acid substitutions, that cause ligand-independent fire leading to human cancer. Alterations in receptor structure including truncation of the ectodomain, may also yield such deregulated signaling. Receptor proteins are overexpressed in many human cancers.
- **Tumor angiogenesis:** Degree of vascularization is the important determinant of tumor growth potential. Tumor angiogenesis plays vital role in tumor growth, invasion and metastasis. In cancer, an '**angiogenic switch**' is almost always activated and remains

'turning on', causing existing normally quiescent vasculature networks near the tumor to continually sprout new blood vessels in the malignant tumor.

TUMOR INVASION AND METASTASIS

Invasion and metastasis constitute important hallmarks of cancer. CSCs proliferate leading to increase in size of malignant tumor that eventually penetrates basement membrane and then invades the surrounding extracellular matrix environment involves several steps. Microinvasion is spread of epithelial malignancies just beyond the point of origin through the basement membrane. Ductal carcinoma *in situ* (DCIS) with microinvasion is a pathologic diagnosis defined by the presence of 1 mm of invasive carcinoma in a background of ductal carcinoma *in situ* in female breasts.

- Microinvasive carcinoma of breast is usually detected by mammography due to abnormal calcifications in associated with DCIS. Cure rate of microinvasive carcinoma is close to 100% following surgical excision. Prognosis may depend on features of ductal carcinoma *in situ*.
- Cancer stem cells first acquire the ability to bind to components of the extracellular matrix (ECM). Tumor–ECM interactions are mediated by the expression of numerous adhesion molecules.
- The tumor undergoes epithelial–mesenchymal transition (EMT). ECM is degraded by proteolytic (MMPs) enzymes released from CSCs.
- After moving through the extracellular matrix environment, the cancer stem cells penetrate or lymphatic channels and venous tributaries by same mechanisms.
- After survival of CSCs in blood circulation from immune system evasion, the CSCs exit the vascular system. CSCs establish micrometastases at the site where it leaves the vasculature.
- These micrometastases grow into grossly evident metastases in organ(s).

TUMOR METASTATIC CASCADE

Tumor metastasis is a process, in which CSCs break away from original (primary) cancer, invade directly in surrounding tissue, travel through lymphatic route to draining lymph nodes, and via hematogenous route disseminate to a new location and establishes a secondary (metastatic) tumor in the new environment of distant organ(s). Most metastatic cancers are manageable, but not curable. Treatment can relieve symptoms, slow cancer growth and improve quality of life. Outline of operational steps is involved in metastases mediated by tumor–host interactions is shown in Fig. 6.7. Tumor development, invasion and metastasis are given in Table 6.5.

- **Metastasis pathways of malignant tumors:** Malignant tumors disseminate by one of five following pathways: tissue invasion, metastasis via lymphatic route to lymph nodes, via hematogenous route to distant organ(s), via transcoelomic route to peritoneum, and surgical implantation.
  - Carcinomas most often metastasize by lymphatic route involving regional lymph nodes, and later via hematogenous route to distant organ(s).
  - Sarcomas disseminate via hematogenous route to distant organ(s). It must be noted that in the lymph nodes, communication between the lymphatic channels and venous tributaries allows cancer stem cells access to the systemic circulation.
  - Virchow node is left (usually) supraclavicular lymph node metastasis from an abdominal malignancy.
- **Metastasis steps:** The five key steps of metastasis include: CSCs invasion, intravasation, circulation, extravasation, and colonization in distant organs. Metastatic cascade is the consequence of chromosomal instability that is caused by continuous errors in chromosome segregation. Metastatic process requires regulation of both metastasis-promoting genes and metastatic suppressor genes.
  - **Metastasis-promoting genes:** Hundreds of genes have been reported to determine invasive potential suggesting that CSCs in primary malignant tumor exhibit metastasis-promoting genes. Most notable genes are the DCC, ABCA13, IAM2, CREBBP, BCL6B and ZNF185 genes, mainly mutated exclusively in metastases and highly likely driver genes of metastatic progression.
  - **Metastasis-suppressor genes:** Metastatic suppressor genes are molecules that inhibit metastatic cascade at a secondary site without affecting the growth of primary tumor. Their specific regulation in the metastatic cascade is critical for the understanding of tumor metastasis. Metastatic-suppressor genes target various signaling pathways at multiple points in the cascade involving RAF/MEK/ERK mitogen-activated protein kinase (MAPK) signaling pathway, G protein-coupled receptor, cell adhesion, cytoskeleton, transcriptional regulation and metastatic susceptibility. A greater understanding of common signaling pathways/molecules targeted by metastasis suppressor could improve metastasis treatment strategies. Metastatic suppressor genes and their mechanism of actions are given in Table 6.6.

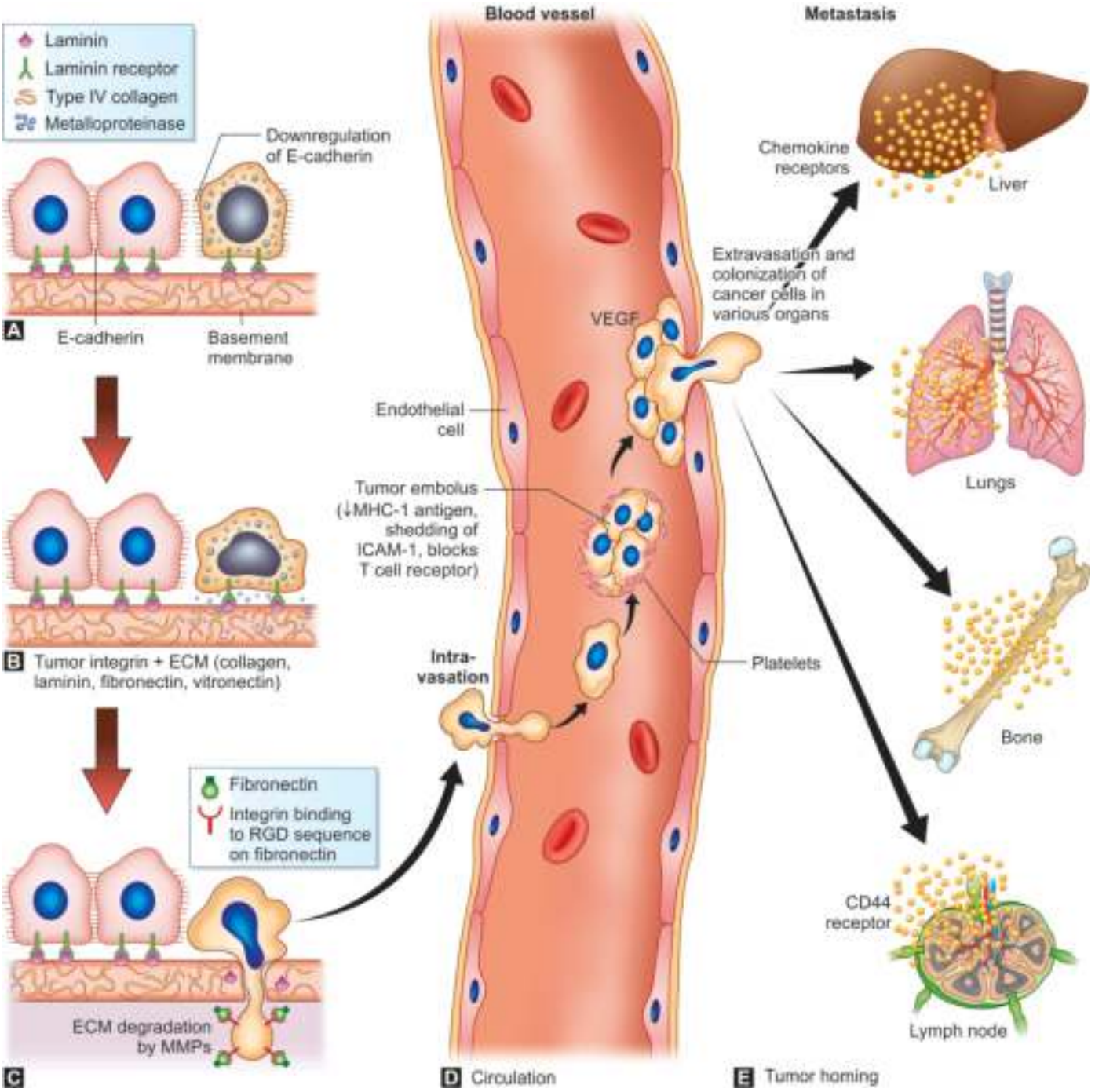


Fig. 6.7: Outline of operational steps involved in metastases mediated by tumor–host interactions. (A) Penetration through basement membranes by tumor cells is preceded by adhesion to basement membrane components. CSCs express receptors for the basement membrane protein laminin and their ligand receptor interaction between laminin and its receptor on CSCs is an important mechanism mediating binding, (B and C) binding of CSCs to connective tissue matrix proteins is followed by proteolytic breakdown of these matrices and detachment of CSCs, which then move through the breaches created by matrix protein-breakdown, (D) intravasation of CSCs, (E) extravasation of cancer stem cells and dissemination to distant organ(s).

- **Metastasis in distant organs:** A number of factors may produce minute clinically inapparent, metastatic foci of cancer stem cells (CSCs) to enter G0 phase of cell cycle, but may be reactivated to enter the cell cycle and form a clinically grossly evident macrometastasis. Micrometastases may also entail a balance between cell proliferation and apoptosis. If this equilibrium is disturbed in favor of tumor cell proliferation, the result may be a grossly evident macrometastasis. **TNM staging** is used to describe most cancers, i.e. (T) tumor size, (N) lymph node involvement and (M) metastasis.

Table 6.5 Tumor development, invasion and metastasis

Tumor Metastasis-promoting and Suppressor Genes	
▪ Tumor metastasis-promoting genes	▪ Tumor metastasis-suppressor genes
Expansive Growth and Invasion of Basement Membrane into the Surrounding Tissue	
▪ Enhanced protease activity (e.g. matrix metalloproteinases—MMPs) ▪ Decreased integrity/strength of the cell-cell contacts (E-cadherin)	▪ Enhanced cell motility interaction with surrounding tissue/ extracellular matrix/stromal cells
Angiogenesis, Intravasation and Transport Cancer Stem Cells (CSCs) within Blood Vessels	
▪ Migration interaction through extracellular membrane (ECM) ▪ Interaction of CSCs with vascular endothelial cells ▪ Interaction of CSCs with cavity mesothelial cells during transcoelomic metastasis	▪ Invasion of CSCs into the blood vessels ▪ Survival of cancer stem cells in circulation from immune system evasion
Arrest and Extravasation of Cancer Stem Cells near Secondary Tissues/Organs	
▪ Interaction of CSCs with vascular endothelial cells ▪ Invasion of CSCs into secondary tissues/organs	▪ Interaction with cavity mesothelial cells during transcoelomic metastasis
Formation of Micrometastasis and Macrometastasis in Secondary Tissues/Organs	
▪ Invasion and migration of CSCs into secondary tissues/organs ▪ Interaction and adaptation to tissue microenvironment	▪ Establishment of new vasculature ▪ Secondary tumor establishment or dormancy

Table 6.6 Metastatic tumor suppressor gene mutations and associated cancers

Functional Categories	Metastatic Tumor Suppressor Gene Mutations	Associated Cancers
Cell adhesion proteins	▪ E-cadherin 1 (CDH1 gene) ▪ DCC (i.e. netrin receptor DCC gene mutation)	▪ Breast carcinoma, gastric carcinoma, colorectal carcinoma, thyroid carcinoma and ovarian carcinoma ▪ Colorectal carcinoma
Apoptosis	▪ Caspase 8 gene ▪ GAS1 (growth arrest specific gene 1) ▪ KAI1 (CD82) gene mutation	▪ Head and neck squamous cell carcinoma ▪ Colorectal carcinoma ▪ Prostatic carcinoma
Cytoskeleton proteins	▪ Gelsolin (actin depolymerization) ▪ RhO/ROCK pathway ▪ DL1	▪ Breast carcinoma ▪ Diffuse gastric carcinoma (linitis plastica) ▪ Lung carcinoma
Tumor dormancy maintenance	KISS1/KISS1R g	Metastatic melanoma
MAPK signaling pathways	▪ MAP2K4 ▪ MKK4 ▪ MAK7 ▪ NM23	▪ Colorectal carcinoma, non-small cell lung carcinoma, melanoma, ovarian carcinoma ▪ Prostatic carcinoma, ovarian carcinoma ▪ Colon carcinoma ▪ Breast carcinoma, melanoma
Transcription regulation	▪ BRMS1 ▪ KLF17 (Krueppel-like factor 17) negative regulator of EMT	▪ Lung adenocarcinoma, breast carcinoma ▪ Metastatic breast carcinoma
Angiogenesis	DRG1	Lung adenocarcinoma
Other metastatic suppressor genes and microRNAs	▪ PTEN upregulation ▪ TXNIP (redox regulation) ▪ CD44 (hyaluronic acid receptor) upregulation ▪ LSD1 (chromatin remodeling) gene ▪ MicroRNA-335 ▪ MicroRNA-126	▪ Prostatic carcinoma, uterine cancer and some brain tumors ▪ Breast carcinoma invasion and metastasis ▪ Molecular marker of CSCs ▪ Breast carcinoma invasion and metastasis of luminal cancer stem cells ▪ Melanoma and nasopharyngeal carcinoma invasion and metastasis ▪ Lung adenocarcinoma

MicroRNA-335 and 126 cause suppression of SOX4 transcription factor, MERTK encoding receptor tyrosine kinase, PTPN2 encoding protein tyrosine phosphatase non-receptor type 2, TNC gene encoding tenascin C.

NOMENCLATURE AND CLASSIFICATION OF TUMORS

TUMOR NOMENCLATURE

Human body is composed of two major classes of tissue: parenchymal tissue and mesenchymal tissue (connective tissue, muscle, bone and blood vessels). The term 'neoplasm' means new growth—synonymous with tumor does not imply benign or malignant, which correlate to the clinical course of the tumor. Tumors are autonomous, progressive (continue to grow in the absence of external stimuli), purposeless and parasitic nature (continue to derive nourishment from host).

- The term 'dysplasia' is a reversible process in a cell or tissue, where nuclear features of malignancy (anisocytosis, poikilocytosis, hyperchromatic nuclei, disorderly arranged unusual number of cells, with prominent nucleoli and abnormal mitotic figures, and cell disorganization—loss of polarity) are observed but general architectural features are benign lesions that regress spontaneously or may transform into malignancy in some cases.
- The term neoplasia refers to the process of tumor growth. Benign tumors of most tissues are usually designated by the suffix-oma. The term 'cancer' refers to malignant tumor. Malignant tumors of the parenchyma are designated as **carcinomas**, while malignant tumors of mesenchymal tissues are designated as sarcomas.
- Anaplastic tumor is poorly-differentiated tumor that exhibits pleomorphism, numerous atypical tripolar or quadripolar mitoses, abnormal nuclear morphology and cell disorganization (loss of polarity).

HISTOGENESIS OF TUMORS

Histogenesis is a method of classifying neoplasms on the basis of the tissue cell of origin and lineage of differentiation, and whether the tumor is benign, borderline (low-malignant potential without stromal invasion) or malignant (*in situ* or invasive carcinoma) based according to their morphological appearance and clinical behavior.

- Tumors are further described by appearance, embryonic tissue of origin (i.e. ectoderm, mesoderm and endoderm), and differentiation. Ectoderm gives rise to skin and nervous tissue. Mesoderm gives rise to muscle, bone and blood-forming cells. Endoderm gives rise to intestine derived pouchings, bronchial tissue, lung, liver and pancreas.
- Tumor is composed of neoplastic element and non-neoplastic supporting connective tissue stroma. Benign tumors are usually well-differentiated and localized, which neither invade surrounding tissues nor metastasize to distant organs(s). Borderline tumors are of low-malignant potential without stromal invasion. Cells that initiate and sustain a malignant neoplasm are called CSCs. Malignant tumors can exhibit a range of differentiation, invade surrounding tissues and metastasize to distant organs(s). There are four main routes of metastasis of malignant tumors—local invasion, lymphatic route, hematogenous route and transcoelomic route.

Benign Tumors

Benign tumors are derived from epithelial and mesenchymal tissues, which stay localized in one place. Benign tumors are slow-growing, well-encapsulated or well-circumscribed, resembling the tissue of origin and localized to the site of origin without invading surrounding tissues, and not metastasizing to distant organ(s).

- In general, cells of benign tumors are well-differentiated, which mimic the structure of their parent tissue of origin, exhibit uniformity in size, shape and nuclear configuration and have relatively infrequent normal type of bipolar mitotic figures.
- In general, benign tumors of most tissues are designated by the 'suffix-oma'. It is worth mentioning that some malignant tumors are also ending with 'suffix-oma', which include melanoma, seminoma (germ cell tumor of testis), dysgerminoma (germ cell tumor of ovary) and mesothelioma (mesothelial cell tumor).
- It is worth mentioning that some non-neoplastic lesions also end with 'suffix-oma' (i.e. hematoma, granuloma, or hamartoma.
- Following surgical excision, some benign tumors (e.g. uterine leiomyoma, pleomorphic adenoma of salivary gland and atrial myxoma) may disseminate to distant organ(s).

Benign Epithelial Tumors

Benign epithelial tumors are essential of two types, i.e. papilloma and adenoma. Papilloma originates from surface epithelium, as the epithelium proliferates, it is thrown into visible finger-like projections, which become complex, which is accompanied by a corresponding growth of supporting connective tissue and blood vessels. Adenoma is derived from the ducts and acini of glands, and proliferation of gland leads to formation of tubules, which ramify and become compound.

Benign Mesenchymal Tumors

Benign mesenchymal tumors are composed of mature connective tissue, i.e. adipose tissue, smooth muscle,

skeletal muscle, lymphatic channel, blood vessel, cartilage, and bone, which include lipoma, leiomyoma, rhabdomyoma, lymphangioma, hemangioma, chondroma and osteoma respectively, which tend to form encapsulated, lobulated masses and compress the surrounding tissues.

Borderline Tumors of Low Malignant Potential

Borderline tumors are of low malignant potential, defined histologically by atypical proliferation of surface epithelial cells, without stromal invasion, e.g. serous, mucinous and endometrioid borderline tumors of ovary, which account for 15% of all primary ovarian neoplasms in women of 20–40 years.

Malignant Tumors

Malignant tumors are derived from any of the three germinal layers (ectoderm, mesoderm and endoderm) and from epithelial tissue (carcinoma derived from squamous, glandular and transitional epithelium lining the organs) and mesenchymal tissue (sarcoma derived from cartilage, bone, smooth muscle, skeletal muscle and blood vessels). Other malignant tumors include neuroectodermal tumors, neuroendocrine tumors, germ cell tumors, hematolymphoid tumors and melanoma.

- **Genetic alterations and cancer:** Mutations may cause gain- or loss-of-function of genes. Malignant tumors occur either due to gain-of-function of oncogenes or inactivation/biallelic loss of tumor suppressor genes. Dominant mutations result from a gain-of-function, some novel characteristics of the protein product. Recessive mutations occur due to loss of function by the polypeptide product.
- **Normal cell is transformed to CSC:** Malignant tumor begins as a single mutated cell. About 30 divisions (doubling time) of transformed CSC take place before the patients develop earliest symptoms. Each division of CSC leads to additional gene mutations. Cancers that are detected late, and tend to have poor prognosis.
- **Rate of tumor growth:** Malignant tumors are rapidly growing, poorly-circumscribed mass, which obtain nourishment from the body and not related to the physiologic needs of the body.
- **Indicators of malignancy:** CSCs possess several unique capabilities such as self-renewal, invasion and metastasis to distant organs(s). Invasion and metastasis are the most important defining indicators of malignancy.
  - Many malignant tumors invade surrounding tissue and metastasize via lymphatic route to lymph nodes (e.g. carcinomas), via hematogenous route (e.g. carcinomas and sarcomas) to distant organ(s) anywhere in the body most commonly in the liver, lungs, brain and bone, and via transcoelomic route to peritoneum (Krukenberg tumor of ovary from metastatic breast carcinoma, gastric carcinoma and colon carcinoma).
  - When malignant tumor metastasizes to distant organ(s), the tissue from which tumor originated is not always apparent from its morphologic properties. In such cases, electron microscopy and immunohistochemistry may aid in detection of correct origin of the tumor.
- **Electron microscopy:** Electron microscopic examination demonstrates desmosomes or special junctional complexes in carcinomas, and melanosomes in melanomas, and membrane-bound granules with dense core in endocrine neoplasms. Electron microscopy remains an invaluable diagnostic adjunct revealing certain subcellular features that indicate a specific line of differentiation in pleomorphic sarcomas of soft tissues (e.g. high-grade fibrosarcoma, pleomorphic liposarcoma and pleomorphic rhabdomyosarcoma).
- **Immunohistochemistry:** Immunohistochemistry technique is an important application of monoclonal antibodies widely used for diagnosis of malignant tumors; as specific tumor antigens are expressed de novo or upregulated in certain human cancers.
  - Immunohistochemistry technique plays an important role in diagnostic and research laboratories to predict prognosis of malignant tumors by detection of enzymes, tumor-specific oncogenes, tumor suppressor genes and proliferation of CSCs.
  - Immunohistochemistry technique is performed to diagnose primary malignant tumor of uncertain origin and metastatic tumor from unknown primary cancer by using a panel of antibodies to resolve diagnostic problem cases.
  - The selection of monoclonal antibodies being made is based on clinical history, morphologic features and results other relevant laboratory investigations. Immunohistochemical stains for intermediate filaments are expressed by CSCs (i.e. cytokeratin, vimentin, desmin, neurofilaments and glial fibrillary acidic proteins).
- **Eradication of malignancy:** Most of the patients with malignant tumors have fatal outcome, whereas benign tumors usually remain localized without morality. Debulking of malignant tumor in stage I done by surgery may result in successful remission. Eradication of the malignant tumor requires removal of the CSCs, which must have the **BMI-1 gene** encoding BMI-1 gene products, which inhibit

tumor suppressors $p16^{INK4A}$ and $p14^{ARF}$. It is worth mentioning that $p16^{INK4A}$ and $p14^{ARF}$ normally function to inhibit the cell cycle.

- **Prediction of therapeutic response to therapy:** Immunohistochemistry technique is widely used to predict therapeutic response in both breast carcinoma and prostatic carcinoma.
  - Growth of breast carcinoma and prostatic carcinoma is under hormone estrogen and androgen respectively. The specific receptors for these growth regulating hormones are located on target CSCs.
  - Tumors expressing high-level of receptor positivity would respond favorably for removal of the source of such hormones, therefore hormonal therapy is administered to lower their levels.
  - Tamoxifen is administered to treat hormone estrogen receptor-positive breast carcinoma. Men have higher levels of androgens, i.e. testosterone and dihydrotestosterone (DHT). Hormonal therapy for prostatic carcinoma can reduce production of androgens by testicles or blocking the action of androgens throughout the body.
- **Screening of cancers:** Goals of screening of cancer is to detect intraepithelial neoplasia (severe dysplasia/carcinoma *in situ*) before it becomes carcinoma, before clinical symptoms appear. Common screening methods for detection of cancers include Papanicolaou smear (cervical carcinoma) and mammography; and prostate-specific antigen analysis and digital rectal examination (prostatic carcinoma); occult blood and colonoscopy (colorectal carcinoma) before it spreads to distant organ(s).

Malignant Epithelial Tumors

Carcinomas, which develop from squamous epithelium or glandular epithelium lining organs that form nests of epithelial cells supported by non-neoplastic stroma. Terminology related to histologic appearance of epithelial tissue-derived malignant tumors includes differentiation, anaplasia, dysplasia (mild/moderate and carcinoma *in situ*/severe dysplasia), and invasive carcinoma.

- Squamous cell carcinoma most often occurs on skin, especially on exposed surfaces, but also develops in other sites covered by stratified squamous epithelium, e.g. lips, tongue, pharynx, esophagus and vagina. In addition, squamous cell carcinoma may occur on surfaces covered by glandular type epithelium through metaplastic transformation as seen in bronchus, uterine cervix and gallbladder.
- Adenocarcinoma may take origin from gland duct, acini or the glandular epithelium of mucous surfaces. On mucosal epithelial surfaces, adenocarcinoma may start as a polypoidal growth, and in compound glands, e.g. the female breast, adenocarcinoma forms an irregular penetrating mass, which exhibits crab-like appearance.
- Transitional cell carcinoma begins in urothelial cells that line urethra, urinary bladder, ureters and renal pelvis. Urothelial cells can change shape and stretch without breaking apart.

Malignant Mesenchymal Tumors

Malignant mesenchymal tissue tumors are referred to as sarcomas, which take origin in soft tissue, bone, cartilage, smooth muscle, skeletal muscle, blood vessels and rarely in viscera. Sarcomas are composed of neoplastic cells intermixed with the connective tissue stroma. Sarcomas are far less common than carcinomas, Unlike the ill-defined growth of invasive carcinomas, sarcomas are well-defined fleshy tumors.

Malignant Tumors Lacking Benign Counterpart

In nomenclature of tumors, many life-threatening malignant tumors lack benign counterpart despite ending in 'suffix-oma'.

- 'Blastomas' with immature embryonic cells resembling fetal analogue occur due to failure of proper differentiation into their intended cell types before birth during embryogenesis or during infancy and early childhood, which include neuroblastoma, retinoblastoma, hepatoblastoma, nephroblastoma (Wilms' tumor), medulloblastoma, ameloblastoma of maxilla, pancreatoblastoma, pleuropulmonary blastoma, gonadoblastoma and glioblastoma multiforme.
- Some malignant tumors ending with 'suffix-oma' include seminoma, dysgerminoma, germinoma, mesothelioma, melanoma, non-Hodgkin's lymphoma and hepatoma.
- Malignant tumors called disease include leukemias, Paget's disease of breast.
- Malignant tumors nominated by scientists' name include Hodgkin's disease, Ewing sarcoma.

Pathology Pearls: Blastomas-derived from Immature Embryonic Cells Resembling Fetal Analogue

- Neuroblastoma
- Retinoblastoma
- Hepatoblastoma
- Nephroblastoma (Wilms' tumor)
- Medulloblastoma
- Ameloblastoma of maxilla
- Pancreatoblastoma
- Pleuropulmonary blastoma
- Gonadoblastoma
- Glioblastoma multiforme

TUMOR CLASSIFICATION HIERARCHY

Tumors may be classified in two ways based on: (a) clinical behavior (i.e. benign, borderline of low malignant potential without stromal invasion and malignant) and (b) histologic origin (i.e. epithelial tissue, mesenchymal tissue, neuroectoderm, neuroendocrine, germ cells and hematolymphoid cells). In clinical practice, there is a spectrum of malignant tumors. Some malignant tumors may grow locally and invade normal surrounding tissues, but never metastasize to distant organs(s). Other malignant tumors produce metastases only after a very considerable time, while some of the malignant tumors metastasize very early in their development. The commonest tumors originate from tissues, which have rapid turnover of cells, and which are exposed to environmental mutagens, e.g. epithelium of mucous membranes, skin, breast, reproductive organs and hematolymphoid tissue. Differences between normal tissue cell and CSC are given in Table 6.7. Tumor classification hierarchy is given in Table 6.8. Principal characteristics of benign and malignant tumors are given in Table 6.9. Classification of common benign and malignant tumors according to their origin are given in Table 6.10. Principal characteristics of carcinoma and sarcoma are given in Table 6.11.

- **Normal cells versus CSCs:** CSCs differ from normal cells in many respects: tumor growth *in vivo*, lack of differentiation (anaplasia), and pleomorphism. Malignant tumors exhibit clonal expansion of cancer stem cells, pleomorphism (varying size and shape), hyperchromatic nuclei, prominent nucleoli,

Table 6.7 Differences between normal tissue cell and cancer stem cell

Characteristics	Normal Tissue Cell	Cancer Stem Cell
Morphologic features		
Differentiation	Well-differentiation	Well-moderate-poor differentiation
Nuclear polarity	Basal polarity	Loss of basal polarity
Nuclear morphology	Normal morphology	Pleomorphic morphology
Nuclear to cytoplasmic (N/C) ratio	Low N/C ratio	High N/C ratio
Nuclear chromatin	Normal chromatin	Hyperchromatism
Nucleolus	Normal	Prominent
Mitotic figures	Bipolar mitoses	Tripolar or quadripolar mitoses
Giant cells	Giant cells absent in normal tissue	Tumor giant cells present in cancers
Molecular features		
Regulation	Normal regulation	Autonomous regulation (uncontrolled cell proliferation)
Genetic stability	Stable	Unstable
Growth factor dependence	Growth factor dependent	Growth factor independent
Density dependent	High	Low
Anchorage dependence	High	Low
Cell-to-cell adhesion	High	Low
Cell-to-cell communication	High	Low
Cell life span	Limited	Unlimited
Antigen expression on cell surface	Absent	Tumor antigen may be present (e.g. carcino-embryonic antigen)
Substance (i.e. protease, hormone) production	Normal	Ectopic production substances
Cytoskeleton composition and arrangement	Normal	Abnormal
Mitochondria number	Numerous mitochondria	Few mitochondria
Rough endoplasmic reticulum (RER) and ribosomes	▪ More prominent ▪ Numerous ribosomes attached to the rough endoplasmic reticulum	▪ Less prominent ▪ Ribosomes dissociated and scattered in the cytoplasm
Chromosomes	Diploidy	DNA polyploidy

Table 6.8 Tumor classification hierarchy

Differentiation of Tumors
▪ Epithelial tumors ▪ Nonepithelial tumors ▪ Mixed tumors (e.g. fibroadenoma of breast, carcinosarcoma, teratocarcinoma, malignant mixed Müllerian tumors of uterus, adenosquamous carcinoma and metaplastic carcinoma)
Embryonic Origin of Tumors
▪ Tumors originate from ectoderm-derived structures, e.g. skin, keratinocytes, sweat glands, breast, salivary glands and nervous system (neuroectoderm) ▪ Tumors originate from mesoderm-derived structures (e.g. bone, cartilage, smooth muscle, fibroblasts, adipocytes, endothelial cells, kidney, testis, endometrium, and blood forming cells) ▪ Tumors originating from endoderm-derived structures, e.g. stomach, small intestine, colon, lung, pancreas, thyroid gland, liver, urinary bladder and prostate gland
Biological Behavior of Tumors
▪ Benign epithelial and mesenchymal tumors are associated with good prognosis ▪ Borderline tumors of ovary (serous cystadenoma, mucinous cystadenoma) have low malignant potential ▪ Malignant epithelial and mesenchymal tumors include carcinoma and sarcoma associated with poor prognosis

atypical tripolar or quadripolar mitotic figures, and chromosomal abnormalities, both structural (e.g. Philadelphia chromosome in chronic myelogenous leukemia) and numerical (e.g. aneuploidy).
  - In contrast to the normal cells, CSCs exhibit loss of functions, simplified cytoplasmic architecture and synthesis of oncofetal proteins such as α-fetoprotein (AFP) and carcinoembryonic antigen (CEA).
- **Benign versus malignant tumors:** Benign tumors are well-differentiated, which resemble the structure of parent tissue and show a remarkable uniformity in size, shape and nuclear configuration, and relatively infrequent bipolar mitotic figures.
  - Malignant tumors tend to be less well-differentiated than those of benign tumors, which show generally haphazard arrangement of tumor cells, variation in size, shape and nuclear configuration, reflecting an increase in chromosomal number and DNA content (aneuploidy) and atypical tripolar or quadripolar mitotic figures.
  - There is a wide spectrum within malignant tumors, from slow-growing and well-differentiated tumors to rapidly growing and highly malignant undifferentiated tumors.
  - Benign tumors do not invade or metastasize to distant organs, whereas primary malignant

Table 6.9 Principal characteristics of benign and malignant tumors

Characteristics	Benign Tumors	Malignant Tumors
General features		
Origin of tumors	▪ Epithelial tissue-derived benign tumors (adenoma, polyp, papilloma) ▪ Mesenchymal tissue-derived (osteoma, fibroma, chondroma)	▪ Epithelial tissue-derived malignant tumors (squamous cell carcinoma, adenocarcinoma, transitional cell carcinoma) ▪ Mesenchymal tissue-derived tumors (osteosarcoma, chondrosarcoma, liposarcoma, fibrosarcoma, rhabdomyosarcoma)
Differentiation	Well-differentiated tumor	Well, moderately and poorly differentiated tumor
Growth rate	Slow-growing tumor remains localized	Rapidly growing tumor usually invades adjacent tissues
Behavior	Expansile sessile or papillary growth growing locally	Fungating, ulcerated or annular tumors invading surrounding tissue
Direction of epithelial growth on skin or mucosal surfaces	Exophytic growth (most often)	Endophytic growth (most often)
Invasion of tumor	Absent (benign tumor grows as a cohesive mass encapsulated by dense connective tissue)	Most often present (malignant tumor destroys surrounding tissue and lacks a well-defined capsule)
Metastases of tumor	Benign tumors most often do not metastasize except tumors metastasize after surgical excision (uterine leiomyoma, cardiac myxoma and pleomorphic adenoma of salivary glands)	Malignant tumor most often metastasizes to distant organ(s), except basal cell carcinoma and glioma of central nervous system

Contd...

Table 6.9 Principal characteristics of benign and malignant tumors (*Contd...*)

Characteristics	Benign Tumors	Malignant Tumors
Effect on host	Slight harmful (can be harmful if, located in brain and airways)	Significant harmful due to invasion and metastasis to distant sites
Gross morphology		
Tumor border	Often well-circumscribed or encapsulated tumor	Irregular poorly-defined or irregular tumor growth and lacks capsule and clear demarcation
Cut surface of tumor	Homogenous cut surface of tumor	Heterogenous cut surface of tumor
Hemorrhage and necrosis on cut surface	Hemorrhage and necrosis rare	Hemorrhage and necrosis common
Ulceration of tumor	Ulceration rare	Ulceration common on skin or mucosal surfaces
Histologic features		
Histologic resemblance to normal tissue	Benign tumor resembles cell of origin (well-differentiated tumors)	Malignant tumor often shows failure of cellular differentiation (may be well-differentiated or moderately-differentiated tumors)
Cells morphology	Cells are uniform throughout the tumor	Pleomorphic cells vary in shape and size in the tumor
Nucleus polarity	Basal nuclear polarity	Loss of basal nuclear polarity
Nuclear morphology	Often normal nuclear morphology	Pleomorphic nucleus morphology with irregular outlines, multiple nucleoli
Nucleocytoplasmic ratio	Nucleocytoplasmic ratio low (1:4 or 6)	Nucleocytoplasmic ratio high (1:1)
Chromatin pattern	Nucleus with normal chromatin	Nucleus with hyperchromatism
Nucleolus	Inconspicuous nucleolus	Prominent nucleolus
Mitotic rate	Low-mitotic rate	High-mitotic rate
Mitotic figures	Bipolar mitoses	Atypical tripolar or quadripolar mitoses
Giant cells	Benign giant cells can be observed in some benign tumors	Bizarre tumor giant cells present in malignant tumors
Tumor ploidy	Chromosomal abnormality without aneuploidy	Usually, aneuploidy

tumors invade the basement membrane of tissues through the action of matrix metalloproteinase (MMP) enzymes, detach from neighboring cells, metastasize via lymphatic route to lymph nodes, via hematogenous route to distant organ(s), or transcoelomic routes to peritoneum to form secondary tumors.

- **Diagnostic significance of classification of tumors:** Distinction between benign and malignant tumors of great importance in diagnosis, treatment, and prognosis. A clear understanding of the histologic classification of tumors is essential for the study of tumors. However, the entire spectrum of tumors arising in various tissues and organs has been difficult to follow a single classification scheme that is either purely morphologic basis or molecular basis. Malignant tumors can exhibit a range of differentiation (well, moderate and poor/undifferentiation).
- **Clinical significance of classification of tumors:** Histologic features of tumors together with information about their respective cell tissue-of-origin, differentiation states and the biologic behaviors (benign versus malignant tumors) are taken into consideration to develop a taxonomy of human tumors that has proven useful for the diagnosis and clinical management of most tumors. Malignant tumors are capable of invasion surrounding tissues and metastasize to distant organ(s). This distinction between benign and malignant tumors is important in clinical practice because metastatic disease is associated with significant morbidity and mortality.

BENIGN EPITHELIAL TUMORS

Benign epithelial tumors are slow-growing, well-circumscribed, encapsulated, resembling to tissue of origin, and most often confined to their site of origin, which neither invade surrounding tissues nor metastasize to distant organ(s). It is worth mentioning that following surgical intervention, tumor cells from

Table 6.10 Classification of common benign and malignant tumors according to their origin

Tissue of Origin	Benign Tumors	Malignant Tumors
Epithelial origin tumors		
Stratified squamous epithelium	Squamous cell papilloma	Squamous cell carcinoma
Basal cell of skin	None	Basal cell carcinoma
Epithelial lining of ducts and glands (glandular epithelium)	▪ Adenoma ▪ Papilloma	▪ Adenocarcinoma ▪ Papillary carcinoma
Respiratory passages	Bronchial adenoma	Bronchogenic carcinoma
Renal epithelium	Renal tubular adenoma	Renal cell carcinoma
Liver cell (hepatocyte)	Hepatocellular adenoma	Hepatocellular carcinoma
Ovarian surface epithelium	▪ Serous cystadenoma ▪ Mucinous cystadenoma	▪ Serous cystadenocarcinoma ▪ Mucinous cystadenocarcinoma
Neuroectoderm (melanocytes)	Benign nevus	Malignant melanoma
Urinary tract surface epithelium	Transitional cell papilloma	Transitional cell carcinoma
Placental epithelium	Hydatidiform mole	Choriocarcinoma
Germ cell of gonads	Benign cystic teratoma (ovary) and extra-gonadal germ cell neoplasms (mediastinum)	Germinoma/seminoma/dysgerminoma, teratomas, embryonal carcinoma, yolk sac tumor and choriocarcinoma
Connective tissue origin tumors		
Fibrous tissue	Fibroma	Fibrosarcoma
Adipose tissue	Lipoma	Liposarcoma
Cartilage	Chondroma	Chondrosarcoma
Bone	Osteoma	Osteosarcoma
Smooth and skeletal muscle tissue origin tumors		
Smooth muscle	Leiomyoma	Leiomyosarcoma
Striated muscle	Rhabdomyoma	Rhabdomyosarcoma
Blood vessels origin tumors		
Lymphatic channels	Lymphangioma	Lymphangiosarcoma
Blood vessels	Hemangioma	Angiosarcoma
Surface covering origin tumors		
Synovium	None	Synovial sarcoma
Mesothelium	Benign fibrous tumor	Malignant mesothelioma
Brain coverings	Meningioma	Invasive meningioma
Hematopoietic myeloid cells, lymphoid cells, histiocytes and primitive cells origin tumors		
Hematopoietic myeloid cells	None	▪ Acute myelogenous leukemia ▪ Chronic myelogenous leukemia
Lymphoid cells	None	▪ Hodgkin's disease ▪ Non-Hodgkin's lymphoma ▪ Malignant thymoma ▪ Multiple myeloma ▪ Acute lymphoblastic leukemia (ALL) ▪ Chronic lymphocytic leukemia (CLL)
Histiocytes	None	Histiocytosis X
Primitive mesenchyme	None	Ewing sarcoma

Contd...

Table 6.10 Classification of common benign and malignant tumors according to their origin (*Contd...*)

Tissue of Origin	Benign Tumors	Malignant Tumors
Tumors with various components usually derived from one germ layer		
Salivary gland	Pleomorphic adenoma	Malignant mixed tumor of salivary gland
Renal analogue	None	Wilms' tumor
Totipotent cells	Benign cystic teratoma ovary	Teratocarcinoma
Nervous system origin tumors		
Primitive neuroectodermal cells	▪ Oligodendroglioma (adults) ▪ Ependymoma (adults) ▪ Meningioma	▪ Astrocytoma ▪ Glioblastoma multiforme ▪ Medulloblastoma ▪ Neuroblastoma (children) ▪ Retinoblastoma
Nerve sheaths	Neurilemmoma	Neurofibrosarcoma
Embryonic tissue origin tumors		
Kidney	None	Wilms' tumor
Liver	None	Hepatoblastoma
Secondary tumors		
Tumors metastasizing to distant sites	None	▪ Carcinoma derived from epithelial tissue ▪ Sarcoma derived from mesenchymal tissue

Tumors eponyms are applied to malignant tumors that cannot be properly classified (e.g. Hodgkin's disease, Wilms' tumor, Ewing sarcoma, Kaposi sarcoma, mesothelioma, melanoma, seminoma, dysgerminoma, chordoma and Burkitt's lymphoma).

Table 6.11 Principal characteristics of carcinoma and sarcoma

Parameters	Carcinoma	Sarcoma
Origin	Epithelial tissue	Mesenchymal tissue
Examples of malignant tumors	Squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, transitional cell carcinoma	Osteosarcoma, chondrosarcoma, fibrosarcoma, rhabdomyosarcoma, leiomyosarcoma, liposarcoma, angiosarcoma, Kaposi sarcoma
Behavior	Malignant behavior	Malignant behavior
Frequency	Common	Relatively rare
Growth rate	Slow growth	Rapid growth
Preferential routes of metastases	Lymphatic route (except renal cell carcinoma, follicular thyroid carcinoma)	Hematogenous route
In situ phase	*In situ* phase present	*In situ* phase absent
Age group	>50 years (most often)	<50 years (most often)
Neoangiogenesis (new blood vessels formed)	Neoangiogenesis confined to the malignant tumor (carcinoma) hence tumor is relatively less vascular	Neoangiogenesis extending from the malignant tumor (sarcoma) into the surrounding tissue hence tumor is more vascular
Histologic examination	Group of cells are separated by connective tissue	Individual cell is separated from adjacent cell by connective tissue
Electron microscopy	Cells express desmosomes and specialized junctional complexes	Cells do not express desmosomes and specialized junctional complexes
Immunohistochemistry	Cytokeratins, epithelial cell membrane (EMA), Ber-Ep4, B72.3, carcinoembryonic antigen (CEA)	Vimentin, desmin, muscle specific actin (SMA), CD99
Prognosis	Relatively good	Poor

Carcinomas often exhibit desmosomes and specialized junctional complexes, which are structures that are not typical of sarcomas or lymphomas.

pleomorphic adenoma of salivary gland may enter circulation and reach distant sites. Benign epithelial tumors can compress surrounding structures resulting in clinical manifestations. Benign epithelial tumors are essentially of two types: papilloma and adenoma, which originate from ectoderm and endoderm. The tumors can be sessile, papillary and polypoid growth on the surface of the skin, larynx, paranasal sinuses, tongue or urinary bladder. Tumors are usually ovoid or rounded in shape. Cut surface of solid and cystic areas but are lacking hemorrhage and necrosis.

Papilloma in Various Organs

Papilloma originates from epithelial lining of skin, larynx, paranasal sinuses, tongue, lactiferous ducts of female breast or urinary bladder. As the epithelium proliferates, it is thrown into visible finger-like projections, which become increasingly complex. The epithelial cell proliferation is accompanied by a corresponding growth of supporting fibrovascular core containing blood vessels.

Breast Intraductal Papilloma

Papilloma may also originate within ducts in the breast, which becomes compressed and exhibits complex structure, which can be located in central and peripheral regions of breast in young women. Peripheral breast papilloma tends to be even smaller in size than a central papilloma. Multiple papillomas can occur in bilateral breasts.

- **Clinical features:** Signs and symptoms depend on location of papilloma in the breast. Females can present with serous discharge in nipple. Some intraductal papillomas containing abnormal cells and multiple intraductal papillomas may have a slightly higher risk of developing breast cancer.
- **Gross morphology:** Tumor is tan-pink, friable, and usually <1 cm in size.
- **Histologic examination:** In general, papilloma consists of fibrovascular core with delicate finger-like projections lined by epithelial cells. Intraductal papilloma is lined by cuboidal to columnar cells toward lumen and outer layer of myoepithelial cells. Histology of intraductal papilloma in female breast is shown in Fig. 6.8.
- **Treatment:** If there is nipple discharge in solitary papilloma, the duct is excised by surgery.

Adenomas in Various Organs

Adenoma is benign tumor derived from the ducts and acini of glands. Proliferation of ducts and acini of glands leads to formation of tubules, which ramify and become compound. The original communication with the parent gland or acinus tends to become lost.

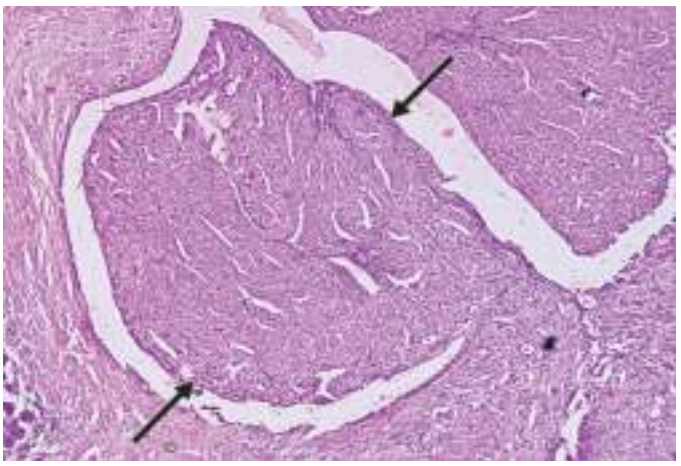


Fig. 6.8: Histology of intraductal papilloma in female breast. Tumor shows multiple branching papillae composed of thin fibrovascular core lined by cuboidal to columnar ductal cells toward lumen and outer myoepithelial cells (arrows) (200X).

- Adenoma is usually pushed upwards into the lumen of the viscus. Most common sites of adenomas include large intestine, salivary glands, thyroid gland, parathyroid glands, pituitary gland and breast.
- In cases, in which retention of secretion results in cyst formation and such tumor is called cystadenoma (ovarian serous, mucinous and endometrioid cystadenomas), which may reach size of 30–40 cm in diameter in the ovary, particularly mucinous cystadenoma is associated with excellent prognosis.

Colorectal Adenomas

Tubular adenoma, villous adenoma, and tubulovillous adenoma arise in the right colon especially in sigmoid colon (40%), left colon (40%) and rectum (20%). Risk of malignant transformation is more in >2 cm sized villous adenoma in rectosigmoid region. Majority of cases have chromosomal instability and demonstrate TP53 gene mutations.

- **Clinical features:** Villous adenoma in the colorectal region can occasionally manifest as a syndrome of severe diarrhea with massive fluid and electrolyte loss. In most cases, colonic/colorectal adenomas are asymptomatic and detected on routine colonoscopy procedure to rule out colon carcinoma. However, some patients can present with blood in the stool or rectal bleeding, abdominal pain, iron deficiency anemia, diarrhea or constipation of more than one week.
- **Gross morphology:** Colorectal adenoma is soft, irregular, red-tan to white mucosal fragments. Specimen is received in 10% formalin. Surgical specimen of tubular adenoma of colon is shown in Fig. 6.9.

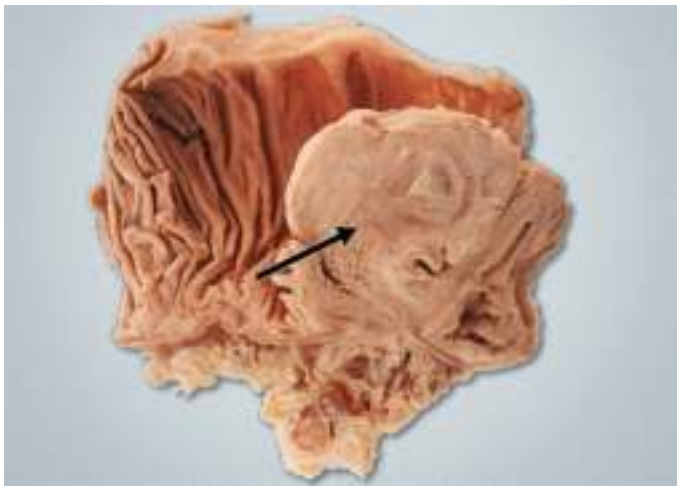


Fig. 6.9: Surgical specimen of tubular adenoma of colon. Tubular adenoma originates in the descending colon (90%) and rectum (50%) compared with other polyps. Tumor has pedunculated polyp (arrow). (*Courtesy:* Dr. Sujata Kanetkar, Professor and Head, Department of Pathology, Krishna Institute of Medical Sciences, Karad, Maharashtra.)

- **Histologic examination:** Colorectal adenomas are categorized based on how they grow: (a) tubular adenoma grows in a round or oval shape, (b) villous adenoma grows in a shaggy, cauliflower-like pattern, and (c) tubulovillous adenoma is a mixture of the two growth patterns such as tubular adenoma and villous adenoma. Villous component architecture resembles small intestinal villi. Histology of tubular adenoma of colon is shown in Fig. 6.10.
- **Treatment:** Endoscopic resection of the colorectal adenoma cures the patient.

Pleomorphic Adenoma of Salivary Gland

Pleomorphic adenoma is most common benign triphasic salivary gland tumor in children and adults. Diagnosis can be rendered on preoperative fine needle aspiration cytology (FNAC) and biopsy, showing metachromatic fibrillary stroma on cytologis examination or triphasic growth pattern on histologic examination of biopsy. Tumor is composed of epithelial (ductal) cells, myoepithelial cells and chondromyxoid stroma.

- **Clinical features:** Pleomorphic adenoma originates from major salivary glands, i.e. parotid gland (85%), submandibular gland, sublingual gland, and minor salivary glands of upper aerodigestive tract. Patient presents with slow-growing, painless and well-circumscribed mass involving major/minor salivary gland.
- **Gross morphology:** Pleomorphic adenoma of salivary gland is well-demarcated, bosselated and gray-white myxoid mass. Recurrent tumor after surgical excision shows numerous myxoid to fibrotic nodules of variable size, giving a shotgun appearance.
- **Histologic examination:** Pleomorphic adenoma of salivary gland has three components: (a) epithelial (ductal) component is forming the inner layer of tubules and cysts, (b) myoepithelial cells are lining the outer layer of tubules and cysts and scattered within the myxoid stroma, which can be spindle shape, stellate shape, epithelioid or clear morphology, and (c) stromal component is typically myxoid, chondroid or myxochondroid, hyalinized or fibrotic.
  - **Metaplastic changes** can be observed, i.e. adipose metaplasia, osseous metaplasia, squamous metaplasia (sometimes with keratinization), sebaceous metaplasia and mucinous metaplasia.
  - **Intravascular permeation** has been reported in small percentage of cases following surgical intervention. Histology of pleomorphic adenoma of salivary gland is shown in Fig. 6.11.

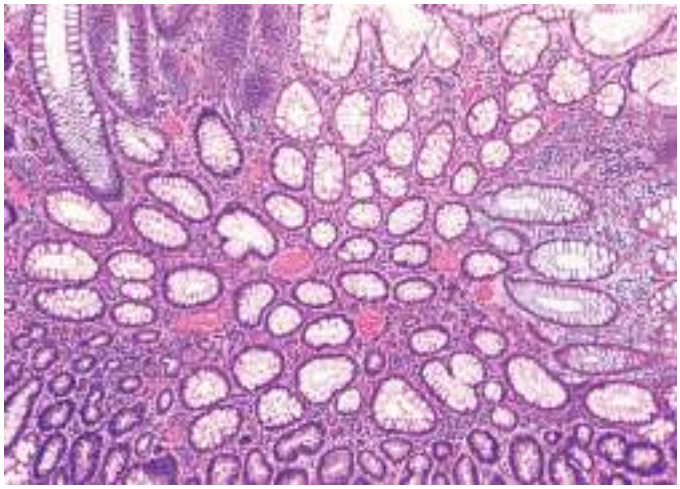


Fig. 6.10: Histology of tubular adenoma of colon. It shows closely packed tubular glands lined by mucus-secreting columnar epithelium (100X).

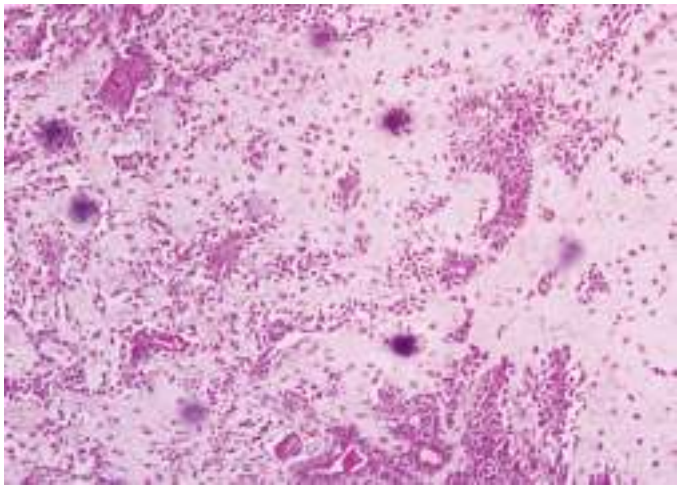


Fig. 6.11: Histology of pleomorphic adenoma of salivary gland. Tumor is composed of gland-like epithelial component surrounded by a myxomatous stroma often containing foci of bone or cartilage (100X).

- **Treatment:** Complete surgical resection of pleomorphic adenoma of salivary gland with negative margin is considered curative. Recurrences are common after incomplete surgical excision. Rarely, on surgical intervention, pleomorphic adenoma may metastasize to distant organs such as bone, sphenoid bone, central nervous system, kidney, liver, lung, lymph node, maxilla, pharynx, and skin. Tumor has benign morphology in primary site as well as metastatic site.

Follicular Thyroid Adenoma

Follicular thyroid adenoma is benign tumor that shows evidence of follicular differentiation, but lacks features of malignancy, and without capsular and vascular invasion.

- **Clinical features:** Patient presents with long-standing solitary thyroid nodule. Patient is most often euthyroid.
- **Gross morphology:** Tumor is solitary, encapsulated and variable size (1–10 cm) with solid fleshy, tan or light brown on cut surface. It is important to exclude malignancy because thyroid carcinoma mimics adenoma.
- **Histologic examination:** Tumor is completely encapsulated by thin capsule and architecturally and cytologically different from surrounding thyroid gland tissue and shows signs of compression. Tumor is composed of closely packed thyroid follicles. Tumor can demonstrate normofollicular, macrofollicular, microfollicular or trabecular/solid sheet patterns.
- **Treatment:** Surgical excision of follicular thyroid adenoma cures the patients.

Parathyroid Adenoma

Most cases of parathyroid adenoma are sporadic. Predisposing factors include radiation exposure and long-term lithium therapy. Parathyroid adenoma may be associated with multiple endocrine neoplasia [MEN-1 (most common), MEN-2A and MEN-2B] and hyperparathyroidism jaw tumor syndrome (HRPT2 gene germline mutation).

- **Clinical features:** Patient presents with symptoms related to hypercalcemia such as nephrolithiasis, osteopenia, osteitis fibrosa cystica, weakness, fatigue and psychiatric disturbances. Blood tests should be done to check the levels of parathormone, calcium, phosphorus and vitamin D. Ultrasonography, CT, MRI and technetium 99 sestamibi scintigraphy (99mTc) are performed to detect parathyroid adenoma.
- **Gross morphology:** Tumor size varies from few mm to more than 10 cm. Cut surface of tumor is solid yellow/tan, and well-circumscribed ovoid nodule without invasion into adjacent structures.
- **Histologic examination:** On histologic examination, tumor is composed of chief cells with round nucleus and granular cytoplasm.
- **Treatment:** Surgical excision of parathyroid adenoma cures the patients.

Pituitary Adenoma

Pituitary adenomas are benign tumors located in the anterior lobe of pituitary gland (i.e. pituitary cell lineage—somatotroph, lactotroph, thyrotroph, corticotroph, gonadotroph and null cell). Abnormal levels of certain hormones may indicate a specific pituitary gland-related syndrome.

- **Clinical features:** Some pituitary adenomas secrete one or more hormones in excess that affect body functions. The symptoms of pituitary adenoma may include headache, vision disturbances, weight gain, easy bleeding/bruising, change in bone structure in the face and hands, menstrual irregularity, lactation and erectile dysfunction.
- **Histologic examination:** Pituitary adenoma is composed of cells with moderate dense or granular cytoplasm, uniform nucleus with stippled chromatin and inconspicuous nucleoli.
- **Biochemical assays:** Blood and urine tests detect abnormal levels of hormones such as growth hormone (GF), prolactin (PRL), insulin-like growth factor 1 (IGF-1), free thyroxine, cortisol and testosterone.
- **Treatment:** Surgical excision of lactating pituitary adenoma cures the patients.

Lactating Adenoma of Breast

Lactating adenoma is well-circumscribed mass in breast in pregnant women and during postpartum period. Tumor may develop in ectopic breast tissue along milk-line extending from axilla to vulva.

- **Clinical features:** Patient presents with slow-growing, painless, soft and mobile discrete. Infarction may lead to pain, tenderness and rapid enlargement.
- **Gross morphology:** Tumor is well-circumscribed, lobulated, solitary or multiple that lacks a true capsule. Cut surface reveals firm/rubbery, gray tan mass usually <5 mm in size.
- **Histologic examination:** Lactating adenoma is composed of cuboidal cells or hobnail-shaped cells with small round nuclei and clear to granular vacuolated cytoplasm, and actively secreting closely packed glands and myoepithelial cell layers separated by thin, delicate connective tissue.
- **Treatment:** Surgical excision of lactating adenoma cures the patients.

Ovarian Cystadenomas

Ovarian cystadenomas are benign epithelial tumors, which vary in size and carry an excellent prognosis.

Ovarian serous and mucinous cystadenomas are most frequent types, whereas endometrioid, clear cell and seromucinous cystadenomas are rare. Despite advances in imaging studies (pelvic ultrasonography, computed tomography, magnetic resonance tomography), definite diagnosis of ovarian cystadenoma is established by histologic examination of surgical specimens.

- **Gross morphology: Serous cystadenoma** ranges in size from 1–30 cm, has smooth external surface and contains thin-walled unilocular/multilocular cysts filled with clear watery fluid. **Mucinous cystadenoma** has smooth external surface and contains thin-walled multilocular cysts filled with mucinous fluid. **Endometrioid cystadenoma** ranges in size from 1–15 cm and is filled with dark brown chocolate hemorrhagic fluid. **Clear cell cystadenoma** ranges in size from 3–15 cm, and has smooth, lobulated external surface. **Seromucinous cystadenoma** is a unilocular cyst with a smooth external surface and may contain serous or mucinous fluid.
- **Histologic examination:** Ovarian cystadenomas are benign tumors lined by epithelial cells with/without minimal cytologic atypia, which carry excellent prognosis. Histologic variants of ovarian cystadenomas include serous cystadenoma, mucinous, endometrioid cystadenoma, clear cell cystadenoma and seromucinous cystadenoma.
- **Treatment:** The management of ovarian cystadenomas depends on symptoms, size of the cystadenoma, age of the patients, clinical history, menopausal status of patient. Unilateral salpingo-oophorectomy or ovarian cystectomy is the adequate treatment of ovarian cystadenomas.

BENIGN MESENCHYMAL TUMORS

Benign mesenchymal tumors are derived from mesoderm and named based on their tissue of origin (e.g. lipoma, fibroma, leiomyoma, benign vascular tumors, rhabdomyoma, chondroma, and osteoma). They are slow-growing, well-circumscribed, encapsulated, resembling to tissue of origin, and most often confined to their site of origin, which neither invade surrounding tissues nor metastasize to distant organ(s). It is worth mentioning that following surgical intervention, tumor cells from leiomyoma and cardiac myxoma may enter blood circulation and reach distant sites. Benign mesenchymal tumors are composed of mature connective tissues, which tend to compress surrounding tissues resulting in clinical manifestations.

Lipoma

Lipomas are most common benign mesenchymal tumors derived from adipocytes that present as soft painless subcutaneous nodules anywhere in the body, i.e. subcutaneous, shoulders, buttocks, proximal extremities, abdominal region, and deep-seated region (intramuscular, parosteal, visceral), where normal adipocytes are present. Subcutaneous lipomas are not usually fixed to the underlying fascia. That is why the fibrous capsule should be surgically removed completely to prevent its recurrence. Lipomas very rarely arise from the viscera.

- **Predisposing factors:** Multiple lipomas are present in 5–10% of affected patients in the settings of familial lipomatosis or genetic disorders (e.g. familial multiple lipomatosis, Gardner syndrome, multiple endocrine neoplasia type 1, Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome, benign symmetric lipomatosis, Proteus syndrome, and Dercum disease).
- **Gross morphology:** Tumor is well-circumscribed, and rounded/lobulated mass that compresses the surrounding tissues. Cut surface reveals homogenous and yellow appearance of <5 cm in size. Surgical specimen of lipoma is shown in Fig. 6.12.
- **Histologic examination:** Tumor is composed of mature, normal-appearing adipocytes with a small eccentric nucleus intermixed among thin fibrous septa containing blood vessels. Lipoma must be carefully distinguished from low-grade liposarcoma. Histology of lipoma is shown in Fig. 6.13.
- **Treatment:** Most lipomas are removed by surgical excision. Recurrences after removal of lipomas are uncommon. Liposuction treatment uses a syringe and needle to remove the fatty tissue.

Uterine Leiomyoma

Leiomyoma (fibroid) is the most common benign tumor of the uterus, usually arising in women of reproductive age. Tumor originates from smooth muscle cells of

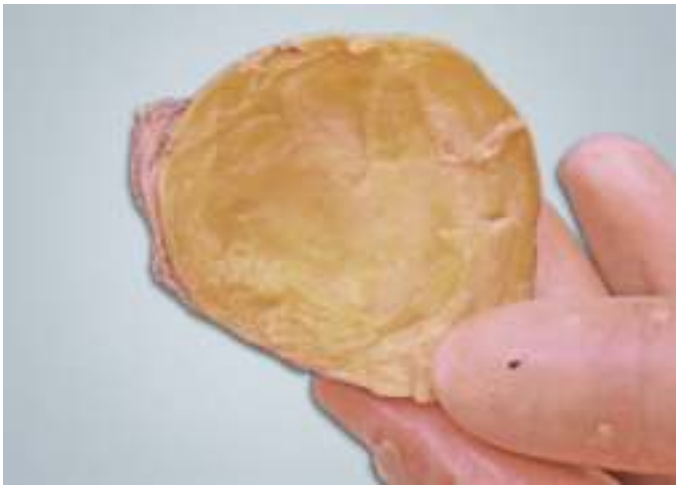


Fig. 6.12: Surgical specimen of lipoma. Tumor is encapsulated varying in size. Cut surface shows yellow and translucent adipose tissue.

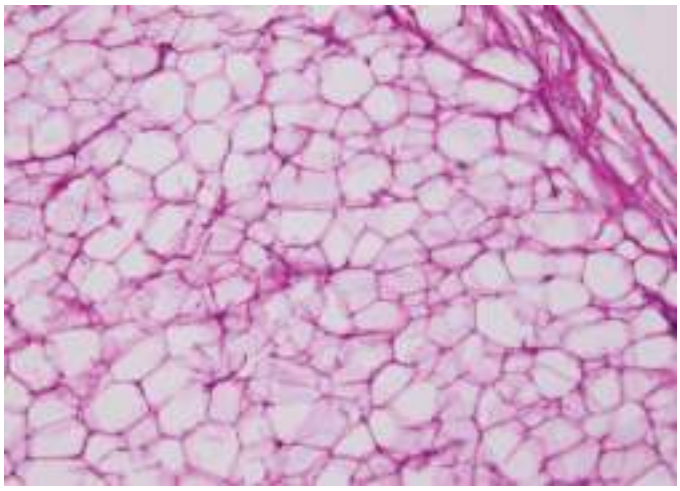


Fig. 6.13: Histology of lipoma Tumor is indistinguishable from normal adipose tissue. Tumor is composed of lobules of well-differentiated unremarkable adipocytes with inconspicuous eccentrically located nuclei intermixed among thin fibrous septa containing blood vessels (400X).

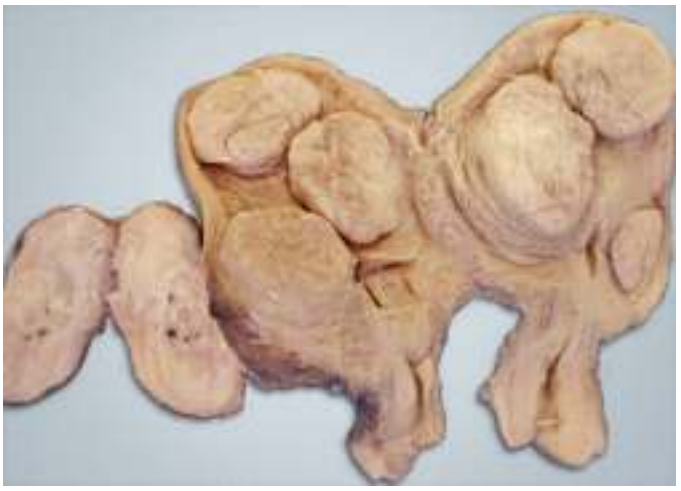


Fig. 6.14: Surgical specimen of uterine leiomyomas. Leiomyoma is a benign mesenchymal tumor derived from smooth muscle and located in the uterus: intramural, submucosal and subserosal. Tumors are most often multiple, well-circumscribed and nonencapsulated. Cut surface of leiomyomas in uterus reveals white or tan-white whorled, firm and bulging above surface.

the myometrium in the uterine corpus (intramural, submucous and subserous regions), cervix and uterine ligaments. Tumor can cause painful menstruation and infertility. Majority of leiomyomas are present within wall (intramural), but some are pedunculated and present in submucosal and subserosal region.

- **Pathogenesis:** Ovarian steroid hormones play key role in the pathogenesis of uterine leiomyomas. Estradiol and progesterone induce mature smooth muscle cells to release mitogenic factors, which stimulate adjacent immature smooth cells leading to uterine leiomyoma with undifferentiated cells that support neoplastic process. Tumor most often regresses after the menopause as a result of decreased estrogens.
- **Molecular/cytogenetic alterations:** Leiomyoma shows chromosomal abnormalities of 12q, 7q and 6p resulting in rearrangement of genes.
- **Clinical features:** Symptoms depend on the size and location of leiomyoma. Patient can present with heavy menstrual bleeding, pelvic pain, frequency of micturition, difficulty in emptying urinary bladder infertility, and backache.
- **Gross morphology:** Tumors are discrete, firm, pale gray, sharply circumscribed and varying in size without encapsulation. Cut section of leiomyoma reveals whorled pattern reflecting the fact that tumor is composed of smooth muscle bundles. Secondary changes in large leiomyomas include yellow-brown to red softening (red cystic degeneration), atrophy, hyaline degeneration, cystic degeneration, fatty change, dystrophic calcification, and chondroid/osteoid metaplasia. Surgical specimen of uterine leiomyomas is shown in Fig. 6.14.
- **Histologic examination:** Tumor is composed of smooth muscle cells arranged in whorled fashion, that resembles the uninvolved myometrium. Individual smooth muscle cells are uniform in size and shape containing oval nucleus and long slender bipolar cytoplasmic processes. Bipolar mitotic figures are sparse in leiomyoma. Histologic examination of uterine leiomyoma is shown in Fig. 6.15.
- **Histologic variants:** Histologic variants of leiomyoma include cellular leiomyoma, leiomyoma with bizarre nuclei, mitotically active leiomyoma, hydropic leiomyoma, apoplectic leiomyoma, lipomatous

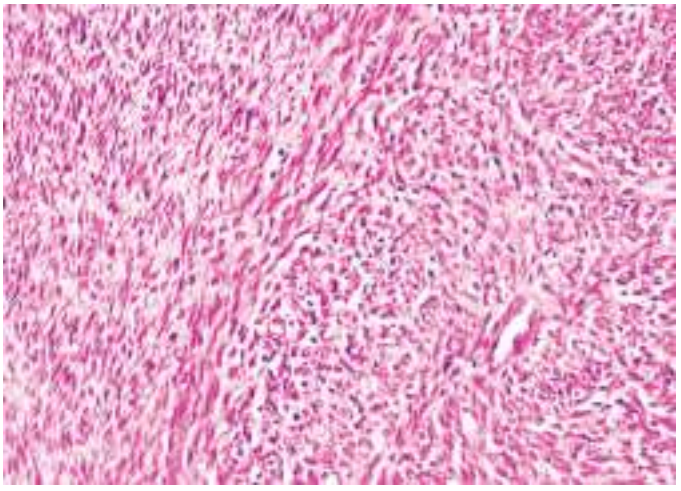


Fig. 6.15: Histology of uterine leiomyoma. Normal myometrium is at the left, and the neoplasm is well-differentiated so that the leiomyoma at the right hardly appears different. Bundles of smooth muscle are interlacing in the tumor mass (400X).

leiomyoma (lipolieomyoma), epithelioid leiomyoma, myxoid leiomyoma, dissecting (cotyledon-like) leiomyoma, diffuse leiomyomatosis leiomyoma, intravenous leiomyoma and metastasizing leiomyoma.

- **Immunohistochemistry:** Neoplastic cells of leiomyoma are immunoreactive with smooth muscle actin (SMA), desmin, muscle specific actin (MSA), h-caldesmon, smooth muscle myosin heavy chain (SMMHC) and estrogen/progesterone receptors.
- **Treatment:** Treatment options for uterine leiomyoma are uterine artery embolization, radiofrequency ablation, laparoscopic or robotic myomectomy, and hysteroscopic myomectomy.

Vascular Tumors

Benign vascular tumors are composed of circumscribed proliferation of predominantly small capillary-sized blood vessels. These are most common tumors of childhood and infancy located in the superficial region of head and neck. Hemangioma most often occurs in infants. Tumors are rare in deep visceral and skeletal regions. Recent studies revealed that benign vascular tumors result from endothelial proliferation with uncontrolled angiogenesis and abnormal function of downstream HIF1A,VEGF and PI3K/AKT signal transduction pathways.

- **Clinical features:** Symptoms and signs depend on size of tumor and its anatomic site. Tumor may compress surrounding structures and cause symptoms in patients. Hemangioblastoma in the brain may cause balance problems, headache, nausea or vomiting. Spinal hemangioblastoma may cause constipation, bowel and urinary incontinence, numbness and muscle weakness.
- **Gross morphology:** Deep-seated vascular tumors are well circumscribed and solid to cystic with dilated blood vessels with/without thrombus formation. Tumor may be polypoid with a stalk. Hemangioblastoma is well-circumscribed, lobulated, gray to brown and solid and cystic mass surrounded by a pseudocapsule.
- **Histologic examination: Capillary hemangioma** consists of capillary-sized vascular channels filled with blood/thrombi separated by scant connective tissue stroma, and lined by single layer of flattened endothelial cells. Histology of capillary hemangioma is shown in Fig. 6.16.
  - Cavernous hemangioma shows predominantly ectatic vascular channels in the dermis and subdermis in pediatric age group during first year of life.
  - Glomeruloid hemangioma resembles glomerular capillaries.

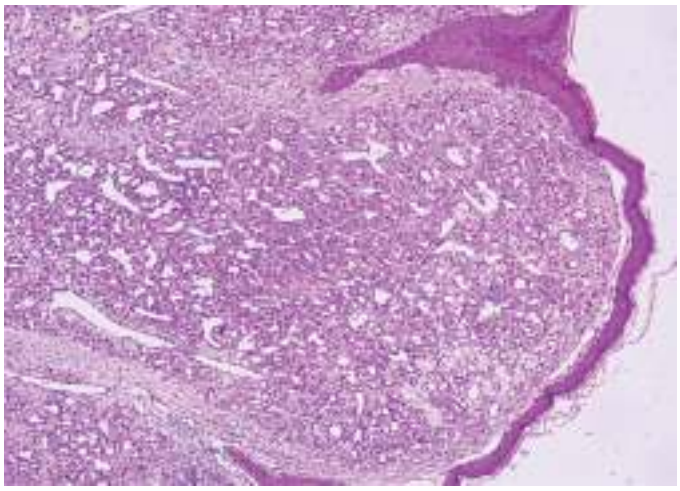


Fig. 6.16: Histology of capillary hemangioma. Capillary hemangioma is composed of closely packed aggregated of thin-walled capillaries filled with blood and lined by single layer of bland endothelial cells (400X).

  - Hemangioblastoma originates from the cells in the lining of the blood vessels of cerebellum, brainstem, spinal cord or retina of adults. Tumor occurring in the setting of von-Hippel-Lindau (vHL) disease in 20–25% of cases is composed of neoplastic stromal cells with mild nuclear pleomorphism, clear cytoplasm and lipid containing vacuoles arranged between numerous thin-walled small blood vessels.
- **Immunohistochemistry:** Immunohistochemical markers for vascular differentiation include CD31, CD34, D2–40 (podoplanin), factor VIII and vWF, *Ulex europaeus*, CD141 and Fli-1.
- **Treatment:** Microsurgical excision of benign vascular tumor using a surgical microscope and fine operating tools cures the patients.

Chondroma

A chondroma is benign tumor of mature hyaline cartilage within medullary cavity, periosteum and soft tissue. Chondroma arises in the region of metaphysis within medullary cavity of the tubular bones of hands and feet, which grows slowly, causes gradual expansion of the bone and can induce pain and pathologic fracture. Less commonly, parosteal chondroma can occur in the long bones, i.e. humerus and femur in children and adults. Rarely, multiple enchondromas occur in the settings of Ollier disease or Maffucci syndrome in children and associated with deformity.

- **Gross morphology:** Tumor has pale blue and glassy appearance due to presence of hyaline cartilage.
- **Histologic examination:** Tumor is lobulated and hypocellular and composed of mononuclear chondrocytes with inconspicuous small nuclei, and arranged in clusters with abundant intercellular

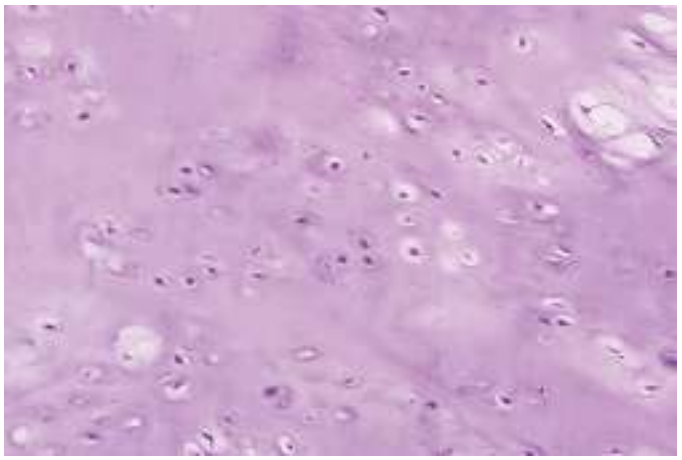


Fig. 6.17: Histologic examination of chondroma. Tumors are composed of well-differentiated hyaline cartilage. Chondrocytes contain small round nuclei with condensed chromatin. These are situated within sharp-edged lacunar spaces (100X).

hyaline cartilaginous matrix. Histologic examination of chondroma is shown in Fig. 6.17.

- **Treatment:** Surgical excision of chondroma cures the patients.

Osteoma

Osteoma is a benign bone forming tumor and composed of mature cortical type or less frequently, trabecular bone. Tumor occurs on the surface of craniofacial skeleton (i.e. paranasal sinuses, orbit, nasal cavity, jaw bones, cranial vault), and rarely it may occur in the long bones. Osteoma is relatively of small size but may produce severe symptoms because of its situation.

- **Gross morphology:** Gross examination reveals well-circumscribed solitary, oval, round or polypoid tumor attached to the underlying bone and cut surface with dense compact bone or trabecular bone.
- **Histologic examination:** Tumor consists of admixture or mature and woven bone patterns with Haversian-like canals with typical cortical type bone architecture that lacks cellular atypia.
- **Treatment:** Surgical excision of osteoma cures the patients.

BORDERLINE TUMORS

Borderline tumors of ovary have low-malignant potential defined histologically by atypical proliferation of cells without stromal invasion in 20–40 years old women, which account for 14–15% of all primary ovarian neoplasms. Examples of borderline ovarian neoplasms include serous tumor (K-RAS mutation), mucinous tumor (K-RAS mutation), endometrioid tumor (β-catenin, PTEN mutation), clear cell tumor and transitional cell tumor (Brenner tumor). Gene mutations in borderline ovarian tumors are given in Table 6.12.

Table 6.12 Gene mutations in borderline ovarian tumors

Histologic Variants	Gene Mutations
Borderline serous ovarian tumor	▪ RAS gene (67%) ▪ BRAF gene (67%)
Borderline mucinous ovarian tumor	RAS gene (>60%)
Borderline endometrioid ovarian tumor	▪ β-catenin (>50%), loss of heterozygosity ▪ PTEN gene (20%) ▪ Microsatellite instability (13%)
Borderline clear cell ovarian tumor	▪ K-RAS gene (5–16%) ▪ Microsatellite instability (13–50%)

- **Serous borderline tumor of ovary:** Borderline serous tumor of ovary most often affects women of 20–50 years of age. It is associated with regional lymphadenopathy and peritoneal 'implants' reflecting 'multifocal origin' rather than metastasis. Tumor shows nuclear atypia and increased mitotic activity and confined to the ovary without stromal invasion. Tumor cells are positive for S-100 calcium binding protein. Prognosis is excellent after surgical excision. Histology of serous borderline tumor of ovary is shown in Fig. 6.18.
- **Mucinous borderline tumor of ovary:** Borderline mucinous tumor of ovary most often affects younger women (mean age 45 years). Tumor is associated with implants (invasive in some cases) in abdomen or pelvic region. K-RAS gene mutation is observed in 60% of cases. It is important to establish a diagnosis by exclusion of metastatic adenocarcinoma of gastrointestinal tract by immunohistochemistry technique using a cytokeratin panel.

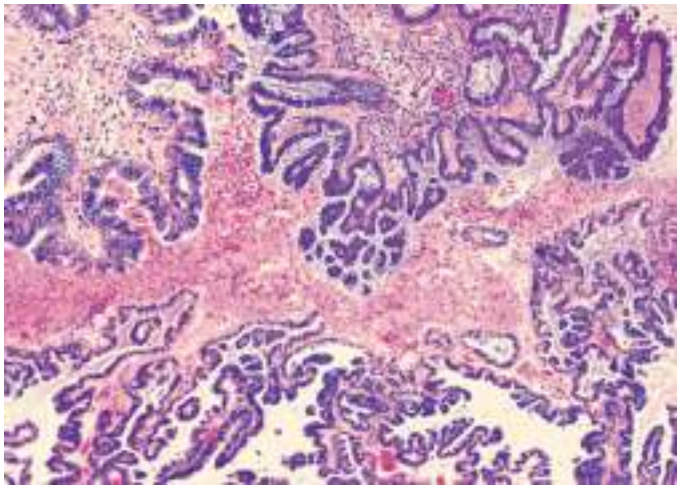


Fig. 6.18: Histology of serous borderline tumor of ovary. Tumor shows varying degree of nuclear atypia and increased mitotic activity confined to epithelium. There is no invasion of stroma by tumor cells (100X).

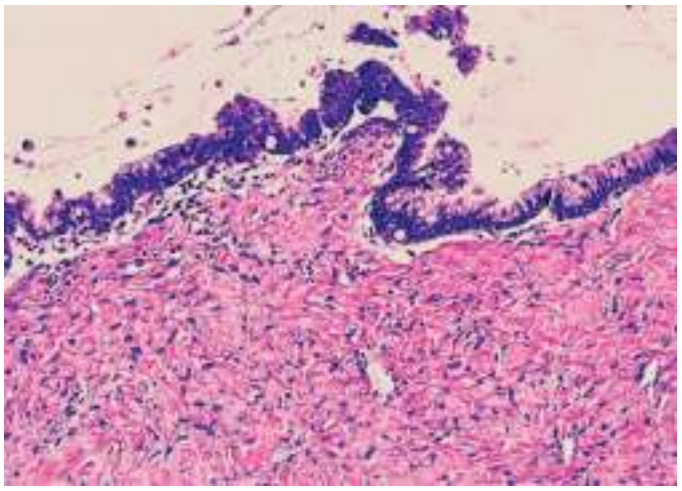


Fig. 6.19: Histology of mucinous borderline tumor of ovary. Tumor shows varying degree of nuclear atypia and increased mitotic activity confined to epithelium. There is no invasion of stroma by tumor cells (100X).

- **Histologic variants:** Borderline mucinous tumor of ovary has distinct histologic variants: intestinal (90%) and endocervical (10%) subtypes. Intestinal subtype coexists with pseudomyxoma peritonei in 70% of cases. The Müllerian subtype—mucinous borderline tumor of ovary is bilateral in up to 40% of cases and may coexist with ipsilateral ovarian or pelvic endometriosis in 20–30% of cases.
- **Histologic examination:** Borderline mucinous tumor of ovary has complex architecture with tufting and villus histology that shows low-grade dysplasia, and mitosis without stromal invasion. Histology of mucinous borderline tumor of ovary is shown in Fig. 6.19.

- **Endometrioid borderline tumor of ovary:** Borderline endometrioid tumor of ovary is characterized by mutations involving the β-catenin gene (50%), PTEN gene (20%) and microsatellite instability (up to 50%). The tumor has potential to progress to low-grade invasive endometrioid carcinoma. Endometriosis and endometrioid adenofibroma serve as precursors of borderline endometrioid ovarian tumor.

MALIGNANT EPITHELIAL TUMORS

Like benign epithelial tumors, carcinomas can arise from squamous epithelium (skin, mouth, esophagus and vagina, as well regions of squamous metaplasia in bronchi or squamocolumnar junction of the uterine cervix) or glandular epithelium (ducts, acini), epithelium (e.g. stomach, colon, lung, breast, endometrium, ovary, pancreas, and prostate), and transitional epithelium (renal pelvis, ureter and urinary bladder). Adenocarcinoma may exhibit acinar, solid, papillary, micropapillary, lepidic with preserved alveolar structure and mucin-secreting (mucinous) patterns. Carcinomas can be further subclassified according to their ability to invade, clinical behavior based on histologic grading of cellular differentiation. Tissue sites of more common histologic types of carcinomas are given in Table 6.13.

- **Rate of growth of malignant epithelial tumors.** Benign epithelial tumors tend to grow slower, whereas malignant epithelial tumors tend to grow more quickly, most often at a rate corresponding to their degree of anaplasia. Growth fraction refers to the proportion of CSCs in the proliferative phase. At the point when most malignant tumors are clinically detected, the growth fraction is less than

Table 6.13 Tissue sites of more common histologic types of carcinomas

Squamous Cell Carcinoma in Tissue Sites
▪ Squamous cell carcinoma of skin
▪ Squamous cell carcinoma of nasal cavity
▪ Squamous cell carcinoma in oropharynx
▪ Squamous cell carcinoma in larynx
▪ Squamous cell lung carcinoma
▪ Squamous cell carcinoma of esophagus
▪ Squamous cell carcinoma of cervix
Adenocarcinoma in Tissue Sites
▪ Breast adenocarcinoma
▪ Esophageal adenocarcinoma in the setting of Barrett's esophagus
▪ Gastric adenocarcinoma
▪ Colorectal adenocarcinoma
▪ Pancreatic adenocarcinoma
▪ Prostatic adenocarcinoma
▪ Endometrial adenocarcinoma
▪ Ovarian adenocarcinoma
▪ Lung adenocarcinoma
Transitional Cell Carcinoma
▪ Transitional cell carcinoma of renal pelvis
▪ Transitional cell carcinoma of ureter
▪ Transitional cell carcinoma of urinary bladder
▪ Transitional cell carcinoma of urethra
Clear Cell carcinoma
▪ Renal cell carcinoma, clear cell variant
▪ Clear cell carcinoma of vagina in daughter [associated with diethylstilbestrol (DES) administration to mother]
Other Type of Carcinomas
▪ Small cell lung carcinoma
▪ Large cell lung carcinoma
▪ Hepatocellular carcinoma
▪ Gastric signet cell carcinoma (linitis plastica)

The epithelia of the body are derived from all three germ cell layers: ectoderm, mesoderm and endoderm. Squamous cell carcinoma of skin arises from ectoderm. Gastrointestinal adenocarcinoma arises endoderm. Renal cell carcinoma and connective tissue sarcoma originates from mesoderm.

20% (i.e. most malignant tumors have their most rapid rate of growth prior to detection).

- **Tumor invasion and metastasis:** Histologic features and rate of growth alone cannot distinguish between benign and malignant tumors. Invasion and metastasis to distant organs are indicators of malignancy, which can distinguish malignant tumors from benign tumors. Malignant tumors are rapidly growing, poorly circumscribed, and invade and destroy surrounding normal tissues, grow quickly and metastasize via lymphatic route to draining lymph nodes, via hematogenous route to distant organ(s) and via transcoelomic route to peritoneum. The metastases spawned by malignant tumors are responsible for almost all deaths from cancer.
  - **Preferential routes of metastasis:** Carcinomas usually metastasize via lymphatic route to draining lymph nodes and via hematogenous route to distant organs (i.e. liver, lungs, bones, brain and adrenal glands).
  - **Rarely metastasizing tumors:** Few organs such as heart, skeletal muscle and spleen do not allow tumor metastasis to grow due to anatomical consideration. Basal cell carcinoma of skin rarely metastasizes.
  - **Non-metastatic malignant tumors:** Some malignant tumors do not metastasize (e.g. gliomas and basal cell carcinoma of skin), but they do invade. Overall, 30% of malignant solid tumors have metastases to distant organ(s) at the time they are clinically detected.
  - **Metastases of solid tumors:** Approximately 30% of newly diagnosed patients with solid tumors (except squamous cell carcinoma of skin and melanoma) most often present with clinically evident metastases. An additional 20% of cases have occult (hidden) metastases at the time of diagnosis.
- **Clinical features:** Malignant epithelial tumors can cause bleeding from ulcerated growth, tissue destruction, obstruction of blood flow in vital organs, and cachexia (severe weight loss and reduction in skeletal muscle mass) and production of tumor specific products/hormones. Poor prognostic factors for carcinomas include large tumor size, deep invasion, perineural invasion, lymphovascular invasion, poor differentiation and immunosuppression.
- **Gross morphologic features:** Desmoplasia refers to the proliferation of non-neoplastic connective tissue in malignant tumor that gives the tumor a firm, fibrous with 'scirrhous appearance' in the settings of infiltrating duct carcinoma of breast (scirrhous type), diffuse type gastric carcinoma (linitis plastica), pancreatic adenocarcinoma and prostatic adenocarcinoma.
  - **Tumor stroma:** Malignant epithelial tumors synthesize growth factors that induce formation of stromal supporting architecture upon which tumors can grow.
  - **Tumor angiogenesis:** Vascularity in malignant epithelial tumors results from elaboration of angiogenic factors that promote angiogenesis. Malignant epithelial tumors must establish a blood supply for their growth. Blood vessels are leaky hence cut surface of malignant epithelial tumor may reveal areas of hemorrhage.
  - **Tumor necrosis:** Necrosis is common in malignant epithelial tumors because CSCs function poorly and cannot maintain their architecture or blood supply or metabolic demands. Necrosis becomes more likely as the size of malignant epithelial tumor increases in size and undergoes dedifferentiation.
- **Tumor invasion assessment on histologic examination:** *Dysplasia* is disordered growth of epithelium with loss of cellular uniformity and architectural orientation that covers the spectrum of changes short of invasive carcinoma in some cases: mild dysplasia, moderate dysplasia and severe dysplasia/carcinoma *in situ*. Dysplasia can be reversible, if inciting agent is removed.
  - Carcinoma *in situ* (intraepithelial neoplasia) involves full-thickness severe dysplasia of the epithelium without invading through the basement membrane. It is frequently observed in the cervix uteri at the junction of ectocervix and endocervix.
  - Microinvasive carcinoma refers to stromal invasion <5 mm beneath the basement membrane without evidence of lymphatic or vascular involvement.
  - Early invasive carcinoma refers to invasion through the basement membrane and surrounding tissue through the action of proteolytic enzymes and detachment of CSCs resulting from lack of surface adhesion molecules such as E-cadherin.
  - Advanced invasive carcinoma disseminates via lymphatic route to draining lymph nodes, blood vessels via hematogenous route (liver, lungs, brain and bone) and via transcoelomic route within peritoneal cavity (Krukenberg tumor of ovary from breast carcinoma, gastric carcinoma and colon carcinoma).
- **Cytomorphologic features:** Histologic features of malignant epithelial tumors include: pleomorphism (variation in nuclear and cytoplasmic shape between cells), increased abnormal tripolar or quadripolar mitotic figures, hyperchromasia (increased nuclear basophilia) and hypercellularity, with loss of normal polarity.

- **Differentiation:** Differentiation is defined how histologically a malignant epithelial tumor closely resembles the structure of normal tissue. Differentiation is used for histologic grading system, which is different for each type of malignant epithelial tumor such as well-moderate-poor differentiation (anaplasia).
- **Pleomorphism:** CSCs often exhibit pleomorphism (i.e. variation in size and shape). Anaplastic tumors contain multinucleated giant tumor cells. Some tumor giant cells contain a single huge polymorphic hyperchromatic nucleus, while other tumor giant cells contain two or more bizarre nuclei.
- **Abnormal nuclear morphology:** Malignant epithelial cells (i.e. CSCs) generally have nuclei, prominent and increased nucleocytoplasmic ratio that approaches 1:1 instead of the normal 1:4 or 1:6.
- **Hypercellularity and loss of polarity:** Normal cells are anchored and oriented to the basement membrane. On contrary, anaplastic cells lose this uniform orientation and grow in a disorganized fashion.
- **Hyperchromasia:** CSCs exhibit hyperchromatism and increased basophilia of the nucleus. Chromatin condensation occurs during the G2/S phase of the cell cycle.

- **Anaplasia:** Anaplasia refers to dedifferentiation (i.e. loss of structural and functional differentiation) of cells during carcinogenesis that is considered a molecular hallmark of cancer.
  - Anaplastic cells appear primitive (undifferentiated) and lack specialization along with a particular tissue cell line of origin.
  - Anaplastic tumor is composed of pleomorphic cells with bizarre hyperchromatic nuclei, abnormal nuclear contours, prominent nucleoli with nucleus to cytoplasmic ratio approaching 1:1, cellular dyspolarity, tumor giant cells with two or more bizarre nuclei. Numerous bizarre tripolar or quadripolar mitotic figures are indicative of proliferative activity in parenchymal cells with increase in number of chromosomes and DNA content (aneuploidy). Anaplastic cells exhibit dark staining on light microscopy in hematoxylin and eosin-stained tissue sections.
  - It is worth mentioning that nuclei in the normal epithelial cells are oriented along the basement membrane, whereas nuclei in anaplastic cells exhibit loss of polarity of nuclei away from basement membrane growing in an anarchic disorganized fashion.
  - Anaplastic tumors are rapidly growing, invading and destroying surrounding normal tissue and metastasizing to distant organ(s). However, extent of metastasis of tumor is used for clinical staging.
- **Atypical mitotic figures:** Atypical mitotic figure (tripolar or quadripolar) count is an important microscopic parameter in tumor classification and prognosis in several human malignancies. Chromatin condensation occurs during the G2/S phase of the cell cycle. Bipolar mitotic figures are demonstrated in proliferating cells in normal bone marrow, intestinal cells, hepatocytes; certain benign tumors and some low-grade malignant tumors, and non-neoplastic proliferations. Counting mitotic figures in hematoxylin and eosin-stained histologic sections are an integral part of the grading system for assessment of prognosis and clinical decisions.
- **Immunohistochemistry:** Panel of immunohistochemical markers are used to diagnose epithelial tissue-derived carcinomas. Immunohistochemical markers on such epithelial-derived malignant tumors can yield conflicting results.
- **Electron microscopy:** Carcinomas exhibit reduced adhesion molecules, free ribosomes, simplification of rough endoplasmic reticulum, pleomorphic mitochondria, and disruption of cytoskeleton (microfilaments, microtubules and intermediate filaments).
  - Classification of pleomorphic malignancies is frequently difficult and important with regard to treatment.
  - Their histologic differential diagnosis is usually wide including sarcomatoid carcinoma, melanoma, anaplastic lymphoma and sarcomas with overlapping histologic appearances (e.g. high-grade fibrosarcoma, myxofibrosarcoma, pleomorphic sarcoma, pleomorphic leiomyosarcoma, pleomorphic rhabdomyosarcoma).
  - In such cases, electron microscopy provides valuable diagnostic subcellular features that indicate a specific line of differentiation.
- **Grading system:** Grading of tumors is based on degree of differentiation, nuclear pleomorphism, size of nucleoli and mitotic activity (atypical tripolar or quadripolar). Well-differentiated carcinoma looks very similar to normal tissue. Moderately-differentiated carcinoma looks something like normal tissue. Poorly-differentiated carcinoma (anaplasia) virtually has no resemblance to normal tissues.
- **Staging system:** Size and spread of malignant tumors determine the stage. *In situ* carcinoma refers to epithelium-derived malignant tumors confined to just the epithelium without invading the basement membrane.
  - **Microinvasion:** Microinvasion refers to spread of malignant epithelial tumor just beyond the point of origin through the basement membrane.

- **Local invasion:** Local invasion refers to spread of malignant epithelial tumor within the organ of origin or contiguous structures.
- **Local spread:** Local spread refers to non-contiguous spread of the malignant epithelial tumor via lymphatic route to draining lymph nodes.
- **Distant metastasis:** Distant metastasis refers to spread of malignant epithelial tumor to distant organ(s) via hematogenous route far away from draining lymph nodes.

Squamous Cell Carcinoma in Various Tissues

Squamous cell carcinoma is a malignant epithelial tumor that begins in the thin and flat squamous epithelium in the tissues. Squamous epithelium forms the surface of the skin, the lining of the hollow organs of the body, and the lining of the respiratory tract (nasal cavity, larynx, lung) and digestive tracts (oropharynx, esophagus), and uterine cervix.

Majority of the squamous cell carcinoma cases occur in various anatomic sites: skin, digestive tract (oropharynx, esophagus), respiratory tract (nasal cavity, lung) and uterine cervix.

Squamous Cell Carcinoma of Skin

Squamous cell carcinoma of skin most often occurs in sun-exposed anatomic sites (e.g. scalp, ear, lip, nose, eyelid) that displays variable degrees of differentiation and cytological features. Well-differentiated squamous cell carcinoma grows slowly. In contrast, poorly-differentiated squamous cell carcinoma spreads early via lymphatic route to draining lymph nodes.

- **Predisposing factors:** Predisposing factors for squamous cell carcinoma of skin are ultraviolet radiation, tobacco smoking, chewing tobacco, marijuana, betel nuts and pan (India), actinic keratosis, xeroderma pigmentosum, chronic skin ulcer, and discharging sinus tract. Epstein-Barr virus is demonstrated in all oral cancers.
- **Pathogenesis:** Cutaneous squamous cell carcinoma develops through a multistep process. Ultraviolet radiation, gene mutations (TP53, CDKN2A, TERT, EGFR, NOTCH1, NOTCH2) and molecular pathways (RAS/RAF/MEK/ERK and PI3K/AKT/mTOR) play important role in its pathogenesis.
- **Gross morphology:** Tumor is most often hyperkeratotic scaly plaque, that may exhibit induration, ulceration and hemorrhage.
- **Histologic examination:** Tumor is composed of cells arranged in irregular strands and columns. CSCs invade basement membrane and underlying connective tissue. Grading of the tumor is based on degree of differentiation and keratinization.
  - Well-differentiated squamous cell carcinoma of skin is slow growing tumor that looks very similar to normal tissue and composed of solid nests of polygonal-shaped cells with distinct borders, intercellular bridges, and pink keratinized cytoplasm. Histology of well-differentiated squamous cell carcinoma of skin is shown in Fig. 6.20.
  - Moderately-differentiated squamous cell carcinoma of skin is composed of uniform cells with indistinct borders arranged in irregular nests, cords and sheets, focal keratinization and atypical mitotic figures.
  - Poorly-differentiated carcinoma (anaplastic carcinoma) of skin virtually has no resemblance to normal tissues. Tumor is composed of small cells with scanty cytoplasm and hyperchromatic nuclei arranged in small nests, cords, sheets and single cells, rare or lack of keratinization and brisk atypical mitotic figures.

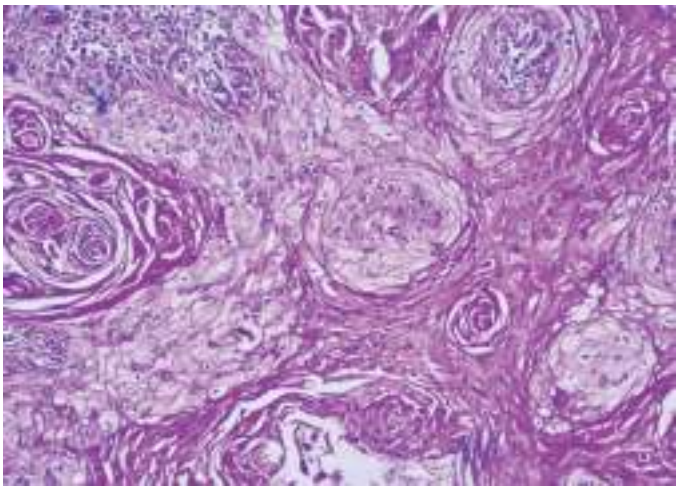


Fig. 6.20: Histology of well-differentiated squamous cell carcinoma of skin. Tumor is composed of solid nests of polygonal-shaped cells with distinct borders, intercellular bridges, pink keratinized cytoplasm and keratin formation (400X).

- **Immunohistochemistry:** Immunohistochemistry technique is usually essential to confirm the diagnosis and exclude melanoma, sarcoma, poorly-differentiated and undifferentiated squamous cell carcinoma. Immunohistochemical markers for squamous epithelial tissue differentiation include cytokeratins, i.e. CK9, CK10, CK11, CK16, CK17 (HMWCK), and epithelial membrane antigen (EMA).
- **Treatment:** Treatment options for high-risk squamous cell carcinoma of skin are Mohs surgery, surgical excision with adequate margins, curettage, electrodessication, radiation therapy; and immunotherapy if inoperable.

Squamous Cell Carcinoma of Oral Cavity and Tongue

About 95% of oral cavity cancers are squamous cell carcinoma. Tumor most often occurs in the floor of mouth, tongue, hard palate, base of tongue.

- **Predisposing factors:** Predisposing factors for squamous cell carcinoma in the region of oral cavity include alcohol consumption, tobacco smoking, tobacco chewing, marijuana, betel nuts and pan (India), Epstein-Barr virus and HPV 6, 8, 11 (10%).
- **Clinical features:** Patient presents with leukoplakia or mass with necrosis, ulcer and rolled border and induration specific for invasive tumor. Tumor spreads locally and via hematogenous route metastasizes to lung, liver, bone and mediastinum.
- **Histologic examination:** Histologic examination reveals verrucoid growth pattern but moderate/marked atypia at base, irregular and infiltrative stromal invasion.

Squamous Cell Carcinoma of Cervix

Squamous cell carcinoma of cervix is most common neoplasm in 40–55 years old women associated with high-risk human papillomavirus (HPV 16, 18), which arises from a precursor lesion at the squamous-columnar junction of the cervix. Grading of cervical squamous cell carcinoma is based on degree of differentiation, nuclear pleomorphism, size of nucleoli, mitotic activity and necrosis and does not correlate with prognosis. Lymphovascular invasion may be present.

- **Predisposing factors:** Predisposing factors are sexual activity with multiple partners, sexual contact with human papillomavirus 16, 18, 31,33 or 45, tobacco smoking, oral contraceptive use and immunosuppression. Steps in evolution of cervical squamous cell carcinoma include: cervical intraepithelial neoplasia (CIN-I, -II, -III), microinvasion, early invasion and advanced invasion stage.
- **Pathogenesis:** Human papillomavirus (HPV 16, 18, 31) encodes E6 and E7 oncoproteins, which bind to p53 and pRB respectively and interfere with normal functions of the tumor suppressors. HPV-E6 oncoprotein induces proteolytic degradation of p53 protein via ubiquitination pathway leading to deregulation of cell cycle, unrestricted cell proliferation and cervical carcinoma. HPV-E7 oncoprotein induces inactivation of pRB that results in overexpression of p16 tumor suppressor protein involved in cell cycle regulation by inhibiting cyclin-dependent kinases (CDKs). The p16 is a surrogative immunomarker for high-risk HPV infection.
- **Clinical features:** Patient in advanced disease presents with abnormal persistent bleeding or spotting between menstrual cycles or postcoital bleeding and pain, pelvic pain, foul smelling vaginal discharge (saprophytic infection), vaginal leakage of stool or urine from a fistula, anorexia, cachexia, weight loss and anemia.
- **Gross morphology:** Tumor is red, friable, indurated, or ulcerated lesion in early stage. Later, tumor is exophytic, nodular, papillary, polypoidal or ulcerated or invasive mass invading into surrounding structures.
- **Histologic examination:** Squamous cell carcinoma of cervix accounts for 80–90% of cases. Grading is based on degree of differentiation and keratinization. Tumor can be well-, moderately-, poorly-differentiated. Tumor is composed of mature atypical squamous cells with abundant keratin pearls, occasional well-developed intercellular bridges, minimal pleomorphism, minimal mitotic activity. Histology of well-differentiated squamous cell carcinoma of cervix is shown in Fig. 6.21. Histologic variants of cervical carcinoma are keratinizing, nonkeratinizing, papillary, basaloid, warty, verrucous, squamotransitional, lymphoepithelial-like and spindled/sarcomatoid.
- **Immunohistochemistry:** Immunohistochemistry technique is usually essential to confirm the diagnosis of poorly differentiated and undifferentiated squamous cell carcinoma. Immunohistochemical markers for cervical carcinoma include HPV, p16, Ki-67, pankeratin (CK5/CK6), AE1/AE3, CEA, p63, CD10, CD146, inhibin and β-hCG.
- **Treatment:** Cervical carcinoma is treated by radical hysterectomy and removal of pelvic lymph nodes with or without radiation to the pelvis, plus chemotherapy.

Squamous Cell Lung Carcinoma

Squamous cell lung carcinoma (SCLC) often occurs in the central region of the lung in the left or right bronchus. Staging is based on CT scan images according to the TNM (tumor-node-metastasis) staging system.

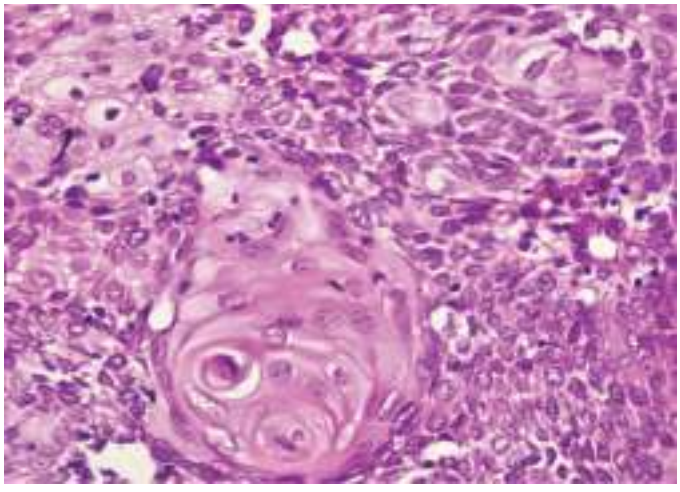


Fig. 6.21: Histology of well-differentiated squamous cell carcinoma of cervix. Tumor is composed of mature squamous cells with abundant keratin pearls, occasional well-developed intercellular bridges, minimal pleomorphism, minimal mitotic activity (400X).

- **Predisposing factors:** Predisposing factors of SCLC are tobacco smoking, exposure to radon gas, asbestos, uranium, arsenic, vinyl chloride, nickel chromates, coal products, mustard gas, chloromethyl ethers, gasoline and diesel exhaust.
- **Clinical features:** Symptoms of SCLC are related to extent of disease, i.e. cough, chest pain, shortness of breath, hemoptysis, wheezing, hoarseness, recurring chest infections, loss of appetite, weight loss, and weakness. Tumor invades adjoining structures, metastasizes via lymphatic route to regional lymph nodes and via hematogenous route to distant organ(s) because of constant flow of lymph and blood through the lungs. Advanced disease can cause bone pain, spinal cord impingement and neurological symptoms (i.e. headache, weakness or numbness of limbs, dizziness, seizures).
- **Gross morphology:** Bronchus exhibits fungating growth towards lumen extending in adjacent structures. Surgical specimen of squamous cell lung carcinoma is shown in Fig. 6.22.
- **Histologic examination:** Squamous cell lung carcinoma is categorized into well, moderately and poorly differentiated, based on amount of keratinization present in predominant component. Histology of well-differentiated squamous cell lung carcinoma is shown in Fig. 6.23.
- **Immunohistochemistry:** Tumor cells in SCLC are positive for p63, CK5/CK6 (85–100%), epithelial membrane antigen (EMA) and thrombomodulin (85–100%). Other variably expressed immunomarkers are CD15, carcinoembryonic antigen (CEA), HPV, p53, p40, S-100 and mesothelin.

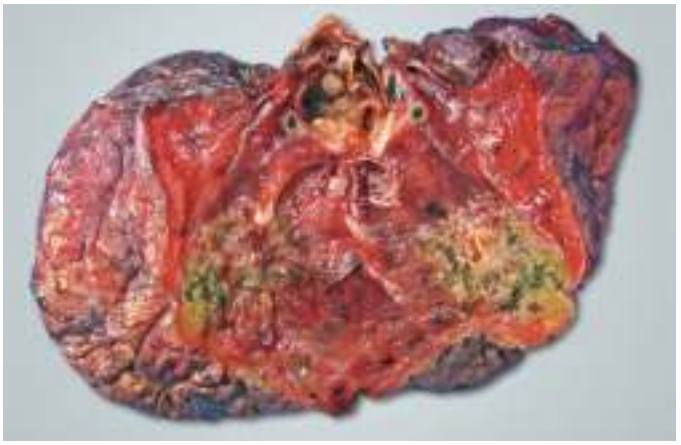


Fig. 6.22: Surgical specimen of squamous cell lung carcinoma. Patient presents with six months history of dysphagia to solids, nausea, weight loss, recent hemoptysis and onset of hoarseness of voice. He has history of chronic cigarette smoking. Chest auscultation reveals diminished breath sounds over right lung fields. Chest radiograph reveals a 8 cm hilar mass on the right. Laboratory studies show serum calcium 15 mg/dl (normal 8.5–10.5 mg/dl).

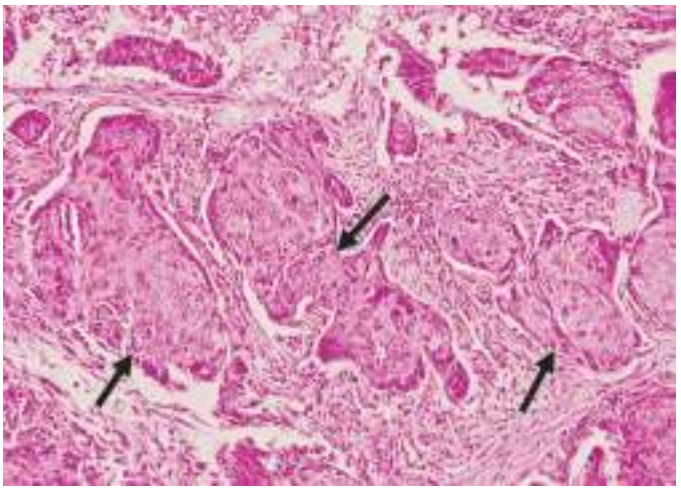


Fig. 6.23: Histology of well-differentiated squamous cell lung carcinoma. Tumor is composed of polygonal tumor cells with pink cytoplasm with distinct cell borders, intercellular bridges, hyperchromatic angular nuclei and many abnormal mitoses (arrows) (400X).

- **Laboratory diagnosis:** Diagnostic modalities for squamous cell lung carcinoma include bronchoscopy, imaging techniques, fine needle aspiration cytology, histologic examination, immunohistochemistry, and molecular testing (PD-L1, EGFR, ALK fusion oncogene and gene mutations).
- **Treatment:** Approved treatment options for squamous cell lung carcinoma include surgery, radiation therapy, chemotherapy, angiogenesis inhibitors and immunotherapy.

Adenocarcinoma in Various Tissues

Adenocarcinoma is a malignant epithelial tumor that begins in the glandular tissue, which lines certain internal organs and makes and releases substances in the body such as mucus, digestive juices and other fluids. Most adenocarcinomas occur in various anatomic sites, i.e. breast, esophagus (in the setting of Barrett's esophagus), stomach, colon, rectum, pancreas, lung, prostate, ovary (surface epithelium-derived) and uterine endometrium.

Breast Ductal Adenocarcinoma

Breast ductal adenocarcinoma arises from epithelial component cells of breast in terminal duct lobular unit (TDLU) or in axilla accessory breast tissue. *In situ* ductal carcinoma of breast does not invade the normal breast tissue, however has significant potential to become invasive carcinoma, that is the reason, *in situ* ductal carcinoma must be adequately treated to prevent the patient from developing invasive ductal adenocarcinoma of breast. CSCs of invasive ductal adenocarcinoma invade tissues outside the normal breast lobules and ducts to grow into underline connective tissue, which has potential to metastasize to draining

lymph nodes and distant organ(s). About 75–80% of breast carcinomas are invasive ductal adenocarcinoma, followed by invasive lobular carcinomas, which account for 10–15% of cases.

- **Predisposing factors:** Most studies have revealed that hormones, reproductive factors, dietary factors and genetic factors increase risk of development of breast carcinoma, which include: (a) prolonged exposure to estrogen in the settings of early menarche, late menopause, nulliparity, post-menopausal women with obesity and estrogen producing ovarian tumors, hormonal replacement therapy, (b) genetic risk factors include familial clustering of breast cancer (BRCA1/BRCA2 germline mutations), Li-Fraumeni syndrome, Cowden disease, heterogenous career for ataxia-telangiectasia, (c) environmental risk factors include obesity, alcohol consumption, and lack of exercise, and (d) other risk factors include atypical proliferative breast disease, carcinoma of endometrium and opposite breast and increased mammographic density.
- **Clinical features:** Patient may present with unifocal palpable lump in the upper outer quadrant of breast, change in the size or shape of the breast, nipple retraction, nipple discharge, change in the color or texture of the skin. In screened populations, mammography reveals as a spiculated mass, architectural distortion with or without calcifications.
- **Gross morphology:** Gross examination may reveal evident tumor nodular mass with an irregular stellate outline/poorly circumscribed mass, firm to hard in consistency on cut surface. On cut surface, tumor may show streaks of chalky white elastic stroma penetrating surrounding stroma (crab-like) and calcification. Large tumor shows areas of hemorrhage and necrosis.
- **Histologic examination:** Histologic grading of breast ductal adenocarcinoma is based on the Nottingham/modified Bloom & Richardson Score: tubule formation, i.e. >75% (1 point), 10–75% (2 points), <10% (3 points); nuclear pleomorphism, i.e. small nuclei similar to normal ductal epithelial cells (1 point), moderate increase in nuclei (2 points), large pleomorphic nuclei (2 points), and mitotic count (1 to 3 points) depending on microscopic field area. Total score is calculated by adding points for tubule formation, nuclear pleomorphism and mitotic count: grade 1 (3–5 points), grade 2 (6–7 points), and grade 3 (8–9 points).
  - Histologic features of breast ductal carcinoma, not otherwise specified vary considerably from case to case. Architecture of tumor varies from sheets, nests, cords, individual cells, clusters with abundant eosinophilic, clear, foamy, granular cytoplasm and uniform to pleomorphic nuclei, but lacks cytomorphologic features of invasive lobular carcinoma of breast.
  - Tubule formations are prominent in well-differentiated breast ductal adenocarcinoma, but absent in poorly differentiated tumors.
  - Mitotic figures are variable from virtually absent to extensive in these tumors. Calcification with variable necrosis is demonstrated in 60% of cases. Perineural invasion is demonstrated in 28% of cases. Histology of breast invasive ductal adenocarcinoma is shown in Fig. 6.24.
- **Laboratory diagnosis:** Screening of patients is done by imaging techniques (mammography, targeted ultrasonography, magnetic resonance imaging), clinical history, clinical examination, fine needle aspiration cytology, surgical specimen examination (gross morphological features, histologic examination and immunohistochemistry), flow cytometry (DNA ploidy), serum tumor markers (CA 15-3, CA 27-29, BRCA, mammaglobin, gross cystic fluid protein 15) and fluorescence *in situ* hybridization technique.
- **Immunohistochemistry:** Breast ductal adenocarcinoma shows positivity for ER/PR (50%), GCDFP (50), mammaglobin (50%), HER2/neu (50%), Ki-67, p53 (poor prognostic marker), CK7 and GATA3.
- **Treatment:** Breast ductal adenocarcinoma is treated by surgical excision, i.e. breast conserving surgery (lumpectomy), mastectomy, with or without axillary lymph node dissection. Patient is given local radiotherapy after lumpectomy or chest wall after mastectomy to reduce local recurrence risk. Systemic chemotherapeutic drugs can be administered in these patients. Selection of treatment is based on tumor size, histologic grade, stage, biomarker status

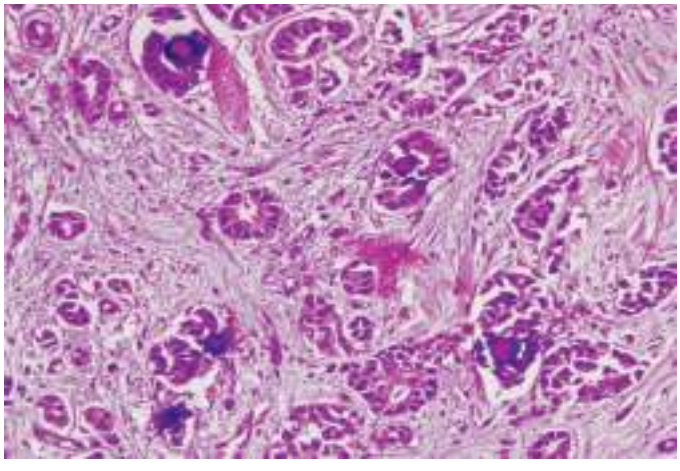


Fig. 6.24: Histology of breast invasive ductal adenocarcinoma. Tumor is composed of atypical pleomorphic cells with hyperchromatic nuclei and arranged in glandular structures, clusters, and sheets invading surrounding connective tissue stroma of breast (400X).

(estrogen receptor, progesterone receptor, HER2/neu status), results of genomic risk assessment (e.g. oncotype DX recurrence Score®, anatomic location, patient's age and heritable breast cancer risk (e.g. BRCA status), prior exposure to chemotherapy or radiation therapy, cosmetic outcome and patient's preference.

Colorectal Adenocarcinoma

More than 90% of colorectal carcinomas are originating from the epithelial cells of the colorectal mucosa of sigmoid colon, ascending colon, transverse colon, descending colon, and cecum. Tumor is detected on colonoscopy and confirmed on histologic examination.

- **Predisposing factors:** Predisposing factors of colorectal adenocarcinoma include inherited polyposis syndromes, Lynch syndrome also known as hereditary nonpolyposis colorectal cancer (HNPCC), inflammatory bowel disease, low-fiber and high-fat diet, red meat consumption, tobacco smoking and radiation therapy applied at abdomen.
- **Molecular genetic alterations:** Colorectal adenocarcinoma arises through chromosomal instability pathway (70–80%) or microsatellite instability pathway (10%). Most commonly mutated genes are APC, TP53 and K-RAS in colorectal adenocarcinoma. Tumor can be screened for microsatellite instability by immunohistochemistry technique to analyze MLH1, MSH2, MSH6 and PMS2. Stage of the disease is the most important prognostic factor.
- **Clinical features:** Right-sided colorectal adenocarcinoma causes rectal bleeding, anemia, weakness, constipation, unexplained weight loss and fatigue. Left-sided colorectal adenocarcinoma causes persistent change in bowel habits (constipation or diarrhea). Poor prognostic factors are positive margins of tumor, advanced stage, higher grade, lymphovascular and perineural invasion, CDX2 loss and high stromal content.
- **Gross morphology:** Tumor in the right colon appears polypoid or fungating or cauliflower-like mass. On the contrary, tumor in rectosigmoid region shows annular lesion that produces "napkin-ring" appearance. Surgical specimen of colon carcinoma is shown in Fig. 6.25.
- **Histologic examination:** Colorectal adenocarcinoma is usually well-differentiated (15–20%) or moderately differentiated (60–70%) or poorly differentiated. Neoplastic glands are often cribriform and filled with necrotic debris in both primary and metastatic sites. Marked desmoplasia is present at the edge of the tumor.
  - Well-differentiated tumor is composed of well-formed glandular structures with uniform, basally oriented nuclei that resemble adenomatous epithelium.
  - Moderately-differentiated tumor is composed of simple or slightly irregular glandular structures.
  - Poorly-differentiated tumor consists of sheets of cells without formation of glandular structures usually in right-sided. Histology of colorectal adenocarcinoma is shown in Fig. 6.26.
- **Immunohistochemistry:** Colorectal adenocarcinoma is positive for immunohistochemical markers CK20, CDX2, CEA, villin, CDX2 and β-catenin.
- **Poor prognostic factors:** Poor prognostic factors of colorectal carcinoma include tumor with positive margins, advanced stage, higher-grade, lymphovascular and perineural invasion, CDX2 loss and high stromal component.

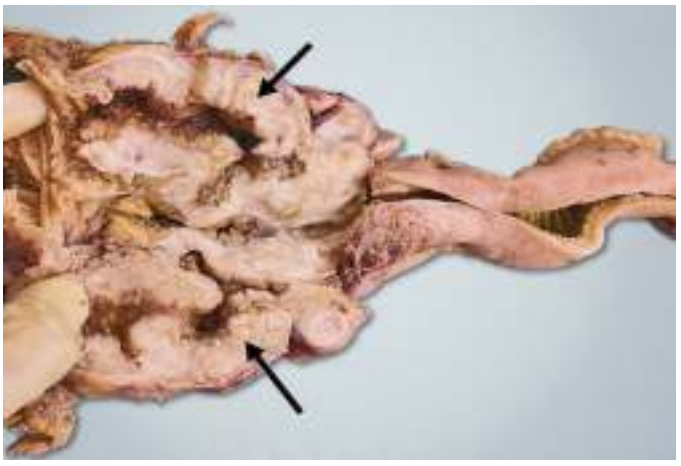


Fig. 6.25: Surgical specimen of colon carcinoma. Cut opened caecum shows fungating and ulcerated growth within lumen. Tumor is gray white firm with areas of hemorrhage and necrosis (arrows). (*Courtesy:* Dr. Sujata Kanetkar, Professor and Head, Department of Pathology, Krishna Institute of Medical Sciences, Krar, Maharashtra.)

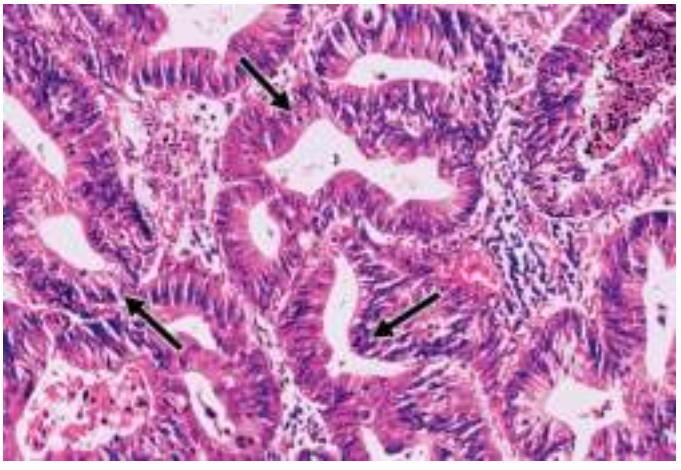


Fig. 6.26: Histology of colorectal adenocarcinoma. Tumor is composed of irregularly distributed tubular cribriform structures with abundant intracytoplasmic pool of mucin in 50% of the tumor area, and intraluminal necrosis in a desmoplastic stroma (arrows) (400X).

- **Treatment:** Patients are treated by combination of surgery, radiation therapy, which can be used to slow the spread of disease.

Intestinal Type of Gastric Adenocarcinoma

Intestinal type of gastric adenocarcinoma is a bulky tumor that can be divided into early and advanced gastric carcinoma. Early intestinal type of gastric adenocarcinoma is defined as invasive gastric adenocarcinoma confined to mucosa and/or submucosa, with or without lymph node metastases irrespective of the tumor size. Tumor can be unifocal in majority of cases or multifocal in some cases. Advanced gastric adenocarcinoma invades the muscular coat or beyond and metastasizes to liver and lung and are associated with poor prognosis.

- **Predisposing factors:** Predisposing factors for intestinal type of gastric adenocarcinoma include *Helicobacter pylori* infection, mucosa-associated lymphoid tissue (MALT) lymphoma, adenomatous gastric polyps, previous partial gastrectomy, pernicious anemia, atrophic gastritis, intestinal metaplasia, exposure to nitrosamines from smoked foods, tobacco smoking and family history of cancer.
- **Clinical features:** Patient presents with epigastric fullness, vomiting and weight loss in advanced stage of gastric adenocarcinoma. Diagnosis is confirmed by esophagogastroduodenoscopy and biopsy. Imaging studies and laparoscopy aid in determining the caner stage. Consequently, staging system dictates the management approach.
- **Gross morphology:** Tumor is large, bulky or ulcerated with raised margins that preferentially involves the lesser curvature of stomach.
- **Histologic examination:** Tumor can demonstrate different histologic patterns such as tubular (grossly polypoidal/fungating growth), papillary (metastases in lymph nodes and liver), and mucinous (signet-ring cells floating in the abundant extracellular mucin pools, which constitute at least 50% of the tumor). Neoplastic intestinal glands resemble colonic adenocarcinoma, which contain apical mucin vacuoles. Tumor can be well-differentiated consisting of tubules with branching and anastomosing structures or poorly differentiated with solid sheets of neoplastic cells.
- **Treatment:** Stage II and III intestinal types of gastric adenocarcinoma are treated by total or subtotal gastrectomy followed by radiation therapy and chemotherapy.

Lung Adenocarcinoma

Lung adenocarcinoma is a type of non-small cell lung carcinoma, which most often occurs in non-smokers. Tumor shows glandular differentiation, mucin production or pneumocyte immunomarker expression. Tumor usually involves lungs (upper lobe > lower lobe, central > peripheral regions. Tumor invades surrounding tissues and metastasizes to brain, bone, liver and adrenal glands in decreased frequency.

- **Molecular/cytogenetic alterations:** Molecular/cytogenetic alterations in lung adenocarcinoma are EGFR mutation, ALK rearrangement, ROS1 gene fusions, RET fusion, HER2/neu amplification, BRAF mutation, NTRK mutation and SMARCA4 mutation.
- **Clinical features:** Patient presents productive cough, hemoptysis, dyspnea, weight loss and chest pain.
- **Gross morphology:** Tumor is well-defined but unencapsulated. Cut surface reveals tan-white that may have central scarring or necrosis.
- **Histologic examination:** Invasive lung adenocarcinoma has main two histologic patterns: mucinous (i.e. acinus, papillary, micropapillary, lepidic, solid) and non-mucinous. Tumor with mucinous histology is composed of glandular differentiation lined by columnar cells with abundant mucin. Tumor-grade is dependent on combination of histologic patterns.
- **Immunohistochemistry:** Neoplastic cells of lung adenocarcinoma are positive for immunohistochemical markers, cytokeratin 7, epithelial membrane antigen (EMA), napsin A, TTF-1, CEA, B72.3, Ber-Ep4 and CD15 (Leu).
- **Electron microscopy:** Tumor cells have short microvilli and reprogrammed cytoskeleton network that aids in the progression, survival, growth, invasion and dissemination to various organs.
- **Treatment:** Treatment of lung adenocarcinoma depends on stage. Tumor without invasion is treated by surgical resection and adjuvant radiation therapy. Inoperable invasive or metastatic tumor is molecular dependent chemotherapy and radiation.

Transitional Cell Carcinoma in Tissues

Transitional cell carcinoma is a malignant tumor derived from urothelial cells (also called transitional cells) that line urethra, urinary bladder, and renal pelvis. Urothelial cells can change shape and stretch without breaking apart.

Transitional Cell Carcinoma of Urinary Bladder

Transitional cell carcinoma (urothelial carcinoma) of urinary bladder arises from transitional epithelium of the urinary tract (e.g. renal pelvis, ureter, urinary bladder).

- **Predisposing factors:** Predisposing factors include tobacco smoking, occupational exposure to β-naphthylamine, *Schistosoma haematobium* infestation, cyclophosphamide and analgesics, radiation

therapy done for cancers of cervix, prostate, or colorectal region, and exstrophy of the urinary bladder (absence of the anterior part of the urinary bladder and abdominal wall).

- **Molecular/cytogenetic alterations:** Mutations of TP53, PIK3CA, RB1 and FGF3 genes involved in cell cycle progression are demonstrated in transitional cell carcinoma of urinary bladder. Aneuploidy in chromosomes 3, 7 and 17 and loss of 9p21 locus is detected by fluorescent *in situ* hybridization (FISH) in majority of cases. Nuclear matric proteins (NMP22) and urinary bladder tumor antigen (BTA) produced by cancer cells are analyzed for surveillance and detection of transitional cell carcinoma of urinary bladder in majority of cases.
- **Clinical features:** Patient presents with hematuria, painful micturition, back pain, unexplained weight loss, and extreme fatigue.
- **Gross morphology:** Tumor ranges from subtle urinary bladder wall thickening, infiltrative, sessile to obvious exophytic fungating or ulcerated mass. Surgical specimen of urothelial carcinoma of urinary bladder is shown in Fig. 6.27.
- **Histologic examination:** Histologic characterization and depth of invasion are the most important factors for determining prognosis. Neoplastic cells are arranged in irregular nests or single cells invading the lamina propria and muscularis propria. High-grade urothelial carcinoma exhibits nuclear pleomorphism, hyperchromasia, high nuclear–cytoplasmic ratio with frequent atypical tripolar or quadripolar mitotic figures. Vascular invasion by cancer stem cells can be demonstrated. Histology of micropapillary urothelial carcinoma with vascular invasion of urinary bladder is shown in Fig. 6.28.
- **Immunohistochemistry:** Positive immunohistochemical markers for transitional cell carcinoma (urothelial carcinoma) are GATA (most sensitive marker), p63, thrombomodulin, uroplakin III, CK7 and CK20.
- **Treatment:** Transitional cell carcinoma of urinary bladder is treated by surgery, intravesical and systemic chemotherapy, radiation therapy, immunotherapy and targeted therapy.

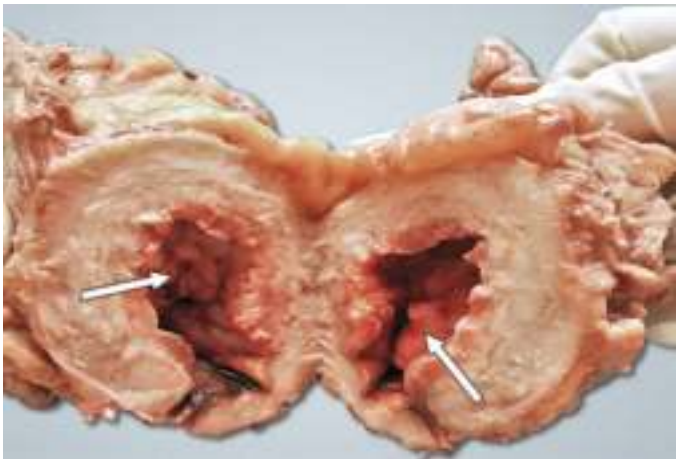


Fig. 6.27: Surgical specimen of urothelial carcinoma of urinary bladder. Cut-opened specimen of urinary bladder shows exophytic, cauliflower-like, fungating growth that fills cavity with areas of hemorrhage (arrows). Macroscopic appearances range from subtle urinary bladder wall thickening to obvious exophytic mass. (*Courtesy:* Senior Professor and Head, Department of Pathology, Govt. Medical College, Srinagar, J&K.)

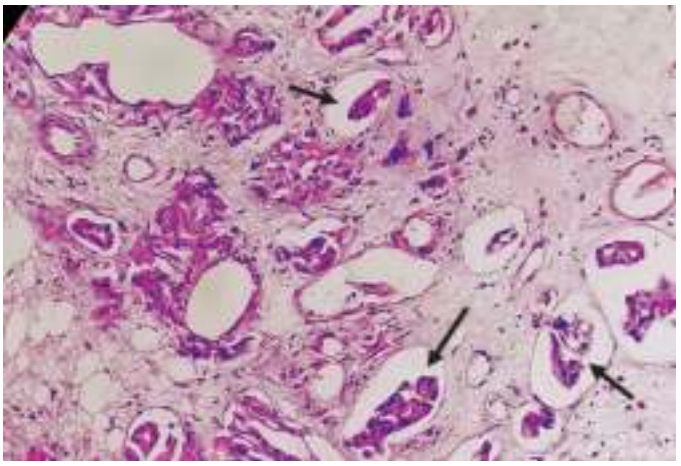


Fig. 6.28: Histology of micropapillary urothelial carcinoma with vascular invasion of urinary bladder. Tumor is composed of neoplastic cells arranged in nests with back-to-back pattern invading blood vessels (arrows) (400X) (*Courtesy:* Senior Professor and Head, Department of Pathology, Govt. Medical College, Srinagar, J&K.)

Clear Cell Carcinoma in Various Anatomic Sites

Clear cell carcinoma occurs most often in the kidney and female reproductive organs (e.g. vagina). Tumor is composed of clear cells when viewed under light microscope.

Renal Cell Carcinoma—Clear Cell Variant

Renal cell carcinoma—clear cell variant is solitary cortical mass that arises in the epithelial cells lining the proximal convoluted tubule in the renal cortex in sporadic tumor. Snake-like masses of tumor may involve the portal vein (35–80%), hepatic vein (20%) or inferior vena cava (similar to small cell lung carcinoma).

- **Predisposing factors:** Predisposing factors include cigarette smoking, obesity, hypertension, long-term hemodialysis and acquired adult cystic kidney disease.
- **Molecular/cytogenetic alterations:** Loss of the vHL protein function usually occurs by deletion or unbalanced transcription, resulting in chromosomal loss of 3p12–26. Second allele of vHL undergoes somatic mutation or epigenetic inactivation through hypermethylation. vHL protein loss

results in accumulation of hypoxia-inducible factor 1α (HIF-1α) that drives transcription of hypoxia-associated genes, including VEGF, PDGFB, GLUT1, TGF-α, erythropoietin and matrix metalloproteinases.

- **Clinical features:** Patient presents with anemia, gross hematuria, flank pain, abdominal mass, weight loss and fever. Classic triad of flank mass, pain and hematuria are present in <10% of cases.
- **Gross morphology:** Tumor is well-circumscribed, expansile in the renal cortex at poles. Cut surface shows golden yellow color due to high lipid content, variegated appearance (i.e. solid and cystic), fibrosis, hemorrhage and necrosis.
- **Histologic examination:** Tumor is composed of compact nests and sheets of cells with clear cytoplasm and distinct membrane arranged in solid, alveolar, acinar or microcystic patterns within delicate stroma. High-grade tumors demonstrate homogenous granular eosinophilic cytoplasm, eccentric nucleus and globular eosinophilic intracytoplasmic inclusions. Histology of renal cell carcinoma—clear cell variant is shown in Fig. 6.29.
- **Immunohistochemistry:** Renal cell carcinoma—clear cell variant is positive for immunomarkers CAIX, PAX8, PAX2, EMA, cytokeratin and vimentin.
- **Treatment:** Patients are managed by surgical resection, immunotherapy with checkpoint inhibitors, monoclonal antibodies against PD1, PDL1 and CTLA4; inhibitors of mammalian target of rapamycin (mTOR) pathways, and tyrosine kinase inhibitors targeting PDGFR and VEGFR.

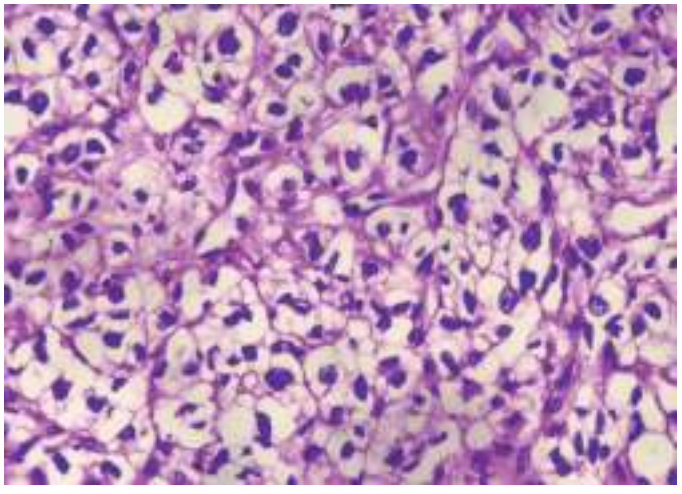


Fig. 6.29: Histology of renal cell carcinoma—clear cell variant. Tumor is composed of cells with clear cytoplasm and distinct membrane with clear to granular eosinophilic cytoplasm arranged in solid nests and sheets, alveolar, microcystic or occasional macrocystic architectural patterns. Presence of network of arborizing small thin-walled blood vessels is important diagnostic feature for cases with granular eosinophilic cytoplasm (400X). (*Courtesy:* Dr. Neha Kumari and Dr. Tushar Kalonia.)

Clear Cell Adenocarcinoma of Vagina

Clear cell adenocarcinoma of vagina develops in daughter due to maternal use of diethylstilbestrol (DES) during pregnancy. Tumor most often involves anterior vaginal wall. About 15–25% of patients remain asymptomatic. Lymphovascular spread involves lymph nodes, lung, peritoneum, omentum, ovary, liver and brain.

- **Pathogenesis:** Diethylstilbestrol (DES) causes persistence of Müllerian epithelium while inducing contact between epithelium and vaginal mesenchyme. Unopposed estrogen and obesity cause increase in the peripheral conversion of steroid hormones to estrone by the aromatase enzyme resulting in hyperestrogenic state.
- **Clinical features:** Patient presents with abnormal vaginal bleeding, malignant pericardial effusion and cardiac tamponade. Per vaginal examination reveals polypoidal and exophytic mass that originated from the anterior wall involving upper two-thirds of the vagina.
- **Gross morphology:** Tumor is superficially located as exophytic polypoidal mass that arises from the anterior wall involving upper two-thirds of vagina.
- **Histologic examination:** Tumor is composed of cuboidal cells with round to irregular hyperchromatic nuclei and conspicuous nucleoli arranged in cystic, tubular, papillary, glandular, and solid patterns. Sometimes, tumor cells contain hobnail type of nuclei protruding into the lumen.
- **Treatment:** Surgery is primary management for low-stage vaginal clear cell adenocarcinomas. Small tumors are managed by local excision, evaluation of retroperitoneal lymph nodes followed by local irradiation to the bed of tumor. Large tumors are treated by neoadjuvant chemotherapy and radiotherapy.

Other Histologic Types of Carcinomas

Other histologic types of carcinomas include diffuse gastric adenocarcinoma (linitis plastica) and conventional hepatocellular carcinoma (HCC).

Diffuse Gastric Carcinoma (Linitis Plastica)

Diffuse gastric adenocarcinoma is an aggressive tumor associated with poor prognosis. Tumor has signet-clear cell morphology, marked desmoplasia, rapid lymphovascular invasion, metastasis involving peritoneum and distant organ(s), and chemoresistance. Tumor can be seen in sporadic and familial (germline mutation in CDH1 gene encoding E-cadherin).

- **Molecular/cytogenetic alterations:** E-cadherin is a calcium-dependent adhesion molecule that

interacts with β-catenin to maintain the structure of epithelium. Germline mutation in E-cadherin gene (CDH1) results in familial diffuse gastric carcinoma (linitis plastica) at an early age.

- **Clinical features:** Patient presents with indigestion, nausea or vomiting, dysphagia, postprandial fullness, loss of appetite, melena, hematemesis and weight loss. Serum carcinoembryonic antigen (CEA) and CA 19-9 levels are elevated in many cases.
- **Gross morphology:** Prophylactic gastrectomy in early-stage disease may not demonstrate any visible lesion. Advanced stage tumor may present as thickening, ulceration or just rigid, leather-like stomach (linitis plastica) due to extensive infiltration by CSCs; that may cause pyloric obstruction. Endoscopy shows flat or depressed lesions with ill-defined border. It is worth mentioning that desmoplasia occurs in the settings of infiltrating duct carcinoma of breast (scirrhous type), diffuse type gastric carcinoma (linitis plastica), pancreatic adenocarcinoma and prostatic adenocarcinoma.
- **Histologic examination:** Tumor is composed of poorly-cohesive cells without well-formed glands that can be either signet-ring cells with a central optically clear, global droplet of cytoplasmic mucin with an eccentrically placed nuclei or differentiated cells, present as scattered individual cells or clusters. Histology of diffuse gastric carcinoma (linitis plastica) is shown in Fig. 6.30.
- **Treatment:** Treatment depends on the extent of the disease, which include surgery, chemotherapy, radiation therapy, chemoradiation, and targeted therapy.

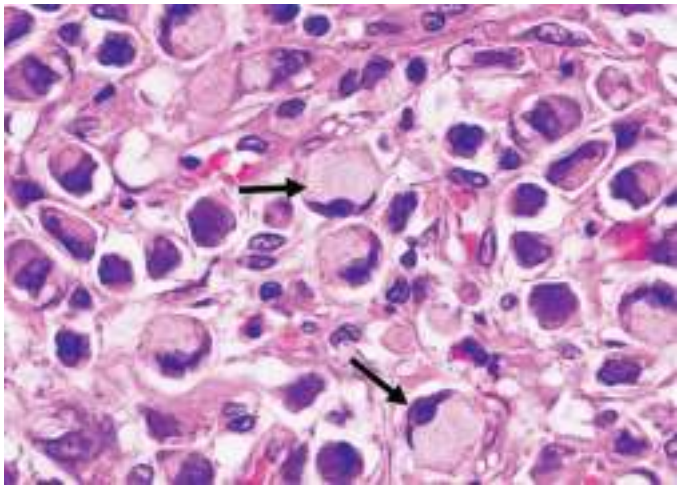


Fig. 6.30: Histology of diffuse gastric carcinoma (linitis plastica). Tumor is composed of poorly-cohesive cells without well-formed glands that can be either signet-ring cells with a central optically clear, global droplet of cytoplasmic mucin with an eccentrically placed nuclei or differentiated cells, present as scattered individual cells or clusters (arrows) (400X).

Conventional Hepatocellular Carcinoma

Conventional hepatocellular carcinoma (HCC) most common occurs in persons with liver disease due to HBV and HCV infection. Stepwise process of hepatocellular carcinoma (low-grade dysplastic nodule → high-grade nodule → early hepatocellular carcinoma → progressive hepatocellular carcinoma) is accompanied by accumulation of molecular alterations such as telomere shortening, TERT activation, cell cycle checkpoint inhibitor inactivation. TERT promoter mutation is a salient event in progression of hepatocellular carcinoma.

- **Predisposing factors:** Predisposing factors include chronic alcohol consumption, HBV and HCV infection, cirrhosis, diabetes mellitus or obesity-related nonalcoholic steatohepatitis (NASH). Other less prevalent risk factors include cirrhosis resulting from primary biliary cholangitis, hemochromatosis, α_1-antitrypsin deficiency and aflatoxin B1 produced by *Aspergillus flavus*. Aflatoxin B1 reacts with DNA to form mutagenic adducts, leading to codon 249 mutation of TP53 gene.
- **Clinical features:** Patient presents with abdominal pain, weight loss, jaundice, ascites, hepatomegaly and splenomegaly. Most metastatic sites in decreasing frequency include lung, portal vein, lymph nodes involvement in portal and abdominal regions.
- **Gross morphology:** Gross examination reveals well-circumscribed solitary mass or multiple discrete nodules that exhibits tan-yellow to green with areas of hemorrhage and necrosis. Background liver is usually cirrhotic.
- **Histologic examination:** Tumor is composed of polygonal cells with nuclear atypia, thick nuclear membrane, prominent nuclei, atypical tripolar or quadripolar mitoses, intracytoplasmic globular hyaline inclusions (Mallory-Denk bodies), and lack of portal triad. Tumor cells are arranged in trabecular (sinusoidal, plate-like) pattern with ≥3 cells much wider than normal liver plate thickness of two cells thick. Presence of sinusoidal vessels surrounding tumor cells is an important diagnostic factor. The reticulin framework is generally reduced or absent. Reticulin stain highlights the thickened hepatocyte plates (≥3 cells). Histology of conventional hepatocellular carcinoma is shown in Fig. 6.31.
- **Immunohistochemistry:** Conventional hepatocellular carcinoma is positive for immunohistochemical stains such as arginase 1, Hep Par 1 (hepatocyte paraffin 1), glypican 3, α_1-antitrypsin, AFP (α-fetoprotein), CEA (carcinoembryonic antigen) polyclonal, CAM 5.2 (CD8/CD18), des-gamma carboxyprothrombin (DCP protein induced by vitamin K absence or antagonist II/PIVKA-2), and TTF-1.

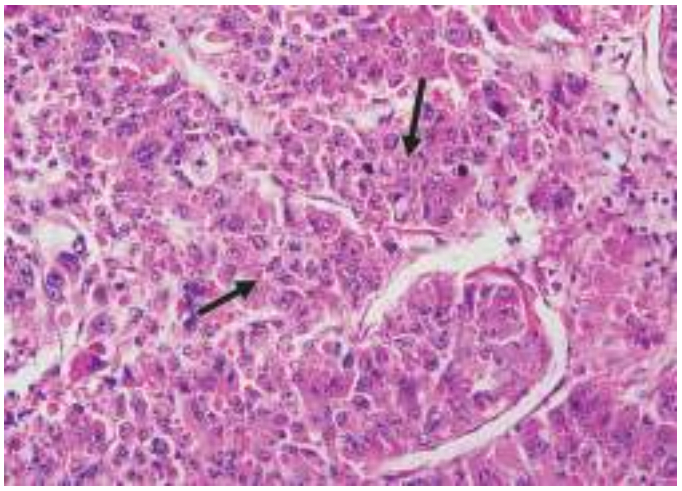


Fig.6.31: Histology of conventional hepatocellular carcinoma. Tumor is composed of liver cords that are much wider than the normal liver plate that is two cells thick. Tumor cells are arranged in trabeculae. There is no discernible normal lobular architecture, though vascular structures are present. It is associated with elevated α-fetoprotein (arrows) (400X).

- **Treatment:** Multiple treatment options are available for conventional hepatocellular carcinoma including curative surgical resection, liver transplantation, radiofrequency ablation, transarterial chemoembolization and systemic chemotherapy (i.e. sorafenib).

MALIGNANT MESENCHYMAL TUMORS

Sarcomas are malignant tumors derived from mesenchymal tissue (adipocyte, fibrous tissue cell, cartilage, bone, fascia, smooth muscle, skeletal muscle, blood vessels and mesothelium). Sarcomas are far less common than carcinomas. Tumors are composed of pleomorphic spindle-shaped cells admixed with supporting mesenchymal stroma and many large thin-walled blood vessels, which are easily invaded by mesenchymal CSCs. Blood-borne metastases to lung are common. In contrast, lymph node involvement is rare in most types of sarcoma. Many highly malignant sarcomas are so poorly differentiated that their specific histologic type cannot be determined. Immunohistochemistry technique, electron microscopy and cytogenetic analysis are helpful in establishing diagnosis by conventional histology alone. Malignant tumors arising from mesenchymal tissue are given in Table 6.14.

Table 6.14 Malignant tumors arising from mesenchymal tissue

Cell Lineage Forming Sarcoma	Malignant Tumor
Smooth muscle cell	Leiomyosarcoma
Striated/skeletal muscle cell	Rhabdomyosarcoma
Cartilage	Chondrosarcoma
Bone osteoblast	Osteosarcoma
Adipose tissue adipocytes	Liposarcoma
Fibrous tissue cell	Fibrosarcoma
Blood vessel endothelial cell	Angiosarcoma and Kaposi sarcoma

Conventional Osteosarcoma

Conventional intramedullary osteosarcoma is primary malignant tumor of bone in 10–20 years of age. Tumor involves long bones near proliferative plates around knee joint (distal end of femur or proximal end of tibia in 60%), hip bones (15%), proximal femur (10%), mandible (8%). Multifocal osteosarcoma may occur in the settings of Paget disease of the bone. Serum alkaline phosphatase is increased two- to three-fold.

- **Predisposing factors:** Predisposing factors for conventional osteosarcoma are giant cell tumor of bone, Li-Fraumeni syndrome (germline TP53 gene mutation), Diamond-Blackfan anemia, Werner syndrome, osteoblastoma, chondroblastoma, metallic and polyethylene implants, osteogenesis imperfecta, familial retinoblastoma, Ollier disease (enchondromatosis), Maffucci syndrome (enchondromatosis associated with hemangiomas), Rothmund-Thomson syndrome and occupational exposure to radium used in watch-dial.
- **Clinical features:** Patient presents with bone pain, swelling and tenderness near the affected region, pathological fracture, fatigue and unexplained weight loss.
- **Radiologic examination:** Tumor has intramedullary permeative growth that replaces medullary spaces and erodes native trabeculae, fills haversian systems, destroys cortex, and invades soft tissue. Serum alkaline phosphatase is increased two- to three-fold. Radiograph shows intramedullary invasive, destructive and blastic intraosseous mass, mineralization, periosteal reactions (sunburst appearance and Codman triangle), cortical permeation, and soft tissue invasion.
- **Gross morphology:** Tumor usually occurs in metaphysis with cortical permeation and soft tissue component that raises the periosteum. Cut surface reveals gritty and mineralized (hard), hemorrhage, necrosis and cystic areas that depends on histologic variant of tumor. Gross morphology of conventional intramedullary osteosarcoma of upper tibia is shown in Fig. 6.32.
- **Histologic examination:** Tumor is composed of pleomorphic neoplastic cells with hyperchromatic nuclei, mitotic figures, formation of variable amount of osteoid, collagen and mineralized bone matrix

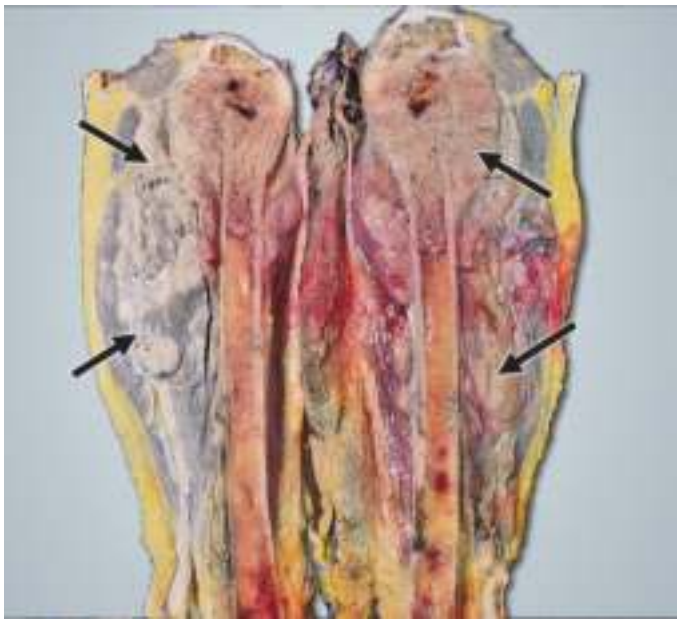


Fig. 6.32: Surgical specimen of conventional intramedullary osteosarcoma. Cut section of the specimen shows intramedullary mass arising from the metaphyseal region of upper tibia, which is permeating within medullary region and surrounding soft tissues (arrows).

in lace-like disorganized woven bone intimately associated with neoplastic cells and broad sheets of bone. Histology of conventional intramedullary osteosarcoma of upper tibia is shown in Fig. 6.33.

- **Histologic variants:** Histologic variants of intramedullary osteosarcomas include: osteoblastic, chondroblastic, fibroblastic, giant cell rich, small cell-rich, clear cell, chondroblastoma-like, malignant fibrohistiocytoma-like and telangiectatic osteosarcoma. Peripheral osteosarcomas occur in parosteal and periosteal region of long bones, and jaw.
- **Treatment:** Conventional treatment for osteosarcoma consists of combination of neoadjuvant and adjuvant chemotherapy and surgery.

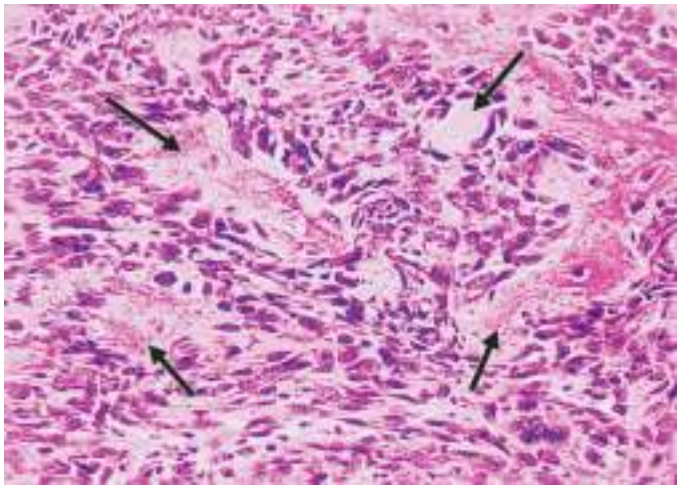


Fig. 6.33: Histology of conventional intramedullary osteosarcoma. Tumor is composed of bone formation by malignant pleomorphic neoplastic cells with hyperchromatic nuclei, numerous mitoses; and formation of 'lace-like' osteoid and calcified bone spicules (arrows) and islands of reactive new woven bone by tumor cells (400X).

Chondrosarcoma

Chondrosarcoma is second most common primary malignant bone tumor characterized by formation of cartilaginous matrix that constitutes 15% of all primary malignant bone tumors. Tumor most often originates in the medulla of flat bones such as pelvic girdle (25%), shoulder girdle (scapula), ribs, and vertebral column.

- **Clinical features:** Patient presents with enlarging lump, pain, and swelling. Skull base tumors induce neurological symptoms. Change in the size and clinical symptoms might be an indicator of malignant transformation in enchondromas and osteochondromas.
- **Radiologic examination:** Radiograph shows popcorn-like calcifications, osteolytic lesions, endosteal scalloping, thickened cortex, cortical erosion or destruction, soft tissue involvement. Cortical destruction and soft tissue extension of pre-existing enchondromas might be indicators of secondary central chondrosarcoma. Thick cartilaginous cap (1.5–2 cm) is demonstrated in secondary peripheral chondrosarcoma. Multilobular appearance is seen in periosteal chondrosarcoma. CT scan and MRI are helpful in showing extent of the tumor.
- **Gross examination:** Tumor has atypical neoplastic hyaline cartilage. Cut surface reveals lobulated appearance, gray-tan, and cystic, myxoid or mucoid material. Mineralization shows chalky calcium deposits. Cortical erosion and soft tissue extension can be seen. Secondary peripheral chondrosarcoma shows thick cartilaginous cap (1.5–2 cm). Periosteal chondrosarcoma appears as a large, lobular mass attached to the surface of bone.
- **Histologic examination:** Tumor is composed of atypical chondroblasts within cartilaginous lacunae. There is increased cellularity showing >2 cells with hyperchromatic nuclei per lacunae. Features of malignancy especially mitoses are best demonstrated at the edge of the tumor. Histology of chondrosarcoma is shown in Fig. 6.34.
- **Treatment:** Conventional treatment for chondrosarcoma consists of surgery, radiation therapy and chemotherapy.

Liposarcoma

Liposarcomas are malignant tumors with differentiation towards adipose tissue that consist of five different

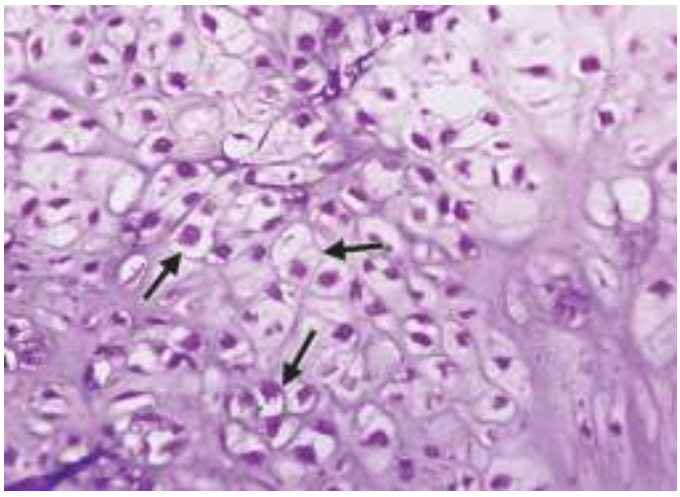


Fig. 6.34: Histology of chondrosarcoma. Tumor is moderately cellular and well-differentiated tumor. It is composed of atypical uniform chondrocytes. Cartilaginous lacunae contain >2 cells with hyperchromatic nuclei. Five-year survival rate is seen in >80% of cases (arrows).

histologic subtypes: well-differentiated, myxoid, round cell, pleomorphic and undifferentiated. Well-differentiated liposarcoma rarely metastasizes and has a good prognosis. Pleomorphic and round cell liposarcomas are highly aggressive tumors, which metastasize early. Primitive adipocyte (lipoblast) is hallmark of liposarcomas. Diagnosis is made with a biopsy showing immature lipoblasts with various cellular atypias depending on the subtype of liposarcomas.

- **Locations:** Liposarcomas are typically seen in patients 50–80 years of age, who present with slow-growing painless mass deep to fascia in lower extremities, upper extremities and retroperitoneum.
- **Clinical features:** Patient presents with a new lump anywhere, or an existing lump that grows persistently, and history of pain in the lump.
- **Molecular/cytogenetic alteration:** Amplification of MDM2 gene and ring chromosome 12 is demonstrated in well-differentiated liposarcoma. Myxoid liposarcoma demonstrates chromosomal translocation t(12;16).
- **Gross morphology:** Tumor is usually large and relatively well-circumscribed. Cut surface may be yellow, fatty, or firm and white with interspersed fibrous septae. Areas of hemorrhage and necrosis may be seen in high-grade tumor.
- **Histologic examination:** Histologic variants of liposarcoma are well-differentiated (low-grade tumor), myxoid (low-to-intermediate grade), round cell (high-grade) and dedifferentiated (low-grade and high-grade) tumors.
  - **Well-differentiated liposarcoma:** Tumor is a low-grade with minimal cellular located in central or retroperitoneal regions. Tumor is composed of atypical lipoblasts in the background mature lipoblasts. Histology of well-differentiated liposarcoma is shown in Fig. 6.35.
  - **Myxoid liposarcoma:** Tumor is low-to-intermediate grade composed of proliferating lipoblasts upon a myxoid stroma matrix.
  - **Round cell liposarcoma:** Tumor is high-grade poorly-differentiated composed small blue cells.
  - **Pleomorphic liposarcoma:** It is high-grade tumor composed of giant lipoblasts with bizarre nuclei.
  - **Dedifferentiated liposarcoma:** Tumor has two histologic variants: low-grade and high-grade dedifferentiated liposarcoma. High-grade dedifferentiated liposarcoma is composed of dedifferentiated mesenchymal heterogenous components such as neural tissue, angioblastic tissue, skeletal muscle, smooth muscle, bone and cartilage. Rhabdomyoblastic differentiation has been associated with poor prognosis.
- **Treatment:** Patients with well-differentiated liposarcoma are treated by marginal surgical excision. Wide surgical resection and radiation is indicated for intermediate and high-grade liposarcomas.

Malignant Vascular Tumors

Malignant vascular tumors have an infiltrative growth and varying degrees of vasoformative features in adults older than 20 years of age. Most common sites are extremities, head and neck region. Immunohistochemical markers for vascular differentiation include CD31, CD34, D2–40 (podopolin), factor VIII and vWF, ulex europaeus, CD141 and Fli-1.

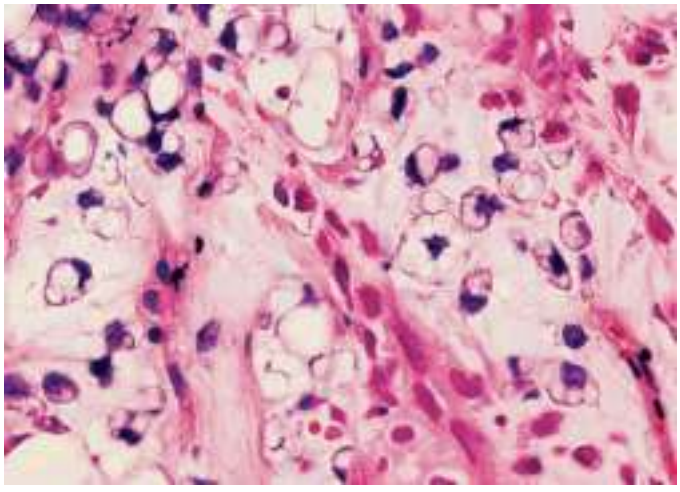


Fig. 6.35: Histology of well-differentiated liposarcoma. Tumor is composed of mature adipocytes of variable size and shape associated with nuclear atypia in adipocytes or spindle cells in the tumor. Spindle cells containing hyperchromatic nuclei tend to be more numerous within fibrous septa. There is presence of varying number of monovacuolated or multivacuolated lipoblasts. It is worth mentioning that mere presence of lipoblasts is not essential to establish the diagnosis (400X).

Epithelioid Hemangioendothelioma

Epithelioid hemangioendothelioma is locally aggressive malignant tumor with metastatic potential involving lymph nodes, soft tissue, bone, liver, lung, and skin. Diagnostic workup includes radiology, histology (biopsy or tumor resection), immunohistochemistry and molecular analysis.

- **Clinical features:** Clinical presentation depends on tumor location. Patient presents with pain, edema or thrombophlebitis due to vascular occlusion, and recurrence after surgery. Tumor may metastasize to lymph nodes and lungs.
- **Gross morphology:** Tumor is poorly circumscribed. Cut surface reveals firm and white-tan mass of variable size up to 18 cm.
- **Histologic examination:** Tumor has histologic variants: (a) WWTR1-CAMTA1 rearranged epithelioid hemangioendothelioma is composed of large atypical endothelial cells with abundant eosinophilic cytoplasm arranged in cords and small nests embedded in a myxohyaline stroma, and (b) YAP1-TFE3 rearranged epithelioid hemangioendothelioma is composed of solid nests or pseudoalveolar formations of epithelioid cells enmeshed in a fibrous stroma.
- **Immunohistochemistry:** Epithelioid hemangioendothelioma is positive for immunohistochemical stains such as CD31, ERG, podoplanin (D2–40), Fli-1, von Willebrand factor, CD34 (can be negative) and CAMTA1 (in WWTR1-CAMTA1 rearranged tumor).
- **Treatment:** Tumor needs wide surgical excision.

Angiosarcoma

Angiosarcoma is a malignant tumor derived from the endothelium of blood vessels and lymphatic channels. It can occur in the skin (face, scalp), breast, liver, spleen and deep tissues. Clinical examination and imaging studies determine size and location of tumor.

- **Locations:** Most common sites of angiosarcoma are skin of the head and neck (60%), extremities, soft tissues, visceral organs, bone and retroperitoneum in adults. Tumor is highly-aggressive with involvement of lymph nodes and distant organ(s).
- **Clinical features:** Patient presents with rapidly growing blue or purple macular, nodular or plaque-like lesion. Advanced disease can show hemorrhage and necrosis. Cervical lymphadenopathy is observed in 10% of cases at the time of presentation.
- **Histologic examination:** Tumor is composed of irregularly shaped anastomosing vascular channels lined by multilayered endothelial cells with nuclear atypia, numerous atypical mitoses and necrosis.
- **Immunohistochemistry:** Tumor is positive for vascular immunohistochemical markers such as CD31, CD34, VEGF, ERG, factor VIII and vWF.
- **Treatment:** Surgical excision is the most common treatment of angiosarcoma.

Kaposi Sarcoma

Classic Kaposi sarcoma and HIV-associated Kaposi sarcoma can involve skin, oral cavity, gastrointestinal tract, lymph nodes, leg, feet, lungs, liver, and spleen. Human herpesvirus-8 (HHV-8) is observed in Kaposi sarcoma patients.

- **Predisposing factors:** Predisposing factors are human immunodeficiency virus (HIV) infection or immunosuppressive drugs administered after organ transplant. Tumor is composed of numerous spindle cells, vascular slits and vascular structures with a predominance of endothelial cells. Some spindle cells can demonstrate nuclear pleomorphism. Extravasated erythrocytes and hemosiderin-laden macrophages are evident.
- **Kaposi sarcoma variants:** Classic Kaposi sarcoma is found most often in elderly men of Eastern European Jewish origin or Italian origin. Patient presents with slow-growing tumor over legs and feet. Epidemic Kaposi sarcoma (HIV-associated Kaposi sarcoma) can involve any part of body such as skin, oral cavity, gastrointestinal tract, lymph nodes, lungs, liver and spleen.
- **Histologic features:** On histologic examination, Kaposi sarcoma demonstrates increased number of spindle cells with vascular slits and vascular structures with a predominance of endothelial cells. Extravasated erythrocytes and hemosiderin-laden macrophages are evident. Some spindle cells can demonstrate nuclear pleomorphism.
- **Treatment:** Classic Kaposi sarcoma is treated by surgery and radiation therapy.

Fibrosarcoma

Fibrosarcoma is a malignant tumor of fibroblasts with herringbone architecture and variable collagen fibers. Tumor can affect both adults and infants. Adult fibrosarcoma involves deep soft tissue of extremities or trunk. Infantile fibrosarcoma occurs before 2 years of age in extremities associated with rapid growth. Tumor can metastasize to lung and bones. Treatment options are surgical excision, irradiation and chemotherapy.

- **Gross morphology:** Adult fibrosarcoma tumor is well-circumscribed but unencapsulated. Cut surface reveals fleshy and white-tan appearance with areas of hemorrhage and necrosis. Infantile fibrosarcoma tumor is poorly-circumscribed and lobulated mass with areas of hemorrhage and necrosis.

- **Histologic examination:** Tumor is composed of numerous small to large fibroblasts arranged in fascicles or herringbone architecture. Tumor cells contain scant cytoplasm, and tapering elongated atypical nuclei with increased granular chromatin, variable nucleoli and numerous abnormal mitotic figures.
- **Histochemistry:** Reticulin stain demonstrates reticulin fibers surrounding each cell. Phosphotungstic acid-hematoxylin (PTAH) demonstrates abundant cytoplasmic fibrils.
- **Immunohistochemistry:** Fibrosarcoma is positive for immunohistochemical stains such as vimentin, type 1 collagen, p53 and high Ki-67.
- **Electron microscopy:** Electron microscopy reveals fibroblasts with prominent rough endoplasmic reticulum but lack myofilaments, external lamina, and distinct myofibroblast.
- **Treatment:** Tumor is treated by surgical excision, irradiation and chemotherapy. Tumor recurs after surgical excision in 50% of cases. Tumor can metastasize to lung and bone. Prognosis is better if tumor is superficial and better differentiated.

Leiomyosarcoma

Leiomyosarcoma is malignant tumor of smooth muscle cell origin *de novo*, and unrelated to pre-existing leiomyoma. Tumor can originate in various anatomic sites, i.e. uterus, soft tissue, blood vessels, retroperitoneum, and skin/subcutaneous tissue.

- **Clinical features:** Patient presents with postmenopausal bleeding, pressure symptoms, pain in pelvis, abnormal vaginal discharge, and a change in urinary bladder or bowel habits.
- **Gross morphology:** Tumor is bulky, fleshy tumor invading into myometrial wall or polypoid tumor projecting into lumen. Cut section reveals tumor is not bulging above the surface. Tumor is invasive and infiltrative growth. Areas of hemorrhage necrosis are present.
- **Histologic examination:** Tumor is composed of spindled/fascicular cells resembling smooth muscle with moderate to marked pleomorphism. Histologic diagnostic criteria include the following features on light microscopy: (a) 10 or more mitoses per 10 HPFs; (b) 5 or more mitoses per 10 HPFs, with nuclear atypia and necrosis; and (c) myxoid and epithelioid leiomyosarcomas with 5 or more mitoses per 10 HPFs.
- **Immunohistochemistry:** Leiomyosarcoma is positive for immunohistochemical stains such as smooth muscle actin (SMA), desmin, muscle specific actin (MSA), h-caldesmon, smooth muscle myosin heavy chain (SMMHC), calponin and ER/PR.
- **Treatment:** Tumor is treated by surgical excision, irradiation and chemotherapy.

Rhabdomyosarcoma

Rhabdomyosarcomas are malignant mesenchymal tumors with skeletal differentiation involving head and neck, uterus, vagina, and urinary bladder, and extremities. Tumors can metastasize via hematogenous route to distant sites. Tumors rarely metastasize to regional lymph nodes.

- **Molecular/cytogenetic alterations:** Chromosomal translocation t(2;13) (q25;q14) is observed in the majority of alveolar rhabdomyosarcoma, and t(1;13) (p36;q14) is found in a smaller subset. The congenital/infantile form of sclerosing/spindle cell rhabdosarcomas demonstrate gene fusions involving VGLL2 and NCOA2 or CITED2 genes.
- **Clinical features:** Although, rhabdomyosarcoma can originate anywhere in the body, tumor is more likely to arise in head and neck region, urinary bladder, reproductive system (i.e. vagina, uterus and testes) and extremities. Signs and symptoms depend on location of tumor.
- **Gross morphology:** Tumor can be well-circumscribed or multinodular with infiltrative borders. Cut surface reveals glistening, gelatinous or gray-white or pink-tan fleshy and bulging appearance. Areas of cyst formation, hemorrhage or necrosis may be present.
- **Histologic examination:** Histologic variants of rhabdomyosarcoma include embryonal rhabdomyosarcoma (botryoid rhabdosarcoma), alveolar rhabdomyosarcoma, pleomorphic rhabdomyosarcoma and spindle cell/sclerosing rhabdomyosarcoma. Tumor cells in rhabdomyosarcoma range from small primitive hyperchromatic round/spindle/stellate cells, and large differentiated ribbon, strap, tadpole, racket-shaped cells with abundant eosinophilic cytoplasm and cross-striations (rhabdomyoblasts), which contain large, round and vesicular nuclei with a prominent nucleolus.
- **Immunohistochemistry:** Immunohistochemical markers for skeletal muscle differentiation include muscle specific actin (MSA), MyoD1 (transcription factor-specific marker), myogenin (transcription factor), desmin, and myoglobin.
- **Treatment:** Patients are treated with surgery and chemotherapy with or without radiation therapy. The histologic type and location of rhabdomyosarcoma influence the survival. The botryoid rhabdomyosarcoma, a variant of embryonal rhabdomyosarcoma has the best prognosis, while pleomorphic rhabdomyosarcoma is most often fatal.

Synovial Sarcoma

Synovial sarcoma is malignant tumor of uncertain lineage of young persons in extremities, and pleuropulmonary parenchyma (10%). It is a misnomer as the tumor does not originate from synovium.

- **Clinical features:** Patient presents with lump in extremities or pleuropulmonary region and related pain in the region.
- **Molecular/cytogenetic alterations:** Chromosomal translocation t(X;18) (p11; q11) with SS18-SSX1 (67%), SS18-SSX2 (33%) fusion gene product is present in more than 95% of cases.
- **Gross examination:** Tumor is large well-circumscribed/lobulated tan-white with subtle invasive growth. Cut surface reveals soft to rubbery with areas of hemorrhage and necrosis. Cystic degeneration and gelatinous areas may be present.
- **Histologic examination:** Synovial sarcoma has three histologic variants: monophasic, biphasic, and poorly differentiated tumors.
  - **Monophasic synovial sarcoma:** Tumor is composed of monomorphic spindled cells with nuclear monotony arranged in fascicular/herringbone growth pattern, atypical mitotic figures, necrosis, hemangiocytoma-like vascular pattern, hyalinized collagen and calcifications.
  - **Biphasic synovial sarcoma:** Tumor is composed of monomorphic spindled cells and epithelial-lineage cells admixed in variable proportions. Epithelial-lineage cells can form glandular structures or solid nests and cords, seldom show squamous metaplasia or granular cell change. Histology of synovial sarcoma is shown in Fig. 6.36.

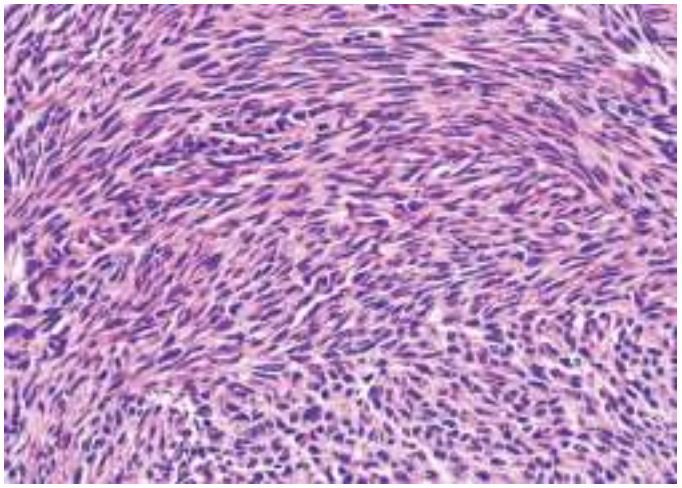


Fig. 6.36: Histology of synovial sarcoma. Tumor shows two histologic patterns depending on the presence of epithelial and spindle cell components in varying proportions: (a) monophasic variant (containing spindle cell component without epithelial differentiation) and (b) biphasic (containing both epithelial and spindle cell components). Biphasic synovial sarcoma usually shows areas of calcification and ossification (400X).

  - **Poorly-differentiated synovial sarcoma:** Tumor is composed of primitive round cells, epithelioid cells or high-grade spindled cells, atypical mitotic figures, frequent rhabdoid change and necrosis.
- **Immunohistochemistry:** Positive immunohistochemical markers for biphasic synovial sarcomas are TLE1, CD99, cytokeratins and epithelial membrane antigen (EMA).
- **Molecular genetic testing:** Chromosomal translocation t(X;18) (p11; q11) with SS18-SSX1 (67%), SS18-SSX2 (33%) fusion gene product is present in more than 95% of cases. Definitive diagnosis of synovial sarcoma often requires molecular genetic testing (RT-PCR and FISH) on small biopsies.
- **Treatment:** Synovial sarcoma is treated by wide surgical excision. A tumor-free margin of 1–3 cm is recommended.

Alveolar Soft Part Sarcoma

Alveolar soft part sarcoma is a malignant tumor of uncertain lineage composed of large polygonal cells with round vesicular nucleus, prominent nucleolus, abundant eosinophilic cytoplasm arranged in pseudoalveolar pattern and PAS diastase resistant positive intracytoplasmic rhomboid- or rod-shaped crystals.

- **Locations:** Tumor predominantly affects the deep soft tissues of the extremities (thigh and buttock), trunk, and internal organs in young adults with female predominance, and head and neck region (tongue and orbit) in young children.
- **Molecular/cytogenetic alterations:** Definitive diagnosis is confirmed by analyzing chromosomal translocation t(X;17) (p11.2; q25) with ASPSCR1-TFE3 fusion gene product. TFE3 nuclear expression is analyzed by immunohistochemistry.
- **Clinical features:** Patient presents with slow growing and painless mass in the region or proptosis in orbital tumor. Tumor is localized in the initial stage. Later, tumor can involve regional lymph nodes and metastasize to distant organ(s).
- **Gross morphology:** Tumor is solid, partially circumscribed mass with fleshy nodules and fibrotic bands with mean 6.5 cm. Cut surface reveals yellow to gray to white-tan tumor with median 6.5 cm size and presence of large blood vessels at the periphery of tumor.
- **Histologic examination:** Tumor is composed of large polygonal cells with round vesicular nucleus, prominent nucleolus, abundant eosinophilic cytoplasm, arranged in pseudoalveolar pattern and PAS diastase resistant positive intracytoplasmic rhomboid- or rod-shaped crystals. Tumor is divided into lobules by thick fibrous septa and rich capillary

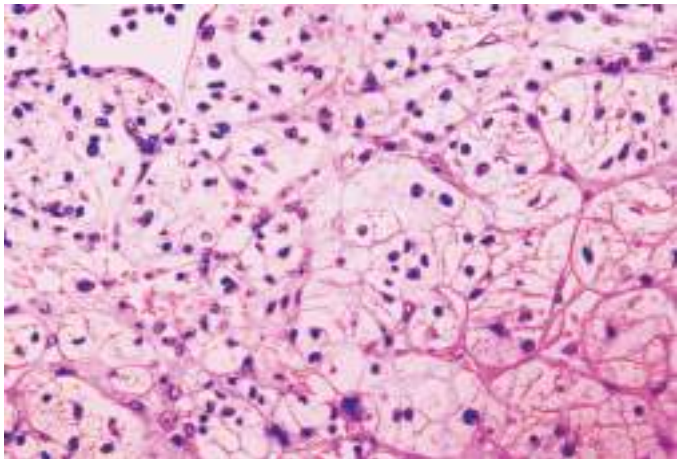


Fig. 6.37: Histology of alveolar soft part sarcoma. Tumor is composed of large uniform epithelioid cells with well-defined borders, centrally placed bland nuclei and abundant eosinophilic cytoplasm somewhat granular and rod-shaped intracytoplasmic inclusions. Neoplastic cells are arranged in solid nests and/or alveolar structures and separated by delicate fibrovascular septa. Loss of cellular cohesion and necrosis of the neoplastic cells in the central region of nests of tumor cells gives pseudoalveolar pattern (400X).

vascular network, dilated veins at the periphery of the tumor. Vascular invasion is common by tumor cells. Histology of alveolar soft part sarcoma is shown in Fig. 6.37.

- **Treatment:** Radical surgical resection of tumor is the treatment of choice. Radiation may reduce the risk of recurrence. Adjuvant chemotherapy does not seem to be effective.

LOCALLY MALIGNANT TUMORS

Groups of slow-growing malignant tumors that spread only by direct invasion in surrounding tissues without dissemination to distant organs. Tumor cells show features of malignancy. Examples of locally-malignant tumors include basal cell carcinoma of skin, dermatofibrosarcoma protuberans, giant cell tumor of bone (osteoclastoma), adamantinoma (tibia or fibula), craniopharyngioma of pituitary gland and carcinoid tumor.

Basal Cell Carcinoma of Skin

Basal cell carcinoma (BCC) is the most common slow-growing locally aggressive malignant epithelial tumor arising from sun-exposed skin on the face near the eyes and nose and trunk regions. Tumor starts as flattened papillary growth which slowly increases in size over months to two years. The surface of tumor breaks down and a shallow, ragged ulcer with pearly edges is formed.

- **Predisposing factors:** Predisposing factors for basal cell carcinoma include ultraviolet radiation, arsenic exposure, blue basal nevus (Gorlin syndrome), xeroderma pigmentosum, oculocutaneous albinism, Bazex-Dupre-Choristol syndrome, Muir Torre syndrome and nevus sebaceous.
- **Clinical features:** Patient with nodular variant of basal cell carcinoma in facial region presents with pearly pink, flesh colored papule or nodule with arborizing and branching blood vessels. Tumor with ulceration has rolled borders. Superficial variant of tumor on trunk region presents with scaly macules, or plaques with erythematous surface.
- **Gross morphology:** Tumor forms nodule, which may extend into subcutaneous tissues. Cartilaginous invasion is unusual.
- **Histologic examination:** Tumor is composed of nests of basaloid cells resembling those of the basal layer of skin with peripheral palisading containing basophilic nuclei, scanty cytoplasm and scattered atypical mitotic figures at the periphery in haphazard arrangement, associated with a fibromyxoid stroma. Histology of basal cell carcinoma of skin is shown in Fig. 6.38.
- **Immunohistochemistry:** Positive immunomarkers are cytokeratins and BerEP4.
- **Treatment:** Treatment options for basal cell carcinoma are surgery, Mohs micrographic surgery, curettage and electrodesiccation, radiation therapy, topical treatment (6-fluorescent, imiquimod), and systemic therapy (Hedgehog pathway inhibitors).

Giant Cell Tumor of Bone

Giant cell tumor of bone (i.e. osteoclastoma) is potentially low-grade rarely metastasizing malignant

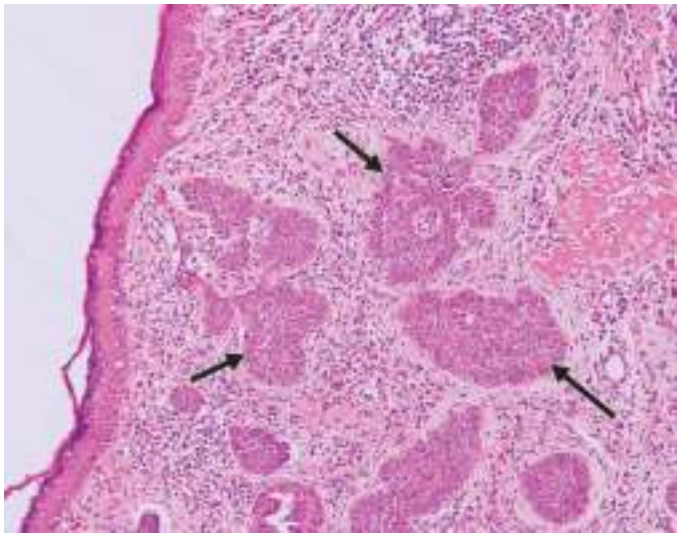


Fig. 6.38: Histology of basal cell carcinoma of skin. Tumor is composed of infiltrative deeply basophilic epithelial (basaloid) cells with narrow rims of cytoplasm arranged in clusters, which are attached to the epidermis and protruding into the subjacent papillary dermis. Nuclei in the periphery of the clustered cells are arranged in palisading manner. Mitotic figures are absent (arrows) (400X).

tumor of monocyte-macrophage lineage that affects 20–45 years of age.

- **Locations:** Locations of giant cell tumor are epiphysis of long bones around knee joint (lower end of femur or upper end of tibia). Tumor can also occur in wrist, upper end of femur, upper end of humerus, spine and pelvis. Local recurrence after curettage is associated with pulmonary metastasis in 2% of cases.
- **Clinical features:** Patient presents with bony mass, pathological fracture, limited movement in the nearest joint, and pain.
- **Molecular alterations:** Giant cell tumor of bone related clonal aberrations occur in a background of epigenetic modifications especially G34W mutation of H3F3A gene.
  - Neoplastic mononuclear stromal cells in the tumor express receptor activator of NF-κB ligands (RANKLs), chemokines and cytokines associated with monocyte recruitment and reactive multinucleated giant cells (osteoclastogenesis).
  - Activation of Wnt/β-catenin pathway, clonal telomeric association and therapeutic radiation are linked to giant cell tumor of bone. Mutations in TP53 gene and H-RAS gene have been demonstrated in malignant giant cell tumor of bone not associated with prior radiation.
- **Radiologic examination:** Conventional radiograph examination shows well-circumscribed osteolytic lesion on epiphyseal region of a long bone in eccentric location giving 'soap bubble appearance'.
- **Gross examination:** Tumor is soft, friable, slightly brownish or red-tan, somewhat poorly defined in the ends of long bone. Surrounding cortex is thinned out and may be destroyed completely. Destructive tumor may extend into the soft tissue. Cut surface of malignant change in giant cell tumor of bone is typically firm and fleshy in consistency. Surgical specimen of giant cell tumor of bone is shown in Fig. 6.39.
- **Histologic examination:** Tumor consists of round to oval and spindled mononuclear neoplastic cells with pale eosinophilic cytoplasm, nuclei with dispersed chromatin and small nucleoli and numerous mitoses, macrophages and uniformly distributed multinucleated giant cells. Numerous mitotic figures are commonly present. Presence of atypical tripolar or quadripolar mitoses should raise suspicion for malignant change in giant cell tumor. Necrosis is present in the setting of pathologic fracture. Histology of giant cell tumor of bone is shown in Fig. 6.40.
- **Treatment:** Without treatment, giant cell tumor of bone will continue to grow and destroy the surrounding bone, so treatment is always essential. Aim of treatment is to excise the tumor, prevent damage to bone and avoid recurrence of the tumor. Surgical treatment is preferred for giant cell tumor of bone. Radiotherapy, tumor embolization and chemotherapy are nonsurgical treatment modalities. Local recurrence after curettage is associated with pulmonary metastasis in ≤2% of cases.

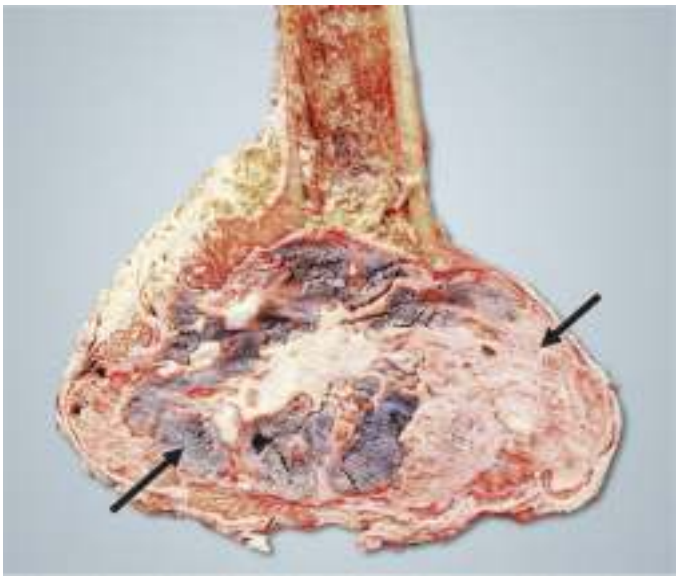


Fig. 6.39: Surgical specimen of giant cell tumor of bone. Tumor is large red brown, fleshy well-demarcated mass. Cut surface shows yellow and/or cystic areas. Areas of hemorrhage and necrosis are common. Hemosiderin deposition and reactive bone formation are demonstrated in the tumor (arrows). (*Courtesy:* Dr. Sujata Kanetkar, Professor and Head, Department of Pathology, Krishna Institute of Medical Sciences, Karad, Maharashtra.)

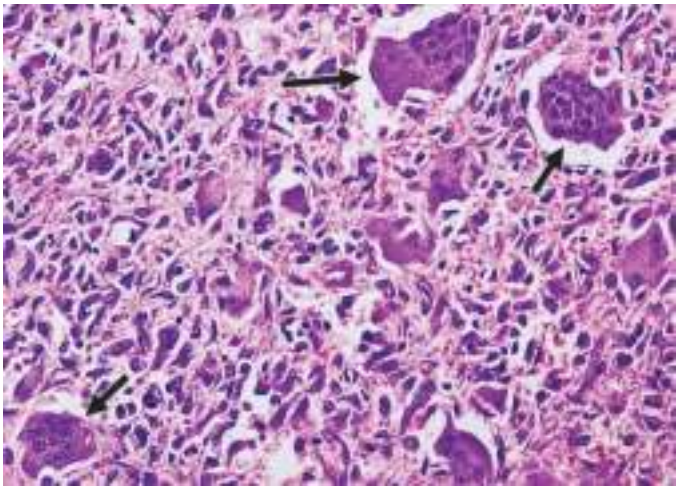


Fig. 6.40: Histology of giant cell tumor of bone. Tumor is composed of ovoid mononuclear cells with indistinct cellular outlines growing in the form syncytium; interspersed with numerous osteoclast-like multinucleated giant cells containing >100 nuclei scattered among mononuclear cells. Mitotic figures are common in mononuclear cells. Morphology of nuclei of stromal mononuclear cells and osteoclast-like multinucleated giant cells are identical (arrows) (400X).

Dermatofibrosarcoma Protuberans

Dermatofibrosarcoma protuberans (DFSP) is locally aggressive mesenchymal neoplasm with fibroblastic differentiation located in skin/cutis. COL1A1-PDGFB fusion gene products are demonstrated by molecular testing in all cases. Tumor demonstrates positivity for immunohistochemical marker CD34.

- **Clinical features:** Patient presents with slow-growing exophytic and nodular cutaneous mass that may progress to fibrosarcomatous transformation.
- **Gross examination:** Tumor may be plaque-like dermal/subcutaneous thickening initially progressing to raised firm multinodular mass of 0.5 to more than 10 cm. Cut surface reveals gray-white, firm-to-myxoid and gelatinous appearance.
- **Histologic examination:** Tumor is composed of monomorphic spindle cells and ovoid to elongated cells with abundant eosinophilic cytoplasm with variable mitotic activity arranged in storiform or whorled pattern; and collagenous or myxoid stroma. Adnexal structures are typically spared. Fibrosarcomatous transformation denotes those with cellular spindle cell fascicles or 'herringbone pattern', greater atypical mitotic figures and positive expression of CD34.
- **Treatment:** Wide local excision is treatment option for localized disease. Advanced disease requires chemotherapy.

Adamantinoma

Adamantinoma is primary biphasic fibro-osseous of bone that involves middle third of diaphysis of tibia or fibula. Multifocal involvement of the tibia is frequently present. Tumor has been reported in the ulna, femur, humerus, radius, ribs, tarsal and metatarsal bones, as well as extraskeletal pretibial soft tissue. Definite diagnosis is established by histologic examination.

- **Clinical features:** Symptoms depend on location and extent of the disease. Patient presents with indolent and nonspecific symptoms as well as slow-growing mass.
- **Gross examination:** Tumor is yellow-gray or gray-white. Cut surface reveals fleshy or firm in consistency. Some tumors may demonstrate straw colored fluid on cut surface.
- **Histologic examination:** Classic biphasic adamantinoma is composed of epithelial and osteofibrous components intermixed with each other in various proportions. Epithelial component is composed of mildly atypical epithelial cells within osteofibrous dysplasia-like stroma forming solid basaloid cells arranged in nest with palisading pattern or less often tubular structures, keratinized squamous nests or spindle cell bundles.
- **Immunohistochemistry:** Positive immunohistochemical stains are cytokeratins (AE1/AE3, CK5, CK14, CK19), vimentin, epithelial membrane antigen (EMA), p63 and podoplanin (D2–40).
- **Treatment:** Tumor is treated by wide local surgical excision. Long-term follow-up is essential due to possibility of late complications.

Craniopharyngioma

Craniopharyngioma is a benign cystic epithelial tumor (WHO grade 1) in suprasellar region, that frequently extends into surrounding structures. Tumor possibly originates from neoplastic transformation of ectodermal-derived epithelial remnant of Rathke's pouch and craniopharyngeal duct.

- **Molecular/cytogenetic alteration:** CTNNB1 (β-catenin) gene mutations and aberrant nuclear expression of β-catenin is demonstrated in up to 95% of cases.
- **Clinical features:** Patient presents with visual disturbances, endocrine deficiencies, failure to thrive, diabetes insipidus, headache, cognitive impairment and personality changes.
- **Radiologic examination:** Preoperative imaging has best diagnostic tool. Radiograph demonstrates multilobulated and multicystic tumor of variable size in extra-axial and suprasellar region. CT scan demonstrates areas of calcifications within tumor.
- **Gross examination:** Tumor is lobular and cystic with calcifications. Cysts contain dark fluid composed of cholesterol and hemorrhage. Tumor can interface with adjacent brain or densely adherent to brain.
- **Histologic examination:** Tumor is composed of well-differentiated squamous epithelium bordered by palisading epithelium, and peripheral loose plumed cells called stellate reticulum, keratin associated with surrounding gliosis and Rosenthal fibers.
- **Immunohistochemistry:** Positive immunomarkers are CK7, CK8, CK9, EMA and β-catenin.
- **Treatment:** Patient is treated with total or partial surgical excision followed by radiation therapy.

Pathology Pearls: Locally Malignant Tumors

- Basal cell carcinoma of skin
- Dermatofibrosarcoma protuberans
- Giant cell tumor of bone (osteoclastoma)
- Adamantinoma
- Craniopharyngioma
- Carcinoid tumor

MIXED BENIGN AND MALIGNANT TUMORS

Mixed benign and malignant tumors have two different morphologic patterns that are derived from the same germ cell layer, which may be benign (e.g. pleomorphic adenoma of salivary gland and fibroadenoma of breast) or malignant (e.g. carcinosarcoma also called malignant mixed Müllerian tumors of uterus), metaplastic carcinoma, mixed germ cell tumor and adenosquamous carcinoma.

Pathology Pearls: Mixed Benign and Malignant Tumors

- Fibroadenoma of breast
- Carcinosarcoma (malignant mixed Müllerian tumors of uterus)
- Metaplastic carcinoma
- Mixed germ cell tumors of gonads
- Adenosquamous carcinoma

Fibroadenoma of Breast

Fibroadenoma is a painless, unilateral, well circumscribed, solid, benign tumor in adolescents and young women within breast or accessory breast tissue in axilla with pushing borders that does not infiltrate the adjacent breast parenchyma. Tumor is composed of proliferation of both glandular epithelial and stromal components of the terminal duct lobular unit (TDLU). Juvenile fibroadenoma generally occurs in younger and adolescent women less than 20 years of age. Complex fibroadenoma affects older women. Myxoid fibroadenoma is associated with Carney complex. Cyclosporin immunosuppression increases risk of fibroadenoma of breast.

- **Clinical features:** Patient most often presents with painless, firm, slow-growing, solitary mobile lump usually <3 cm in diameter, which can be multiple and bilateral in breast or accessory breast tissue in axilla.
- **Gross morphology:** Tumor is well-circumscribed, unencapsulated firm, ovoid mass with bosselated and lobulated bulging above the cut surface with slit-like spaces. Cut surface may be mucoid, and fibrotic appearance. Tumor may undergo calcification.
- **Histologic examination:** Fibroadenoma of breast is a biphasic benign tumor composed of both glandular epithelial and interlobular stromal components of the terminal duct lobular unit (TDLU). Tumor is composed of proliferation of both glandular epithelial and stromal components of the terminal duct lobular unit. Glandular component has two growth patterns: (a) intracanalicular pattern consists of glands compressed into linear branching structures by proliferating stroma, and (b) pericanalicular pattern consists of glands retained open lumens by separated by expanded stroma. Glandular components have intact myoepithelial cells. Stromal component consists of collagen and spindle-shaped cells with elongated or ovoid nuclei. Histology of breast fibroadenoma is shown in Fig. 6.41.
- **Treatment:** Treatment options are conservative management, local surgical excision and follow-up.

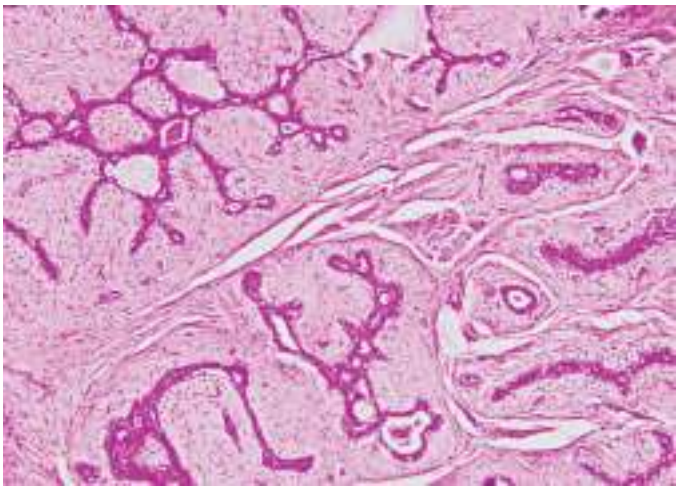


Fig. 6.41: Histology of breast fibroadenoma. Tumor shows intracanalicular and pericanalicular patterns. Glands compressed by stroma result in slit-like spaces known as intracanalicular pattern. Ducts not compressed by stroma have round shape known as pericanalicular pattern (100X).

Carcinosarcoma (Malignant Mixed Müllerian Tumor)

Carcinosarcoma (malignant mixed Müllerian tumor) is biphasic high-grade malignant tumor epithelial and mesenchymal (homologous or heterologous) components. Sarcomatous component is derived from the carcinomatous component as a result of metaplasia/transdifferentiation (EMT: epithelial to mesenchymal transition). Terminology used for carcinosarcoma are mixed Müllerian tumor, malignant mesodermal mixed tumor or metaplastic carcinoma. Tumor is sporadic that almost affects postmenopausal woman (mean age: 65 years).

- **Locations:** Tumor most frequently occurs in the uterine corpus, cervix, ovaries, fallopian tubes, vagina, peritoneum and extragenital sites.
- **Pathogenesis:** Carcinomatous components of tumor convert themselves to sarcomatous components of tumor via epithelial–mesenchymal transition (EMT). This theory is supported by EMT gene signature scores as a result of epigenetic changes at microRNA promoters and mutations and amplification of histone octamer genes.
- **Predisposing factors:** Predisposing factors for malignant mixed Müllerian tumor include tamoxifen use, pelvic radiation therapy, prolonged estrogen exposure, nulliparity, obesity and diabetes mellitus.
- **Clinical features:** Patient presents with vaginal bleeding, abdominal lump and pelvic pain, extrauterine metastasis (≤45%) and distant metastasis (10%).

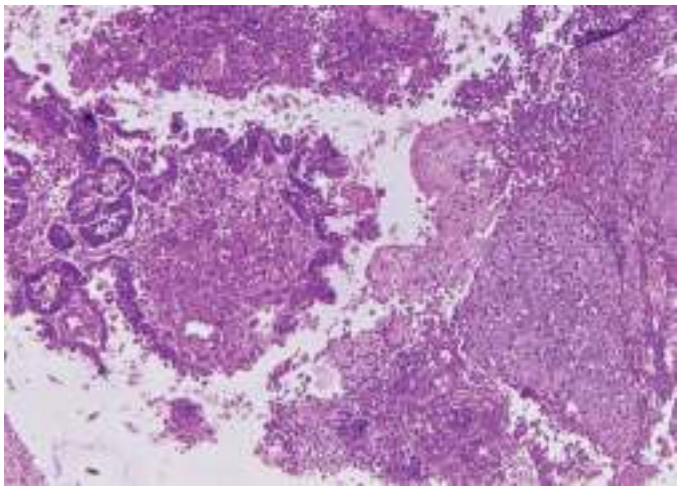


Fig. 6.42: Histology of malignant mixed Müllerian tumor with heterogenous differentiation. Endometrial curettings from 68 years postmenopausal female shows malignant Müllerian tumor composed of juxtaposed areas of adenocarcinoma and chondrosarcoma components (400X).

- **Prognostic factors:** Stage of disease is the most consistent independent predictor of outcome. Five-year survival for stage 1 disease is over 80%. Presence of heterogenous elements is poor prognostic factor in early-stage disease. Carcinomatous component tends to spread via lymphatic route. Sarcomatous component spreads via hematogenous route to lungs.
- **Gross examination:** Polypoidal tumor with extensive invasion into muscle and extension beyond organ is observed in the surgical specimen. Cut surface reveals fleshy, bulky and friable with hemorrhage and necrosis.
- **Histologic examination:** Tumor is biphasic malignant tumor composed of juxtaposed carcinomatous and sarcomatous components. Histology of malignant mixed Müllerian tumor with heterogenous differentiation is shown in Fig. 6.42.
  - **Carcinomatous component:** Carcinomatous component is most often either endometrioid carcinoma, or high-grade serous carcinoma (50–70%) or hybrid morphology tumor. Lymphovascular invasion is more common in carcinomatous element.
  - **Sarcomatous component:** Sarcomatous element is most often spindle and pleomorphic that consists of heterologous elements (i.e. rhabdomyosarcoma, chondrosarcoma, liposarcoma, osteosarcoma, angiosarcoma). Other types of sarcomatous element are less common.
- **Immunohistochemistry:** Immunohistochemistry technique can be used to demonstrate heterologous elements in carcinosarcoma. Positive immunohistochemical markers for carcinomatous component of tumor are PAX8, EMA and cytokeratins. Aberrant p53 is expressed in both serous and high-grade endometrioid carcinomas. Endometrioid component of tumor shows ER and PR positivity. Sarcomatous component of tumor shows aberrant p53 expression. Rhabdomyosarcoma component expresses desmin and myogenin. Chondrosarcoma and liposarcoma components show S-100 protein positivity.
- **Treatment:** Treatment consists of total abdominal hysterectomy and bilateral salpingo-oophorectomy with pelvic lymphadenectomy, radiation therapy and chemotherapy.

Metaplastic Carcinoma of Breast

Metaplastic carcinoma of breast is characterized by the histologic presence of two or more cellular components, commonly a mixture of epithelial and mesenchymal components including but not restricted to spindle, chondroid and osseous cells and other cells. When breast carcinoma lacks features of ductal carcinoma *in situ* or invasive ductal carcinoma, a panel of immunohistochemical markers (cytokeratins and p63) are required to confirm the presence of epithelial differentiation.

- **Clinical features:** Patient presents with large-sized breast lump with low-to-high grade biological behavior.
- **Gross examination:** Tumor is well circumscribed, solid and firm with mean 4 cm in diameter. Cut section reveals gray-white and glistening surface in areas of squamous and chondroid differentiation.
- **Histologic examination:** Tumor is composed of heterogenous elements: (a) epithelial component (low-to-high grade adenosquamous carcinoma), and (b) heterogenous mesenchymal components (i.e. chondroid or osseous-derived malignant element). Histology of breast metaplastic carcinoma is shown in Fig. 6.43.

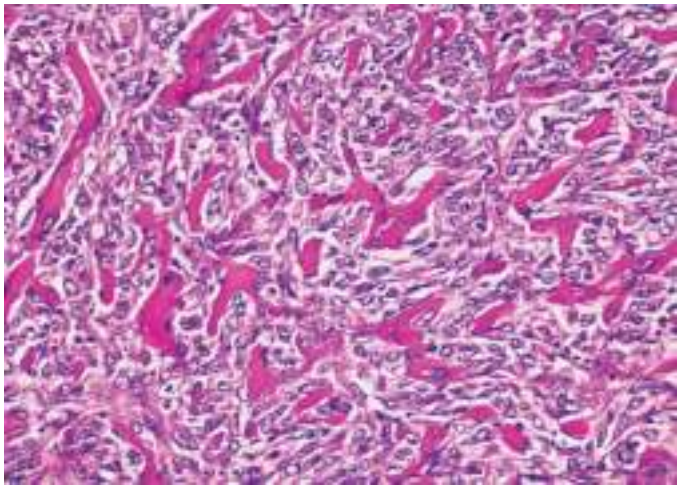


Fig. 6.43: Metaplastic carcinoma of breast. Tumor is composed of heterogenous malignant mesenchymal components such as osseous element and spindle cells (400X).

- **Immunohistochemistry:** Positive immunohistochemical markers include broad spectrum cytokeratin (AE1/AE3, CAM 5.2, OSCAR:75–90%), high-molecular-weight cytokeratin (CK5/CK6, CK14:70–75%), and low-molecular-weight cytokeratin (CK8/CK18, CK19:35–60%).
- **Treatment:** Metaplastic carcinoma of breast is treated by mastectomy with or without radiation therapy and chemotherapy. Tumor has poor response to neoadjuvant systemic therapy. Complete response to neoadjuvant systemic therapy is seen in 10–15% of cases.

Adenosquamous Carcinoma in Various Organs

Adenosquamous carcinoma is defined as tumor in which both glandular and squamous elements are clearly recognizable histologically malignant. Tumor can occur in lung, cervix, endometrium, breast, esophagus, colon and gallbladder, salivary gland and pancreas. Tumor disseminates via lymphatic, perineural and hematogenous routes. Clinical features depend on location of tumor and extent of invasion and metastasis. Adenosquamous carcinoma of cervix is composed of glandular and squamous components. Histology of adenosquamous carcinoma of cervix is shown in Fig. 6.44.

Mixed Germ Cell Tumors of Gonads

Mixed germ cell tumors contain more than one germ cell component. The most frequent combinations are embryonal carcinoma with seminoma, teratoma, or yolk sac tumor. However, any combination can be seen.

- Common combinations include: (a) teratoma + embryonal carcinoma + endodermal sinus tumor (yolk sac tumor), (b) seminoma + embryonal carcinoma, and (c) teratoma + embryonal carcinoma.

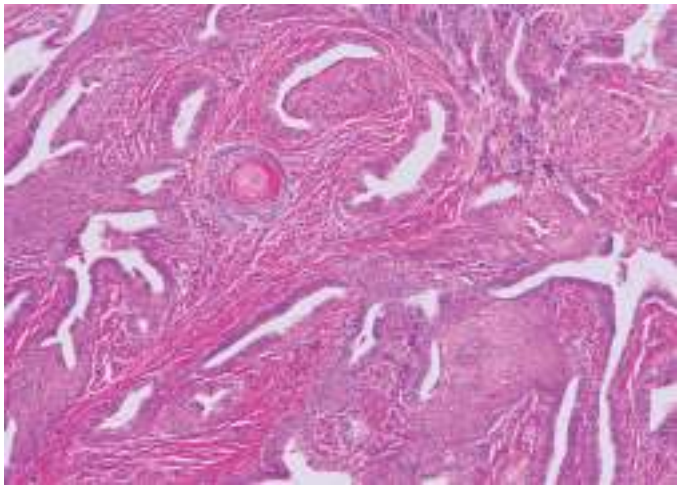


Fig. 6.44. Histology of adenosquamous carcinoma of cervix. Tumor arises from reserve cells in cervix with favorable prognosis. Tumor is composed of glandular and squamous components (400X).

- Tumor contains more than two components. It may be noted that seminoma with syncytiotrophoblastic cells is not a mixed germ cell tumor and is classified as a seminoma. Even though, tumor contains more than one component because its biologic behavior is similar to seminoma. Mixed germ cell tumor is heterogenous, multinodular solid tumor with foci of hemorrhage and necrosis.

GERM CELL TUMORS

Germ cell tumors are broadly divided in two classes: seminomatous (germinoma in pineal gland, suprasellar region, seminoma in testis, dysgerminoma in ovary) and nonseminomatous germ cell tumors (teratoma, yolk sac tumor, embryonal carcinoma, and choriocarcinoma).

- Germinoma is an intracranial counterpart of seminoma in testis and dysgerminoma in ovary.
- Mixed germ cell tumors have two or more than two different germ cell components.
- Patients with gonadal germ cell tumors have better prognosis than those with extragonadal mediastinal germ cell tumors, and survival exceeds 90% in localized tumors.
- Treatment options for germ cell tumors include surgical removal of the tumor, chemotherapy with drugs and radiation therapy. Seminoma in testes, dysgerminoma in ovary, intracranial germinoma and other extragonadal tissues are highly radiation sensitive tumors associated with good prognosis.

Germinoma/Seminoma/Dysgerminoma

Germinoma is an intracranial counterpart of seminoma in testis and dysgerminoma in ovary. Seminoma can occur in anterior mediastinum, and retroperitoneum. Presence of syncytiotrophoblast giant cells is associated with elevated hCG levels in some cases.

Teratomas

Teratomas contain tissues from the three germ cell layers: ectoderm, mesoderm and endoderm. Teratomas can be located in ovary, testis and extragonadal sites mainly in the midline structures, e.g. brain (pineal gland, suprasellar region), nose, beneath tongue, neck region, mediastinum, retroperitoneal region and sacrococcygeal region. Mediastinal teratoma may present with cough, dyspnea or cyanosis. Benign cystic teratoma (also known as dermoid cyst) is a benign tumor of ovary, which consists of well-differentiated derivatives of germ cell layers (i.e. ectoderm, mesoderm and endoderm) developing as hair, muscle, teeth or bone. Prepubertal and post-pubertal testicular mature and immature teratomas have malignant behavior. As there is great risk of torsion of benign cystic teratoma, hence,

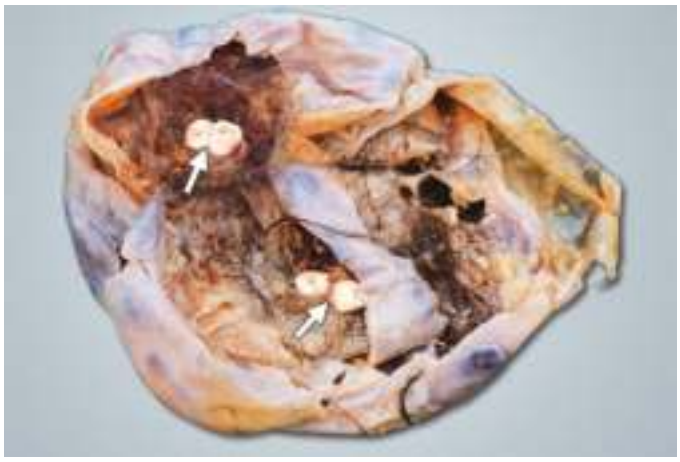


Fig. 6.45: Surgical specimen of benign (mature) cystic teratoma of the ovary. Cut surface shows cyst filled with hair, teeth and adipose tissue (arrows).

it should be surgically excised. Surgical specimen of benign (mature) cystic teratoma of the ovary is shown in Fig. 6.45. Histology of benign (mature) cystic teratoma of the ovary is shown in Fig. 6.46.

Yolk Sac Tumor

Yolk sac tumor is malignant primitive germ cell tumors of ovary, testis and extragonadal tissues such as retroperitoneum, mediastinum and pineal as well as more exotic locations. Elevated serum α-fetoprotein (AFP) should raise the suspicion for a yolk sac component. Tumor may occur in as pure yolk sac tumor or mixed with another germ cell tumor (e.g. teratoma and embryonal carcinoma) in pediatric age group.

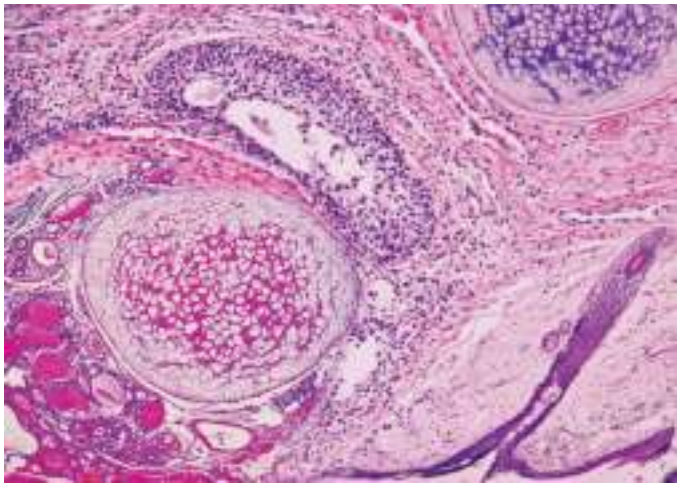


Fig. 6.46: Histology of benign (mature) cystic teratoma of the ovary. Outer surface of mature cystic teratoma of ovary is composed of ovarian hyalinized stroma. The cavity is lined by skin, and cutaneous structures. The skin is composed of keratinized squamous epithelium and contains sebaceous and eccrine glands associated with adipose tissue. Some tumors may demonstrate lipogranulomatous, fat necrosis-like, sieve-like pattern. Cyst contains hair and other dermal appendages (400X).

Embryonal Carcinoma

Embryonal carcinoma is a germ cell tumor that occurs in the ovary and testis in young age group. Pure embryonal carcinoma is extremely rare and most often found as a part of a mixed germ cell tumor. Serum levels of β-hCG and α-fetoprotein can be elevated. OCT4 immunohistochemical stain is positive in embryonal carcinoma and negative in yolk sac tumor, hence OCT4 is the best discriminatory stain.

Choriocarcinoma

Choriocarcinoma is highly malignant germ cell tumor characterized by the presence of syncytiotrophoblastic and cytotrophoblastic elements, and biochemically by the production of the human chorionic gonadotropin (hCG). Tumor originates in the testis or ovary, uterus or midline structures with pluripotent germ cells. Women may develop choriocarcinoma following hydatidiform mole, normal pregnancy or spontaneous abortion. Tumor metastasizes to lungs, vulvovaginal region, brain and liver. Tumor occurs in young boys associated with elevated hCG, and impotence associated with poor prognosis.

PRIMITIVE NEUROECTODERMAL TUMORS

Primitive neuroectodermal tumors are a group of highly-malignant tumors composed of small round cells of neuroectodermal origin that arise in central nervous system, autonomic nervous system and outside central and autonomic nervous systems. Primitive neuroectodermal tumors (PNETs) include glioblastoma multiforme, astrocytoma, epen dymoma, medulloblastoma (rosettes in 33% cases), oligodendroglioma, retinoblastoma, neuroblastoma and Ewing sarcoma. PNETs exhibit great diversity in their clinical manifestations and pathologic similarities with other small cell tumors. Neuroectodermal neoplasms are given in Table 6.15.

Neuroblastoma

Neuroblastoma is a primitive neoplasm of neuroectodermal origin. Tumor occurs anywhere in distribution of neuroendocrine system (adrenal medulla, sympathetic ganglia) followed by soft tissue, retroperitoneum, and mediastinum. Tumor most often affects children under 5 years with peak incidence in the first 3 years of life. Tumor secretes catecholamines, hence urine analysis reveals high levels of catecholamines or their metabolites (dopamine, vanillylmandelic acid, homovanillic acid).

- **Clinical features:** Clinical features depend on location and extent of tumor. Deteriorating health, malnourishment and pain indicate metastatic disease. Patient often presents with a large abdominal

Table 6.15 Neuroectodermal neoplasms

Tumor	Cell of Origin	Comments
Astrocytoma	Astrocyte (type of glial cell)	Astrocytes normally provide support to neurons in central nervous system and tumor arising from astrocyte is called astrocytoma
Glioblastoma multiforme	Astrocyte (type of glial cell)	Highly aggressive astrocytoma
Medulloblastoma	Granular cells of cerebellum	Granular cells are present at the lower level of cerebellar cortex
Ependymoma	Cells lining the ventricles of the brain	Ventricles are fluid-filled cavities of brain, tumor arising from cells lining the ventricles is called ependymoma
Oligodendroglioma	Oligodendrocytes covering axons	Oligodendrocytes are similar to Schwann cells that construct insulating myelin sheath around axons in the brain
Meningioma	Arachnoid cells of meninges	Meninges cover the brain, tumor arising from arachnoid cells is called meningioma
Schwannoma	Schwann cells around axons	Schwann cells around axons construct insulating myelin sheath in the peripheral nervous system
Retinoblastoma	Cone cell in the retina	Photosensor for color vision during day-light
Neuroblastoma	Cells of the peripheral nervous system	Neuroblastoma arises from cells of the sympathetic nervous system

mass, malaise, fever, pallor and loss of weight. Neuroblastoma spreads to the liver, bones in skull, orbit, scalp, lymph nodes, subcutaneous tissue, intraspinal extension or kidney. Bone metastases are multiple and sometimes symmetric.

- Hutchinson's type of neuroblastoma is characterized by left side primary tumor and spreading upward by lymphatic route and metastases in the orbit and skull.
- Pepper type of neuroblastoma is characterized by primary tumor as well as secondaries especially liver metastases on right side of body.

- **Radiologic examination:** Abdominal contrast enhanced CT scan demonstrates irregular-shaped lobulated and heterogenous mass, calcification, hemorrhage and necrosis.
- **Gross examination:** Tumor is well-circumscribed ovoid to multilobated. Cut surface reveals areas of hemorrhage with vague and bulging lobules.
- **Histologic examination:** Tumor is composed of small, round blue tumor cells (neuroblasts) with '**salt and pepper chromatin**', inconspicuous nucleoli, and minimal cytoplasm, forming characteristic rosette-like structures (Homer-Wright pseudorosettes) around small vessels. Histology of neuroblastoma is shown in Fig. 6.47.
- **Immunohistochemistry:** Tumor cells of neuroblastoma show positivity for immunohistochemical markers neuron-specific enolase (NSE), neurofilament, chromogranin A, and synaptophysin.
- **Treatment:** Several types of treatment can be used for neuroblastoma which include surgery, chemotherapy, radiation therapy, high-dose chemotherapy and stem cell transplant, and immunotherapy.

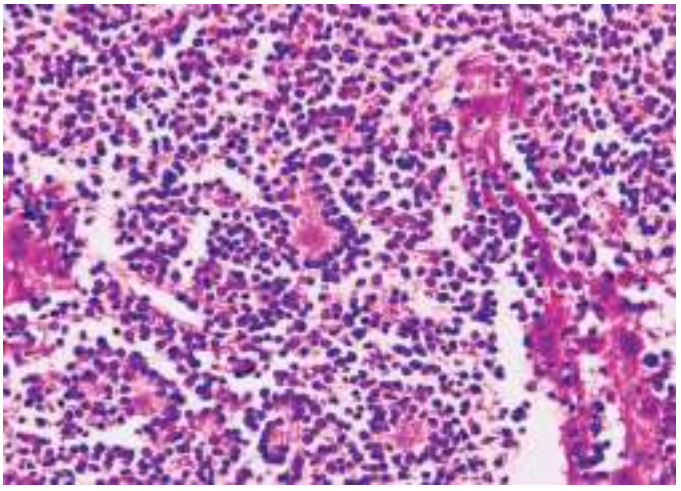


Fig. 6.47: Histology of neuroblastoma. Tumor is composed of small round blue cells (neuroblasts) and Homer-Wright pseudorosettes. Tumor cells contain round to oval nuclei with stippled salt and pepper chromatic and scanty cytoplasm (400X).

Retinoblastoma

Retinoblastoma is the most common intraocular malignancy in children caused by mutational inactivation of both alleles of RB1 gene, which maps to chromosome 13q14 and encodes retinoblastoma protein (pRB) that acts a tumor suppressor. Normally, RB1 gene is essential for the normal differentiation and growth of retinal stem cells. Tumor arises from the nuclear layer of the retina, with different growth patterns: endophytic, exophytic and mixed.

- **Clinical features:** Signs of retinoblastoma include white pupil, called leukocoria, strabismus, painful blind eye and loss of vision.

- **Radiologic examination:** Mineralization is common within human retinoblastoma, that can be diagnosed by ultrasound, CT scan and MRI scan. Exposure to radiation is not preferred especially in patients with RB1 germline mutation predisposed to second malignancy.
- **Gross morphology:** Cut surface of tumor reveals white, brain-like appearance with chalky areas of dystrophic calcification and yellow necrotic areas. Choroid and optic nerve extension can often be observed on gross examination.
- **Histologic examination:** Tumor is composed of small round blue cells with hyperchromatic nuclei, scant cytoplasm, high nuclear to cytoplasmic ratio, variable necrosis and calcifications. Tumor differentiation can be determined by the presence of rosettes. Well-differentiated retinoblastoma shows 'Homer-Wright rosettes'. Less-differentiated retinoblastoma shows 'Flexner-Wintersteiner rosettes' and fleurettes. Poorly-differentiated retinoblastoma comprises anaplastic cells without rosettes or fleurette formation with nuclear pleomorphism and atypical mitotic figures.
- **Treatment:** Several types of treatment for retinoblastoma are surgery (enucleation), radiation therapy, laser therapy (photocoagulation or thermotherapy), cryotherapy, and chemotherapy.

NEUROENDOCRINE TUMORS

Neuroendocrine tumors may synthesize a number of hormones. Selected examples of neuroendocrine tumors are carcinoid tumors, medullary thyroid carcinoma, pheochromocytoma, Merkel cell carcinoma, small cell lung carcinoma and large cell neuroendocrine tumor of lung. Recent 2022 WHO classification of neuroendocrine tumors is given in Table 6.16.

- **Tumor markers:** Many tumor markers are used for diagnosis and follow-up of neuroendocrine tumors such as serum calcitonin in medullary thyroid carcinoma, serum catecholamines, and urinary 5-hydroxyindoleacetic acid (5-HIAA) in carcinoid tumor, serum insulin in insulinoma, gastrin, pancreatic polypeptide and glucagon specific to one subtype of exocrine neuroendocrine tumor of pancreas. Tumor markers

Table 6.16 Recent 2022 WHO classification of neuroendocrine tumors

Neuroendocrine Neoplasm	Classification	Diagnostic Criteria
Gastrointestinal and panreatobiliary tract		
Well-differentiated neuroendocrine tumor (NET)	▪ NET grade 1 ▪ NET grade 2 ▪ NET grade 3	▪ Mitoses (<2/2 mm²) and/or Ki-67 (<3%) ▪ Mitoses (2–20/2 mm²) and/or Ki-67 (3–20) ▪ Mitoses (>20/2 mm²) and/or Ki-67 (>20)
Poorly-differentiated neuroendocrine tumor (NET)	▪ Small cell NET ▪ Large cell NET	▪ Mitoses (>20/2 mm²) and/or Ki-67 (>20) ▪ Mitoses (>10/2 mm²) and/or Ki-67 (>20, often >70) and large cell morphology
Upper aerodigestive tract and salivary glands		
Well-differentiated neuroendocrine carcinoma (NEC)	▪ NET grade 1 ▪ NET grade 2 ▪ NET grade 3	▪ Mitoses (<2/2 mm²) and/or necrosis, Ki-67 (<20) ▪ Mitoses (2–10/2 mm²) and/or Ki-67 (<20) ▪ Mitoses (>10/2 mm²) and/or Ki-67 (>20)
Poorly-differentiated neuroendocrine carcinoma (NEC)	▪ Small cell NET ▪ Large cell NET	▪ Mitoses (>10/2 mm²) small cell morphology and/or Ki-67 (>20) ▪ Mitoses (>10/2 mm²) larger cell morphology and/or Ki-67 (>20)
Lung and thymus gland		
Well-differentiated neuroendocrine tumor (NET)	▪ Typical carcinoid/NET grade 1 ▪ Atypical carcinoid/NET grade 2 ▪ Carcinoids/NETs with elevated mitotic counts and/or Ki-67 proliferative index	▪ Mitoses (<2/2 mm²) and lack of necrosis ▪ Mitoses (2–10/2 mm²) and/or necrosis (usually punctate) ▪ Mitoses (>10/2 mm²) and/or Ki-67 (>30)
Poorly-differentiated neuroendocrine carcinoma (NEC)	Small cell lung carcinoma	▪ Mitoses (>10/2 mm²) often necrosis and small cell morphology ▪ Mitoses (>10/2 mm²) and virtually presence of necrosis and large cell morphology
Thyroid gland		
Medullary thyroid carcinoma (MTC)	▪ Low-grade MTC ▪ High-grade MTC	▪ Mitoses (<5/2 mm²) and lack of necrosis and/or Ki-67 (<5) ▪ At least one of the following features: Mitoses (≥5/2 mm²), Ki-67 (≥5), necrosis

Table 6.17 Tumor markers in neuroendocrine tumors in clinical oncology

Neuroendocrine Tumor	Serum and Urine Markers
Carcinoid tumor	▪ Serum chromogranin A ▪ Urinary 5-hydroxyindoleacetic acid (5-HIAA)
Nonfunctional carcinoid tumor	▪ Serum chromogranin A ▪ Serum neuron-specific enolase (NSE)
Small cell lung carcinoma	▪ Serum chromogranin A ▪ Serum synaptophysin ▪ Serum neuron-specific enolase (NSE)
Medullary thyroid carcinoma	▪ Calcitonin ▪ Serum chromogranin A ▪ Carcinoembryonic antigen (CEA)
Insulinoma	▪ Serum insulin ▪ Serum C-peptide ▪ Serum proinsulin ▪ Serum neuron-specific enolase (NSE) ▪ Serum chromogranin A
Nonfunctional pancreatic neuroendocrine tumor	▪ Serum pancreatic polypeptide ▪ Serum neuron-specific enolase ▪ Serum chromogranin A
Gastrinoma	▪ Serum gastrin ▪ Serum chromogranin A
Pheochromocytoma	▪ Urinary metanephrine ▪ Serum neuron-specific enolase ▪ Serum chromogranin A

in neuroendocrine tumors in clinical oncology are given in Table 6.17.

- **Immunohistochemistry:** General immunohistochemical markers for various neuroendocrine tumor subtypes include chromogranin A, synaptophysin and neuron-specific enolase (NSE).

Carcinoid Tumor

Carcinoid tumor is slow-growing neuroendocrine tumor originating in gastrointestinal tract (70%), i.e. stomach, appendix, small intestine, colon, rectum, respiratory tract (25%), i.e. bronchopulmonary region, and rarely ovaries, testicle and kidneys. Carcinoid tumors are classified based on embryonic site of origin: (a) carcinoid tumors in foregut with trabecular pattern (stomach, respiratory tract), (b) carcinoid tumors in midgut with polygonal cells arranged in sheets (small intestine, appendix), and (c) carcinoid tumors in hindgut (distal colon, rectum, genitourinary tract) with similar morphologic pattern and demonstrated by silver staining.

- **Clinical features:** In normal persons, biologically active polypeptides and biogenic amines (5-hydroxytryptamine, 5-hydroxyindoleacetic acid, dopamine, histamine), polypeptides (chromogranin A, neurotensin, vasoactive amines, motilin, somatostatin, substance P), tachykinins (kallikrein, neuropeptide) and prostaglandins are normally inactivated in the liver. Most patients become symptomatic due to increased level of serotonin. Serotonin is an end product of tryptophan metabolism. Serotonin undergoes oxidative reactions that lead to the formation of 5-hydroxyindoleacetic acid (5-HIAA) with the help of aldehyde dehydrogenase, which is then excreted in the urine.
  - **Midgut carcinoid tumor:** Midgut carcinoid tumor secretes bioactive products, which causes carcinoid syndrome in patient with liver metastasis. Active bioactive products, i.e. biogenic amines, polypeptides (tachykinins, serotonin, histamine, kallikrein and prostaglandins) directly enter the systemic circulation, escaping the first-pass metabolism of the liver as these bioactive products are not inactivated due to deranged liver function. Patient presents with carcinoid syndrome characterized by flushing over face, neck and upper trunk (85%), wheezing (10–20%), diarrhea (80%), malabsorption, pellagra (secondary to niacin), tricuspid valvular disease (60–70%), and fatigue. Midgut carcinoid tumors are frequently multiple exhibiting multicentricity associated with mechanical obstruction of the small intestine as a result of extensive mesenteric fibrosis.
  - **Foregut and hindgut carcinoid tumors:** Carcinoid tumors of the foregut and lungs lack the enzyme aromatic L-amino acid decarboxylase, which is essential for metabolism of 5-hydroxytryptophan to serotonin. Foregut and hindgut carcinoid tumors do not produce and bioactive products hence they rarely cause carcinoid syndrome. However, lung carcinoid tumor secretes histamine, which can cause flushing and pruritus.
- **Diagnostic modalities:** Diagnostic modalities for carcinoid tumors include testing for biochemical markers followed by localization of tumors with radiologic and endoscopic studies: 24-hour urine for 5-hydroxyindoleacetic acid (5-HIAA), immunomarker chromogranin A, radiographic imaging and histologic examination of bronchoscopy biopsy.
- **Gross examination:** Tumor is well circumscribed but not encapsulated. Cut surface reveals white or pale-yellow tan and very firm due to marked desmoplastic reaction.
  - Gastric carcinoid tumor is usually large and solitary.

- Appendiceal carcinoid tumor is discovered as incidental finding.
- Bronchopulmonary carcinoid tumor originates from Kulchitsky cells of bronchopulmonary mucosa, which is well-circumscribed tan-colored, sessile or pedunculated with partial or complete obstruction.

- **Histologic examination: Typical carcinoid tumor** is well-differentiated with size >5 mm and composed of cytologically uniform bland cells with polygonal shape, round to oval nuclei with 'salt and pepper chromatin' and inconspicuous nucleoli arranged in nests, trabecular and pseudoglandular pattern, that exhibit minor cellular atypia and <2 mitoses/2 mm^2 and absence of necrosis. Pathologic diagnostic criteria of atypical bronchopulmonary carcinoid tumor depend on the presence of morphology and at least one of the following: (a) necrosis or (b) 2–10 mitoses per 10 high power fields. Histology of carcinoid tumor of ileum is shown in Fig. 6.48.
- **Immunohistochemistry:** Chromogranin A, synaptophysin, CD56 and neuron-specific enolase (NSE) are diffusely and strongly positive in carcinoid tumors. Ki-67 proliferative index is useful to discriminate between typical and atypical carcinoid, and high-grade small or large cell neuroendocrine tumor. TTF-1 and RB are useful immunomarkers of bronchopulmonary carcinoid tumors.
- **Treatment:** Surgical approach is the primary therapy for localized and locoregional resectable disease.

Small Cell Lung Carcinoma

Small cell lung carcinoma (SCLC) is high-grade neuroendocrine tumor in tobacco smokers associated with invasion and metastasis via lymphatic route to lymph nodes, and via hematogenous route to liver, adrenal glands, bone marrow and brain. Snake-like masses of tumor may involve the portal vein (35–80%), hepatic vein (20%) or inferior vena cava (similar to renal cell carcinoma). Tumor is treated by combined chemotherapy and radiotherapy.

- **Clinical features:** Patient presents with cough, dyspnea, weight loss and paraneoplastic syndromes like Cushing syndrome (ACTH secretion), syndrome of inappropriate antidiuretic hormone secretion (SIADH), Lambert-Eaton syndrome, peripheral neuropathy, cerebellar degeneration, gynecomastia and testicular atrophy (gonadotropin-like hormone).
- **Gross morphology:** Tumor is centrally located in the hilar along peribronchial region. Gross examination reveals white-tan, soft, friable and necrotic tumor with fleshy appearance on cut surface.
- **Histologic examination:** Tumor is composed of round/oval blue cells with minimal cytoplasm, usually small- to medium-sized with finely dispersed chromatin, molding, smudging and high mitotic rate but without distinct nucleoli. Tumor cells are arranged in sheets, clusters, ribbons, and rosettes. Thin, scant and delicate fibrovascular stroma present. 'Azzopardi phenomenon' can be demonstrated as basophilic nuclear material lining blood vessel walls.
- **Electron microscopy:** Electron microscopy of tumor reveals neurosecretory granules, which are present in cytoplasm with perinuclear hallo.
- **Immunohistochemistry:** SCLC is positive for immunohistochemical markers such as synaptophysin and chromogranin A (90–100%). Other immunomarkers such as CD56, CD57, neuron specific enolase (NSE), TTF-1, and napsin.

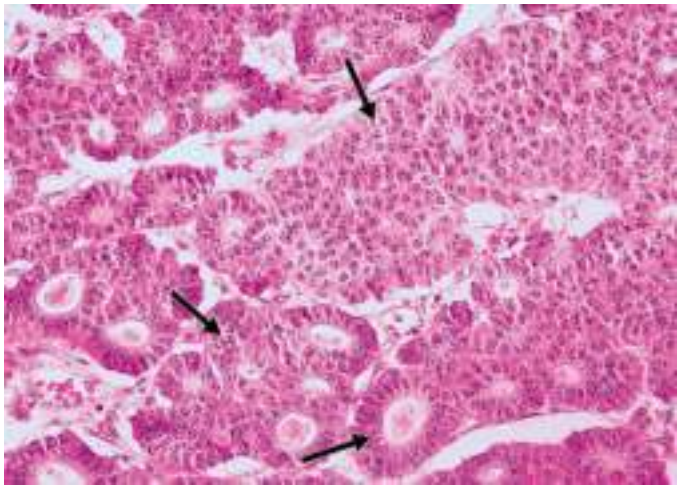


Fig. 6.48: Histology of carcinoid tumor of ileum. Tumor is composed of uniform cells with stippled chromatin (arrows) (400X).

Medullary Thyroid Carcinoma

Medullary thyroid carcinoma is neuroendocrine tumor derived from C cells of ultimobranchial body of neural crest, which secretes calcitonin. Tumor can be either sporadic (70%) or familial (germline mutation of RET gene in 30%) in the settings of multiple endocrine neoplasia 2A or 2B (MEN-2A or 2B), familial medullary thyroid carcinoma, von Hippel-Lindau disease or neurofibromatosis (NF).

- **Clinical features:** Patient presents with painless thyroid mass, and/or lymph node involvement (75%). Serum calcitonin level is correlated with tumor burden. Patient with metastasis may present with severe diarrhea and flushing. Tumor may secrete ACTH or corticotropic releasing hormone leading to Cushing syndrome.

- **Gross examination:** Sporadic medullary thyroid carcinoma is well-circumscribed and unencapsulated, gray-tan and firm mass. Familial medullary thyroid carcinoma is generally bilateral and multifocal. Cut surface of large tumor shows areas of hemorrhage and necrosis.
- **Histologic examination:** Tumor is composed of round, polygonal, plasmacytoid with round nuclei and fine to coarse chromatin, arranged in nests, cords and follicles. Tumor stroma demonstrates amyloid deposits from calcitonin, prominent vasculature and calcifications.
- **Histochemical stain:** Congo red stain demonstrates amyloid in tumor stroma of medullary thyroid carcinoma.
- **Immunohistochemistry:** Medullary thyroid carcinoma is positive for immunohistochemical markers such as calcitonin, carcinoembryonic antigen (CEA), PAX8, calcitonin gene-related peptide, ACTH, somatostatin, gastrin releasing peptide, neurotensin, chromogranin A, synaptophysin, and neuron-specific enolase (NSE).
- **Biochemical modalities:** High serum calcitonin and CEA levels are demonstrated in untreated patients with medullary thyroid carcinoma. Patients are monitored to analyze their levels.
- **Treatment:** Surgical thyroidectomy with neck dissection is performed base on serum calcitonin. Total thyroidectomy with cervical lymphadenectomy is done for lymph node positive patients.

Pheochromocytoma

Pheochromocytoma is a neuroendocrine tumor, which originates from chromaffin cells of the adrenal medulla that affects fourth- and fifth-decade persons. The tumor cells have traits of both hormone-producing endocrine cells (catecholamines, i.e. adrenaline and noradrenaline during stress), and nerve cells.

- **Pathogenesis:** Tumor is most often sporadic, associated with hereditary syndromes (30%) in the settings of von Hippel-Lindau syndrome, multiple endocrine neoplasia 2 (MEN2), neurofibromatosis type 1 (NF1) and familial paraganglioma. Malignant pheochromocytoma occurs in 10% of cases. Uncontrolled surges of extra catecholamines (adrenaline and noradrenaline) released by pheochromocytoma increase blood pressure and heart rate. Hormonal surges can still lead to life-threatening complications, i.e. cerebral stroke, myocardial infarction or sudden cardiac death.
- **Clinical features:** Patient presents with classic triad of episodic headaches, sweating and increased heart rate in 30% of cases. Other clinical manifestations are palpitations, postural hypotension and paroxysmal hypertension. Subclinical pheochromocytoma remains asymptomatic.
- **Gross examination:** Tumor is well-circumscribed and unencapsulated mass. Cut surface reveals solid, white to red-brown and hemorrhagic tumor with a mean weight of 150 g. Malignant tumor weighs 280 g.
- **Histologic examination:** Tumor is composed of large, polygonal, uniform or extensively vacuolated cells with abundant fine granular red-purple cytoplasm, round to oval nuclei, prominent nucleoli and arranged in nests, solid sheets or trabecular pattern.
- **Immunohistochemistry:** Positive immunohistochemical stains for pheochromocytoma are synaptophysin, chromogranin A, S-100, GATA3, CD10, and vimentin. SDHB is usually positive unless in SDHB deficient tumor.
- **Diagnostic modalities:** Tumor secretes catecholamines (i.e. noradrenaline and adrenaline or dopamine) or their metabolites. Catecholamines and their metabolic products are detected in the blood or urine via high performance chromatography or tandem mass spectroscopy. Chromogranin A secreted by chromaffin cells is detected in serum in 90 of cases. Computed tomography (CT) imaging can detect lesion 5 mm. Magnetic resonance imaging (MRI) is done to evaluate metastatic disease.
- **Treatment:** Surgical resection of pheochromocytoma tumor is done via total adrenalectomy unless bilateral tumors. Chemotherapy is administered in unresectable malignant tumor.

Pathology Pearls: Paraganglioma (Neuroendocrine Tumor)

- Paraganglioma is a slow-growing neuroendocrine tumor that can originate in the abdomen (most common), along sympathetic nervous system or parasympathetic nerves in the head and neck, carotid artery, chest, pelvis and urinary bladder. Tumor remains localized (60%) and metastasizes (40%) to distant organs.
- Like pheochromocytoma, sympathetic paraganglioma almost always secretes catecholamines. Sympathetic nervous system regulates body's responses to stress and activity.
- Parasympathetic paraganglioma occurs in the head and neck regions, which usually does not produce catecholamines. Parasympathetic nervous system regulates the body functions during rest.

Merkel Cell Carcinoma

Merkel cell carcinoma is highly aggressive primary neuroendocrine carcinoma of skin affecting elderly and immunocompromised persons. Tumor occurs in the

settings of polyomavirus (most often), and exposure to sun ultraviolet radiation (head and neck > extremities > trunk).

- **Gross examination:** Tumor is fleshy to tan-brown with expansile, nodular or diffusely infiltrative growth with variable ulceration within dermis and variably in subcutis.
- **Histologic examination:** Tumor is composed of small round cells with round to oval nuclei, nuclear molding, finely dispersed chromatin with **salt** and **pepper appearance,** scant cytoplasm, atypical mitotic figures arranged in sheets and trabeculae.
- **Immunohistochemistry:** Merkel cell carcinoma is positive for immunohistochemical markers such as cytokeratins (CAM 5.2, AE1/AE3, CK20), chromogranin, synaptophysin, neuron-specific enolase (NSE), and CD556. MCPyV is positive nuclear immunostain. Tumor can express PAXA5, BCL-2, CD99, Fli-1 and TdT.
- **Treatment:** Treatment consists of wide local excision in negative sentinel lymph node patient with or without adjuvant radiotherapy. Advanced tumor is treated by immune checkpoint blockade therapy, targeting PDL1/P1 signaling pathway. Chemotherapy is ineffective and reserved for palliation.

HEMATOLYMPHOID MALIGNANCIES

The hematopoietic cells are derived from the embryonic mesoderm. Hematolymphoid malignancies have no benign counterparts, which include leukemias, multiple myeloma, Hodgkin's disease and non-Hodgkin's lymphoma.

Leukemias

Leukemia is a heterogenous group of hematologic malignancies that originate from dysfunctional proliferation of developing leukocytes in the bone marrow. It can be classified by either acute myelogenous leukemia (AML-M0-to-AML-M7) or acute lymphoblastic leukemia (ALL-L1, ALL-L2, ALL-L3), or chronic myelogenous leukemia (CML) and chronic lymphocytic leukemia (CLL). Leukemic cells can infiltrate liver, spleen, lymph nodes, central nervous system, testes and other organs.

- **Acute myelogenous/lymphoblastic leukemias:** According to revised 2016 WHO classification of acute leukemias (acute myelogenous leukemia and acute lymphoblastic leukemia), diagnostic criteria are the presence of ≥20% blast cells as the cut off percentage in bone marrow and peripheral blood. Patient suffering from acute leukemias presents with weakness, lethargy, fatigue, dyspnea, fever, weight loss or easy bruising or bleeding. Blasts may also infiltrate lymph nodes and organs resulting in lymphadenopathy and hepatosplenomegaly. Bone marrow infiltration with blasts can result in bone pain.
- **Chronic myelogenous/lymphocytic leukemias:** CML is pluripotent hematopoietic stem cell disorder with chromosomal translocation t(9,22) (q34.1;q12.2) and formation of the Philadelphia (Ph) chromosome, containing the BCR-ABL1 fusion gene. CML occurs in three different phases: chronic phase (bone marrow, blood and spleen), accelerated phase and blast phase (spleen, liver, lymph nodes, skin and soft tissue). CLL is a malignant disorder of mature B cells with characteristic immunophenotype showing peripheral lymphocytosis ($\geq 5 \times 10^9$/L) with or without lymph node or extranodal manifestations.

Multiple Myeloma

Multiple myeloma is a neoplastic proliferation of plasma cell that can commonly result in multiple skeletal lesions, hypercalcemia, renal insufficiency, anemia and immunosuppression.

- **Diagnostic criteria:** Diagnostic criteria of multiple myeloma include: presence of plasma cells ≥10% in trephine bone marrow biopsy or biopsy proven bony/extramedullary plasmacytoma and ≥1 of the CRAB features and multiple myeloma defining events. CRAB features of end-organ damage include: high calcium level (C), renal insufficiency, anemia (A), bone pain (B) and ≥1 osteolytic lesion on radiograph, CT scan or PET/CT scan.
- **Treatment:** Treatment is usually chemotherapy and radiation. Surgery management is indicated for associated pathologic fractures.

Lymphomas

Lymphomas are malignancies that develop from lymphocyte (i.e. Hodgkin's disease and non-Hodgkin's lymphoma), which mainly involve the lymph nodes. WHO categorizes Hodgkin's disease into two subtypes: (a) classical Hodgkin's disease (i.e. nodular sclerosis, mixed cellularity, lymphocytic depletion and lymphocytic rich), and (b) nodular lymphocytic-predominance.

- **Diagnostic criteria:** Hodgkin's disease is marked by the presence of neoplastic Reed-Sternberg cells (RS cells), whereas Reed-Sternberg cells are absent in non-Hodgkin's's lymphoma.
  - Reed-Sternberg cells in Hodgkin's disease (HD) include classical Reed-Sternberg cells (mixed cellularity HD), mononuclear Reed-Sternberg cells (mixed cellularity HD), pleomorphic Reed-Sternberg cells (lymphocytic depletion HD), lacunar Reed-Sternberg cells (nodular sclerosis HD), and lymphocytic–histiocytic (popcorn) Reed-Sternberg cells (nodular lymphocytic predominance HD).

- Reed-Sternberg cells are CD30 and CD15 positive except in the Hodgkin's disease (nodular lymphocytic predominance). Nodular lymphocytic predominance Hodgkin's diseases are positive for CD20 and CD45.

- **Lymph nodes involved:** Hodgkin's disease most often involves lymph nodes in neck, axillary region and chest. In contrast, non-Hodgkin's lymphoma involves lymph nodes throughout the body.

MISCELLANEOUS TERMINOLOGY RELATED TO NEOPLASMS

Miscellaneous terminology related to neoplasms is used by pathologists such as congenital melanocytic nevus, hamartomas, choristomas and polyps in various organs. Hamartoma is not a neoplasm, which consists of a disorganized collection of tissue, normally found in the organ. Choristoma is not a neoplasm, which is composed of ectopic fairly normal tissue located at a site where it normally is not present. **Polyp** refers to mass projecting from a mucosal surface, which may or may not be a neoplasm.

Hamartoma

Hamartoma (from Greek *hamartia* meaning '**fault defect**' and *oma*, denoting a tumor) is benign appearing non-neoplastic tissue composed of mature but disorganized cells of tissues indigenous to the particular organ, where growth occurs. It is considered a developmental error and can occur at a number of sites. Hamartoma contains varying combinations of mature cartilage, ducts or bronchi, connective tissue, blood vessels, and lymphoid tissue. Developmental remnants may be considered hamartomatous if they form discrete tumor-like masses. Hamartomatous syndromes are given in Table 6.18.

- **Congenital melanocytic nevus:** Congenital melanocytic nevus represents an aggregate of pigment cells since birth over trunk and limbs, that are normally dispersed in the skin. Nevus can be flat or raised or thickened that shows tan to black discoloration. Excessive hair growth can occur within the nevus.
- **Hamartomas in Peutz-Jeghers syndrome:** Peutz-Jeghers syndrome is an autosomal dominant disorder due to LKB1/STK11 gene in young children 10–15 years of age. Patient presents with multiple hamartomatous polyps in small intestine (most common), stomach, and colon in decreasing frequency; melanotic pigmented macules (1–5 mm) on lips, perioral skin, hands and genitalia. Peutz-Jeghers syndrome is linked to increased risk for cancers of gastrointestinal tract, thyroid, breast, lung, pancreas, gonads and urinary bladder.
- **Pulmonary hamartoma:** Pulmonary hamartoma presents as a nodule composed of cartilage, bronchial epithelium, and smooth muscle cells. It occurs in adults over 40 years of age, with a peak incidence

Table 6.18 Hamartomatous syndromes

Syndrome	Gene Locus	Clinical Features	Predisposition to Cancers
Juvenile polyposis	▪ SMAD4 (18q21.1) ▪ BMPRIA (10q22.3)	Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu) especially with SMAD4 gene mutation	▪ Colorectal carcinoma ▪ Gastric carcinoma ▪ Pancreatic carcinoma
Peutz-Jeghers syndrome	STK11 (18p13.3)	▪ Small intestinal hamartomas that may obstruct or bleed ▪ Hyperpigmented macules mouth, buccal mucosa, eyes, nostrils, perianal regions	▪ Thyroid carcinoma ▪ Ovarian carcinoma ▪ Lung carcinoma ▪ Pancreatic carcinoma ▪ Breast carcinoma
Cowden's syndrome (PTEN hamartoma syndrome)	PTEN (10q23.31)	▪ Mucocutaneous features in ≤80% ▪ Macrocephaly ▪ Mixed colonic polyposis	▪ Thyroid carcinoma ▪ Breast carcinoma ▪ Endometrial carcinoma ▪ Brain cancer ▪ Renal cell carcinoma
PTEN-hamartoma syndrome (Bannayan-Riley-Ruvalcaba syndrome)	PTEN (10q23.31)	▪ Microcephaly ▪ Lipomatosis ▪ Pigmented macules of the glans penis	▪ Thyroid carcinoma ▪ Breast carcinoma ▪ Endometrial carcinoma ▪ Brain cancer ▪ Renal cell carcinoma
Hereditary mixed polyposis syndrome	▪ BMPRIA (10q22.3) ▪ GREM1	▪ Atypical polyposis with juvenile polyps, adenomas ▪ Hyperplastic and inflammatory adenomas	Colorectal carcinoma

in the sixth decade of life. It constitutes 10% of coin lesions in lungs with popcorn pattern of calcification, discovered as incidental finding on chest radiograph. Immunohistochemistry technique demonstrates expression of smooth muscle actin and S-100 in spindle cells. In males, the epithelial cells express ER, PR and androgen receptors. Histology of pulmonary hamartoma is shown in Fig. 6.49.

- **Bile duct hamartoma:** Bile duct hamartoma is asymptomatic lesion also known as von Meyenburg complex that shows irregularly dilated bile ducts. It is important to distinguish this disorder from multiple hepatic metastases. Women are more affected than men. Histologic examination of the hamartomatous nodule shows proliferations of bile ducts. Histology of bile duct hamartoma is shown in Fig. 6.50.

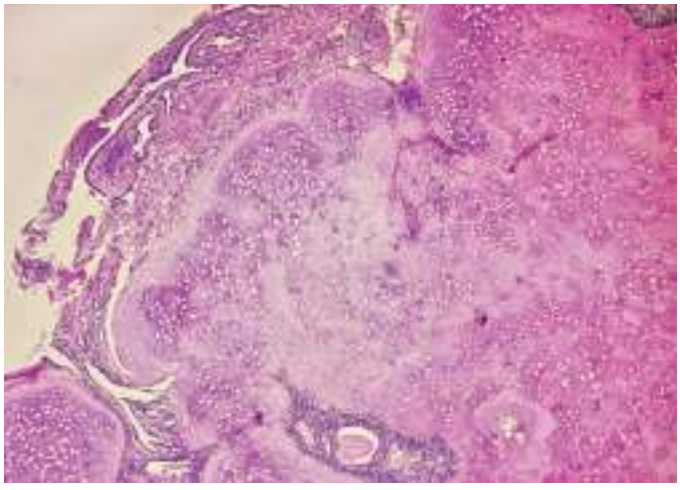


Fig. 6.49: Histology of pulmonary hamartoma. Tumor is composed of cartilage, bronchial epithelium, and smooth muscle cells (100X).

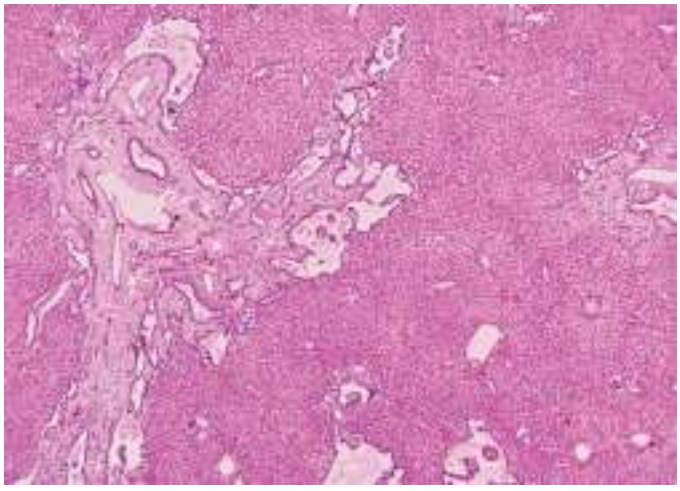


Fig. 6.50: Histology of bile duct hamartoma. Multiple bile duct hamartomas are also known as von Meyenburg complex. Bile duct hamartoma is composed of small disorganized clusters of dilated cystic bile ducts lined by columnar cells enclosed within fibrocollagenous stroma (100X).

Table 6.19 Selected examples of choristomas

Microscopic Tissue	Located in Organs
Pancreatic tissue	Stomach intestine, liver
Gastric mucosa	Meckel's diverticulum
Adrenal rest	Beneath renal capsule
Ectopic brain tissue	Nasal cavity
Splenic tissue	Peritoneal cavity

Choristoma

Choristoma is ectopic islands of normal mature tissue derived from germ cell layers misplaced within another organ, e.g. pancreatic tissue within the wall of the stomach.

- About 85% of osseous and cartilaginous choristomas occur on tongue (dorsal posterior third near foramen cecum) especially in adults. Presence of ectopic islands of normal mature tissue derived from germ cell layer in an organ in a disorganized mass is called choristoma. Selected examples of choristoma are given in Table 6.19.
- Patient presents with firm, exophytic, symptomatic nodule covered by intact oral mucosa. Base of the lesion may be sessile or pedunculated. Glial choristoma of palate has been reported in one-month infant. Buccal choristoma has been reported in a five-year old child. Surgical resection remains the best treatment to prevent recurrence of choristoma.

Surgical Pathology: Choristoma

Gross Morphology

- Choristoma is firm mass covered by oral mucosa with smooth contour usually measuring less than 1 cm. Careful histologic examination of choristoma of >2 cm size is essential.
- Oral choristomas can be classified according to types of tissues they constitute: salivary gland choristoma, cartilaginous choristoma, osseous choristoma, lingual thyroid choristoma, lingual sebaceous choristoma, glial choristoma, gastric mucosal choristoma and respiratory mucosal choristoma.
- Cartilaginous choristoma is composed of mature hyaline cartilage in fibrous tissue that resembles perichondrium; usually multilobulated chondrocytes lacking atypia.
- Osseous choristoma is composed of dense mature bone with compact osteocytes.

Histologic Examination

- Choristoma should be differentiated from cartilaginous metaplasia beneath ill-fitted denture and pleomorphic adenoma of thyroid gland.

- Pancreatic tissue can be located in wall of gastric, intestine and liver.
- Gastric tissue is demonstrated in Meckel's diverticulum.
- Adrenal rest may be located beneath renal capsule.
- Splenic tissue may be located in the peritoneum.

Polyps in Various Organs

Polyp is a mass that projects above a mucosal surface to form a macroscopically visible structure. A polyp is a descriptive term; the mass causing the polyp may or may not be a neoplasm. Polyps are distinguished by their growth patterns (i.e. sessile or pedunculated). Sessile polyps are flat and they grow directly out of the surrounding tissue. Pedunculated polyps have a stalk, that is attached to the mucosa. Polyps occur on surface lining of nasal cavity, gastric region, large intestine and uterine endometrium.

Nasal Polyps

Nasal polyps are soft painless, benign growths on the lining of nasal passages or paranasal sinuses. They hang down like grapes, and frequently occur in childhood. Nasal polyps arise from wall of maxillary antrum, extending through maxillary ostium into nasal cavity. These may project into choanae or nasopharynx.

- **Predisposing factors:** Predisposing factors for nasal polyps include chronic inflammation, bronchial asthma, allergic rhinitis, sensitivity to nonsteroidal anti-inflammatory drugs (NSAIDs), cystic fibrosis and Churg-Strauss syndrome or immune system disorders.
- **Clinical features:** Patient presents with sensation of nasal block, breathing through mouth, running nose, postnasal drip, nasal congestion, snoring, and headache.
- **Gross morphology:** Nasal polyp has long narrow stalk with firm fibrous body.
- **Histologic examination:** Nasal polyp is composed of thin surface, stroma with stellate cells, less edema and fewer glands, prominent blood vessels and numerous eosinophils.
- **Treatment:** Nasal polyps may recur after surgery.

Endometrial Polyps

Endometrial polyps are hyperplastic overgrowth of endometrial tissue that forms a localized projection as polypoidal or pedunculated mass into the endometrial cavity. Polyp originates from anywhere in the endometrium in the posterior surface of uterine cavity, followed by anterior, lateral and uterotubal junction.

- **Predisposing factors:** Predisposing factors for endometrial polyps include increased endogenous estrogen and exogenous estrogen administration.
- **Clinical features:** Patient can present with irregular or heavy periods, bleeding or spotting between menstrual periods or abnormal vaginal bleeding after menopause in 25% of patients.
- **Gross morphology:** Endometrial polyp is usually solitary, 0.3–12 cm size, smooth surface, sessile or broad base, pedunculated or attached to the endometrium by thin stalk. Cut surface reveals soft, cystic, firm, and fibrous appearance.
- **Histologic examination:** Endometrial polyp is composed of glandular architecture out of menstrual cycle phase with the background endometrium and supporting fibrous stroma rich in collagen with abundant extracellular connective tissue that contains thick-walled blood vessels secondary to complex interaction of leuteinizing hormone, follicle-stimulating hormone, estrogen and progesterone hormones.
- **Treatment:** Postmenopausal polypectomy of hysterectomy is performed for symptomatic polyp(s).

Gastric Polyps

Gastric polyps are arising from epithelium include fundic gland polyps, hyperplastic polyps and adenomatous polyps. Fundic gland polyps are small and smooth flat benign lesions detected on endoscopy.

- **Hyperplastic gastric polyps:** Hyperplastic gastric polyps are strongly linked to chronic gastritis, *Helicobacter pylori*-induced gastritis and pernicious anemia, and which may undergo malignant change.
- **Adenomatous gastric polyps:** Adenomatous gastric polyps are the most common neoplastic polyps found in the gastric antrum, which may transform to malignant phenotype.

Colonic/colorectal Polyps

Colonic/colorectal polyps are growths that appear on the mucosal surface of the colon varying in size and number. Polyps are distributed throughout the large intestine, i.e. right colon (40%), left colon (40%) and rectosigmoid region (20%). Colonic/colorectal neoplastic polyps include: (a) benign hyperplastic colonic polyps without undergoing malignant change, (b) adenomatous polyps in the colon greater than 2 cm in size may be precursor of colon carcinoma, and (c) malignant colonic polyps are diagnosed by histologic examination.

FEATURES USED TO DISTINGUISH BENIGN TUMORS FROM MALIGNANT TUMORS

DISTINGUISHING FEATURES OF BENIGN AND MALIGNANT TUMORS

Benign tumors tend to grow slower; and malignant tumors tend to grow more quickly, often at a rate corresponding to their degree of anaplasia. Histologic features are reliable indicators of malignancy in many organs, which do not always distinguish benign tumors from malignant tumors in the endocrine glands.

- Benign tumors are well-circumscribed with distinct borders and smoothzz on cut surface. On the other hand, gross appearance of cancers is highly variable, consistent with the diversity of their origin, size, and biological behavior. Cut surface of malignant tumors may be firm, hard (scirrhous)/soft in consistency, homogeneous/heterogeneous texture, solid/cystic appearance, pale/dark color, and discolored by endogenous pigments, and hemorrhage and/or necrosis. Overall, 30% of malignant solid tumors have metastases in organ(s) at the time clinical presentation. Invasion and metastasis are indicators of malignancy associated with poor prognosis.
- In the biology of malignant tumors, cancer stem cells (CSCs) differ from normal cells in their biological properties in many aspects in respect of (a) tumor growth *in vivo*, (b) tumor structure, (c) tumor degree of differentiation, (d) tumor nucleocytoplasmic features, (e) tumor growth rate, (f) tumor cellularity and cell polarity, (g) tumor atypical mitotic figures, (h) monoclonal expansion of CSCs, (i) telomerase activity, (j) tumor angiogenesis, and (k) tumor invasion of basement membrane of tissues, through the action of matrix-metalloproteinase enzymes, detachment of CSCs from neighboring cells due to lack of surface adhesion molecules such as E-cadherin, and (l) tumor metastasis via lymphatic route to draining lymph nodes, hematogenous route to distant organs (lung, liver, bones and brain), and transcoelomic route to ovary (Krukenberg tumor).
- Cancer stem cells (CSCs) also differ from normal cells in many aspects in respect of tumor growth *in vitro*: (a) lack of contact inhibition, (b) exhibition of anchorage-independent growth in soft agar, and (c) exhibition of growth factor-independent tumor growth (autocrine stimulation).
- The term '**anaplasia**' refers, which implies dedifferentiation (loss of the structural and functional differentiation) of normal cells during tumorigenesis, which is almost always indicative of malignancy. Anaplastic cells exhibit pleomorphism, hyperchromatic nuclei, prominent nucleoli, nucleocytoplasmic ratio approaching 1:1, atypical mitotic figures (tripolar, quadripolar), chromosomal structural abnormalities (Philadelphia chromosome in chronic myelogenous leukemia/acute lymphoblastic leukemia), and chromosomal numerical abnormalities (aneuploidy).

TWO BASIC CELLULAR COMPONENTS IN TUMORS

Majority of benign and malignant tumors have two basic components: (a) proliferating neoplastic cells that constitute their parenchyma; and (b) supportive stroma made up of connective tissue and blood vessels. Parenchymal cells have the ability to divide, however supporting connective tissue stroma contains blood vessels, which provide nutrition to the parenchymal cells. Hematopoietic lineage-derived tumors lack supporting connective tissue stroma.

Benign Tumors: Two Basic Components

Benign tumors consist of two distinct components: parenchyma neoplastic (neoplastic cells), and non-neoplastic supporting connective tissue stromal components. They lack invasion in surrounding tissues metastasis. Benign tumors are well-differentiated, which resemble to their normal tissue of origin.

Malignant Tumors: Two Basic Components in Solid Organs

Most solid malignant tumors consist of two distinct components: parenchyma neoplastic (neoplastic cells) and non-neoplastic supporting connective tissue stromal components.

- **Tumor parenchyma:** Tumor parenchyma is made up of neoplastic cells that determines the biologic behavior of the tumor. The stromal cells work in conjunction with the parenchymal cells. The tumor parenchymal cells are responsible for the progression and invasiveness of malignant tumor.
- **Tumor non-neoplastic supporting connective tissue stroma:** Non-malignant and extracellular matrix (ECM) constitute the stroma in the tumor. Tumor connective tissue stroma is induced by the CSCs that contains newly formed blood vessels (tumor angiogenesis induced by angiogenic growth factors), that provide nutritional support and remove waste products. Tumor stroma is made up of two types of cells: (a) resident cells (cancer-associated fibroblasts, endothelial cells, pericytes, CSCs, mesenchymal cells), and (b) non-resident cells (T cells, B cells,

natural killer cells, myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages). In case of leukemias, blood serves as stroma, although an additional stromal response, i.e. angiogenesis may develop in the bone marrow.

Pathology Pearls: Stromal Desmoplasia in Solid Malignant Tumors

- Most infiltrating carcinomas (i.e. invasive ductal adenocarcinoma, i.e. scirrhous carcinoma of female breast, desmoplastic melanoma, cholangiocarcinoma, pancreatic carcinoma and diffuse gastric carcinoma—linitis plastica) induce production of a dense fibrous stroma (desmoplasia) composed of fibroblasts, vascular channels, immature cells and extracellular matrix (ECM) in varying proportions and minimal cellular infiltrate. Desmoplasia in patients with malignancy has long been associated with a poor clinical outcome.
- Stromal desmoplasia has been considered to be a response of invasive cancer stem cells (CSCs) in supporting stroma of the malignant tumor.
- Recent studies revealed that desmoplasia is the result of coordinated changes in several stromal cells under the control of CD36 gene product, whose expression results in a decrease in accumulation of adipocytes, and increase in dedifferentiation of cancer stem cells (CSCs) into fibroblasts, which themselves synthesize more collagen fibers leading to increased deposition of extracellular matrix (ECM) within malignant tumor
- Presence of desmoplasia in breast cancer detected in the form of mammographic density is one of the strongest risk factors for infiltrating duct carcinoma of female breast. On the other hand, medullary carcinoma of female breast lacks desmoplasia and hence exhibits soft consistency.

DEGREE OF DIFFERENTIATION IN TUMORS

During embryonic development, cells become more differentiated and specialized that reflect their specific functions. Differentiation is a term used to describe the extent to which appearance of tumor resembles corresponding their normal parenchymal tissue of origin. Cancer stem cells (CSCs) lose the ability to differentiate and enter a state called anaplasia.

Degree of Differentiation in Benign Tumors

All benign tumors tend to grow slowly and remain localized at the site of origin. Many benign tumors are enclosed by a capsule consisting of connective tissue derived from the structures immediately surrounding the tumor. Well-encapsulated tumors are not anchored to their surrounding tissues. Benign tumors are well-differentiated, which closely resemble to the tissue of origin on histologic examination.

Degree of Differentiation in Malignant Tumors

Malignant tumors are often poorly differentiated/undifferentiated (loss of resemblance to corresponding original parenchymal tissue, called anaplastic tumors), grow rapidly with many atypical mitoses (tripolar or quadripolar mitotic figures), which exhibit invasive growth without capsule and frequently metastasize via lymphatic route to draining lymph nodes and via hematogenous route to distant organ(s).

- On histologic examination, some malignant tumors can exhibit well-differentiation (close resemblance to their tissue of origin), moderate differentiation (intermediate resemblance to their tissue of origin) and poor differentiation. Invasion and malignancy are reliable indicators of malignancy.
- Histologic differentiation is used in grading system of malignant tumors. Differentiation often predicts responsiveness to certain therapies such as estrogen receptor inhibitor such as tamoxifen drug in treatment of infiltrating ductal carcinoma of breast.
  - Keratin pearl is a characteristic feature of a well-differentiated squamous cell carcinoma originating from skin, mouth, upper respiratory tract, and lungs. Squamous cell lung carcinoma synthesizes various ectopic hormones such as ACTH, parathyroid-like hormone, insulin and glucagon.
  - Well-differentiated and moderately-differentiated adenocarcinoma show glandular histology. If malignant tumors outgrow their blood supply, these may undergo necrosis on cut surface.

NUCLEOCYTOPLASMIC FEATURES IN TUMORS

Normal cell contains cell membrane, nucleus and cytoplasm. The cell nucleus contains the majority of the cell's genetic material in the form of multiple linear DNA molecules organized into structures called chromosomes. The nuclear pore complex is the only known gateway between the cell nucleus and the cytoplasm.

Nucleocytoplasmic Features in Benign Tumors

Benign tumors exhibit nucleocytoplasmic ratio close to the normal cells 1:4 or 1:6 and normal bipolar mitotic spindles (bipolar mitoses). On contrary, malignant tumors are composed of pleomorphic cells with hyperchromatic nuclei due to abundant DNA content, prominent nucleoli with high nucleocytoplasmic ratio 1:1, atypical mitotic figures (tripolar or quadripolar) and loss of polarity (disturbed orientation and tendency to form anarchic disorganized growth).

Nucleocytoplasmic Features in Malignant Tumors

In the pathology laboratory, surgical specimens are scrutinized by pathologist to observe nucleocytoplasmic

features to distinguish benign tumors from malignant tumors. Malignant tumors exhibit nucleocytoplasmic ratio 1:1, irregular nuclear membrane, atypical mitotic spindles (tripolar or quadripolar mitoses), and abnormal distribution of chromatin.

- Cytomorphologic nuclear abnormalities in cancer stem cells (CSCs) occur resulting from gene mutations, abnormal expression of genes, dysregulation of signal transduction pathways, alterations in nuclear envelope proteins and abnormal distribution of chromatin, and aneuploidy. Nuclear morphologic abnormalities are analyzed by examination of body fluids, fine needle cytology aspirates and biopsies.
- Cytomorphologic features are important in evaluation of tissue biopsies, where nuclear morphologic features are assessed in conjunction with architectural features such as growth pattern of CSCs in conjunction with desmoplastic stromal response.
- Intranuclear inclusions are important diagnostic features of many benign and malignant tumors (e.g. adrenal cortical adenoma, papillary thyroid carcinoma, melanoma, meningioma, hepatocellular carcinoma, pancreatoblastoma, pulmonary blastoma, fetal type adenocarcinoma, lobular carcinoma of breast and ovarian serous carcinoma). Intranuclear pseudoinclusions represent invaginations of cytoplasm into the nucleus, which are appreciated in cytologic preparations than in frozen sections or paraffin-embedded hematoxylin and eosin-stained tissue sections under light microscope thus forming an important cytological feature.

GROWTH RATE IN TUMORS

Benign tumors tend to grow at slower rate and stay in their primary locations without invading other sites of the body. In contrast, malignant tumors tend to grow rapidly, most often at a rate corresponding to their degree of anaplasia, which destroy surrounding normal tissues and metastasize via lymphatic route to lymph nodes, via hematogenous route to distant organs (liver, lungs, brain and bone) and via transcoelomic route. Growth rate of tumors depends on cell cycle, blood supply and hormones. As the epithelial cells have a shorter cell cycle than connective tissue cells, thus tumors of epithelial origin grow more rapidly than do tumors of connective tissue origin.

Growth Rate in Benign Tumors

Tumor develops when cells in the body divide and grow at an excessive rate. A tumor is a heterogenous mass of cells derived from a single ancestor cell.

- Most benign tumors have slow growth rate.
- A few benign tumors have variable growth rate (e.g. uterine leiomyoma, pituitary adenoma).
- Uterine leiomyoma has variable growth rate depending on circulating estrogen levels. Increased estrogen levels increase the size of leiomyoma, while decreased estrogen levels after menopause leads to decrease in size of leiomyoma.
- Pituitary gland adenoma shrinks in size due to compression of blood vessels by progressive enlargement of tumor in sella turcica.

Growth Rate in Malignant Tumors

Growth fraction refers to the proportion of CSCs in the proliferative phase. At the point, when most malignant tumors are clinically detected, growth fraction is less than 20% (i.e. most malignant tumors have their most rapid growth rate prior to detection).

- At least 30 doubling times of cancer stem cell (CSC) are essential for a malignant tumor to be clinically evident. Exposure to carcinogens result in the transformation of a normal cell to malignant phenotype. Malignant tumors continue to grow with further mutations, only if available nutrients, oxygen, and blood supply are adequate and the immune system fails to recognize or respond to CSCs.
- Growth rate is variable and dependent on differentiation of malignant tumors. Anaplastic cancers have marked increase in growth rate. Growth rate is faster in leukemias, non-Hodgkin's lymphoma and lung carcinoma than breast carcinoma and colon carcinoma.
- CSCs in tumors synthesize their own growth factors, which act in autocrine fashion. There is increased expression of growth factor receptors on the cell membrane of these rapidly growing CSCs. Increased growth factor and growth factor receptors further enhance proliferation of CSCs.
- Chemotherapy is targeted on rapidly proliferating CSCs. Debulking of malignant tumor is done by surgery or radiotherapy. Human cancers synthesizing growth factors with autocrine actions are given in Table 6.20.

CELLULARITY AND CELL POLARITY IN TUMORS

Cell divisions are controlled by internal and external positive and negative signals. In the **asymmetric** division, a stem cell produces one differentiated cell and one stem cell. In the **symmetric division,** a stem cell produces two differentiated cells or two stem cells, which plays an important role in development and homeostasis. Pathologists play important role in molecular diagnostics beyond identification of correct cells for analysis. Accurate cellularity not only ensures

Table 6.20 Human cancers synthesizing growth factors with autocrine actions

Ligand (Growth Factor)	Receptor	Human Cancer Type
HGF	Met	Miscellaneous endocrine tumors, breast carcinoma, lung carcinoma, osteosarcoma
IGF2	IGF-1R	Colorectal carcinoma
IL-6	IL-6R	Multiple myeloma, hereditary nonpolyposis colon cancer (HNPCC)
IL-8	IL-8R	Urinary bladder carcinoma
NRG	ErbB2α/ErbB3	Ovarian carcinoma
PDGF-BB	PDGFRA, B	Osteosarcoma, glioma
PDGF-C	PDFGRA, B	Ewing sarcoma
PRL	PRLR	Breast carcinoma
SCF	KIT	Ewing sarcoma, small cell lung carcinoma
VEGF-A	VEGFR (FLT-1)	Neuroblastoma, prostatic carcinoma, Kaposi sarcoma
TGF-α	EGFR	Squamous cell lung carcinoma, breast carcinoma, prostatic carcinoma, pancreatic carcinoma, mesothelioma
GRP	GRPR	Small cell lung carcinoma

sufficient material for required analytic sensitivity, but also provides a quality assurance of the molecular assays.

Cellularity in Tumors

Normal cells are anchored and oriented to the basement membrane; and CSCs lose this uniform orientation, which grow in a disorganized fashion. Histologic cellularity assessment of residual tumors in post-surgical tissues is used to analyze a malignant tumor's response to a therapy. Correct cellularity assessment of malignant tumors increases the chances of getting an appropriate treatment and facilitates patient's survival.

Cell Polarity in Tumors

Cell polarity is characterized by differences in structure, composition and function between at least two poles of a cell.

- **Physiologic state:** The correct establishment and maintenance of cell polarity in epithelial cells are crucial for normal cell physiology and tissue homeostasis. Apical-basal cell polarity and cell-to-cell adhesion are tightly interconnected. Functionally, **apical-basal polarity** has two fundamental roles in epithelial cells that are intimately linked to tumor suppression. Cell-to-cell adhesion and apical-basal cell polarity are tightly interconnected.
- **Pathologic state:** Loss of cell-to-cell adhesion and apical-basal cell polarity in epithelial cells, tissue disorganization and excessive cell growth are commonly observed in malignant epithelial tumors and correlates with their migration into adjacent tissue surrounding by epithelial–mesenchymal transition (EMT), and metastasis to distant organ(s).
  - Asymmetric cell division has recently emerged as a major regulatory mechanism that regulates stem cell numbers and differentiation.
  - **Front-to-back** polarity exists in migrating cells, which allow cells to adhere and detach from extracellular matrix (ECM) and migrate as isolated cells as well as in clusters during tumorigenesis.

MITOTIC FIGURES IN TUMORS

In normal eukaryotic cells, mitotic cell division typically occurs in a bipolar fashion resulting in two daughter cells with identical nuclear genomes. This restricted polarity is based on tight control of the centrosome cycle so that no more than two chromosomes are concurrently active during mitosis.

- Normally, bipolar mitoses are seen in rapidly proliferating cells in normal bone marrow, intestinal cells, hepatocytes.
- The centrosome is the main microtubules (MT)-organizing center in eukaryotic cells, which plays important roles in polarity, migration and cell division. During the cell cycle, centrosomes duplicate only once during synthetic (S) phase to ensure that at mitotic onset a cell carries two centrosomes, which serve as main microtubule-organizing centers to form mitotic spindle cell at the poles in distinct eukaryotic cell lineages.

- Mitotic activity is a measure of how fast tumor cells are dividing and growing. Careful scrutiny of mitotic figures is important in evaluation of proliferative activity as the counting methodology.

Landscape of Centrosome Abnormalities in Benign Tumors

Increased mitotic activity occurs in myometrial smooth muscle and intramural leiomyoma (up to 15 bipolar mitoses/10 high power fields) over the course of menstrual cycle. Leiomyoma with increased mitotic activity must not have any significant atypia or tumor necrosis. Mitotic activity should be reported as an average over per 10 high power field by using the 40X objective on conventionally figured light microscopes.

Landscape of Centrosome Abnormalities in Malignant Tumors

The centrosome plays an important role in polarity, migration and cell division. Centrosomal defects in human cancers can be classified as structural or numerical aberrations. It is worth mentioning that pyknotic nuclei in smooth muscle can be mistaken as mitoses by an inexperienced pathologist. Ki-67 is a proliferative marker that has some prognostic significance in leiomyosarcoma. Ki-67 immunohistochemical marker is helpful in exclusion of pyknotic nuclei and diagnosing malignancy.

Structural Aberrations in Centrosome and Human Cancers

Structural defects in centrosome can be divided into two groups: (a) defects in centriole structure, and (b) defects in the amount of pericentriolar material (PCM). A centrosome consists of two centrioles embedded in a matrix of proteins known as the pericentriolar material (PCM), which serves as a platform for protein complexes that regulate organelle trafficking, protein degradation and spindle assembly. The origin of centriole structural defects in human cancer is still not clear.

Numerical Aberrations in Centrosome and Human Cancers

Centrosome amplification is hallmark of cancer and has been associated with high-grade tumors and poor prognosis. A number of mechanisms can lead to centrosome amplification, including cytokinesis failure, mitotic slippage, cell–cell fusion, overduplication of centrioles and *de novo* centriole assembly.

- The number of underlying mechanisms may explain the diversity of proteins encoded by oncogenes and tumor suppressor genes and centrosome amplification in human solid malignant tumors breast, prostate, urinary bladder, colon, ovary and pancreas and hematolymphoid neoplasms (Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma and leukemias).
- Similarly, loss of the tumor suppressor BRCA1 gene leads to centrosome amplification via increasing the levels of the pericentriolar material (PCM) and γ-tubulin. In urothelial cancers, centrosome amplification is a strong predictor of tumor recurrence, highlighting its potential as a biomarker for advanced disease.
- In cancers of breast, prostate and head and neck, centrosome amplification is correlated with lymph node and distant metastases, further reinforcing its association with disease progression.
- Counting abnormal mitotic figures (tripolar, quadripolar) in hematoxylin and eosin-stained tissue sections are an integral part of evaluation and grading system of malignant tumors by pathologists for assessment of prognosis and clinical decisions. An elevated mitotic index indicates rapidly dividing cells, which is an important prognostic factor predicting both overall survival and response to chemotherapy in most malignant tumors.
- Anaplastic tumors display abnormal mitotic figures (i.e. tripolar, quadripolar, and multipolar spindles). Atypical tripolar mitosis in high-grade carcinoma is shown in Fig. 6.51.

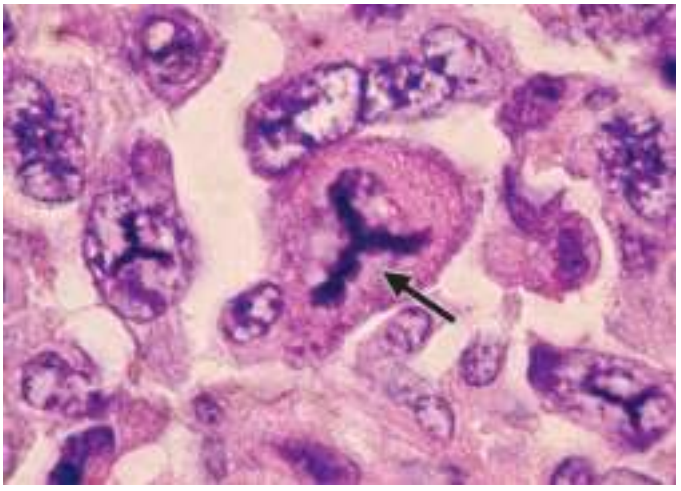


Fig. 6.51: Atypical tripolar mitosis in high-grade carcinoma. Tumor is composed of pleomorphic cells with hyperchromatic large nuclei and high nuclear to cytoplasmic ratio, and atypical tripolar mitotic figure (400X) (arrow).

TUMOR MONOCLONAL PROPERTIES: ANALYSIS TECHNIQUES

Heterogenous malignant tumors invariably develop from the clonal expansion of one genetically damaged precursor cell (clonal progeny), i.e. malignant tumors

are monoclonal rather than polyclonal. Polyclonal cell proliferation is always non-neoplastic in nature. Transformation of a clonal cell population to a malignant tumor is usually a multistep process. Monoclonal properties of malignant neoplasms are shown in Fig. 6.52.

- Successive proliferation of transformed cells leads to form subclones harboring additional different driver and passenger gene mutations with high proliferative potential, which differ from the original normal precursor cell. Eventually, some clonally expanded cells acquire a fully malignant tumor to produce an invasive malignant phenotype. This type of clonal evolution continues in established malignant tumor so that not all cells in a malignant tumor are genetically identifiable, e.g. different growth patterns, and different expression of proteins.
- Evolution of different clonal and subclonal cell properties during carcinogenesis often leads to development of more clinically aggressive malignant tumor. Heterogenous malignant tumor is formed via genetic and epigenetic changes. Similarly, therapeutic maneuvers such as chemotherapy and radiation therapy may be selected for malignant tumor subclones that are resistant to these therapeutic treatment options. For example, patients with estrogen positive breast cancer respond differently to tamoxifen anti-estrogen therapy.
- In addition to monoclonal CSCs, all solid malignant tumors consist of stromal component that provides a supporting extracellular matrix and blood vessels that are essential for malignant tumor growth beyond microscopic 1–2 mm size.
- Growth factors and cytokines secreted by CSCs stimulate stromal component resulting in characteristic histologic appearance called tumor desmoplasia. This type of stromal reaction is characteristic features of most invasive malignant tumors.
- Stromal and vascular cells are not directly derived from CSCs. Malignant tumor-associated vasculature is abnormal and leaky. In some situations, different properties of malignant tumor-associated vasculature may provide therapeutic targets (e.g. blocking tumor angiogenesis).

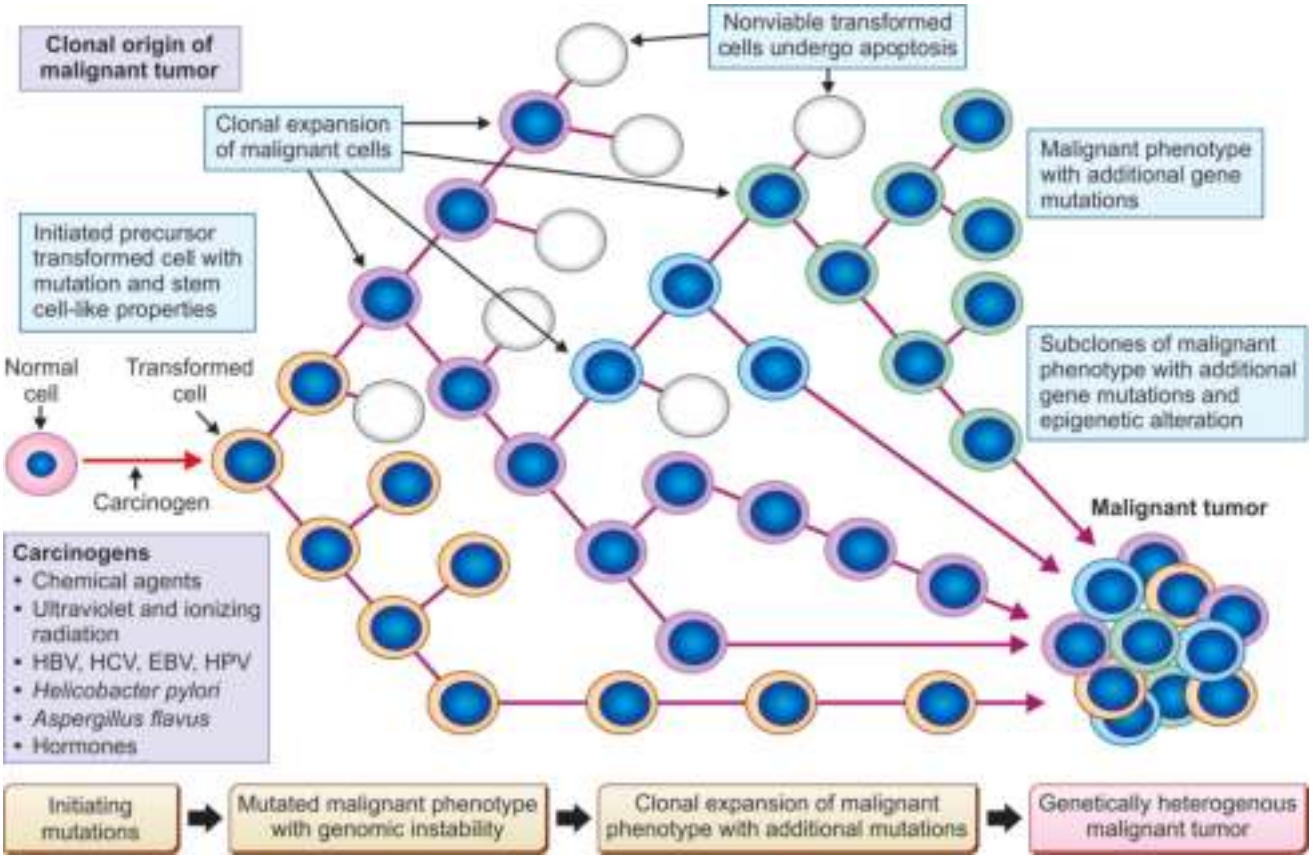


Fig. 6.52: Monoclonal properties of malignant neoplasms. Exposure to carcinogenic stimulus, single aberrant cell undergoing malignant transformation reproduces itself into a monoclonal heterogenous population of tumor cells harboring different mutations with high proliferative potential forming malignant tumor. Some of transformed cells undergo apoptosis. As the tumor grows, it becomes a complex population of clonal cells, each clone with different behavior. With time, it becomes difficult to eliminate transformed cells with chemotherapeutic agents (e.g. tamoxifen for breast carcinoma), because different patients respond differently to the treatment.

- The clonal property of tumors is important to understand carcinogenesis and discovery of new important genes such as retinoblastoma (RB) tumor suppressor gene model. Further research into clonal property of malignant tumors will provide new insights into malignant tumor development and growth as well provide clonal markers relevant to the clinical diagnosis and follow-up of individual cases. Three major approaches to assess monoclonality of malignent tumors include X chromosome inactivation, lymphocyte analysis and somatic mutation in genes.

G6PD Isoenzyme (A or B) Analysis Technique in Tumors

Monoclonal property is assessed by studying glucose-6-phosphate dehydrogenase in selected benign and malignant tumors. Some women are heterozygous for having two alleles of glucose-6-phosphate dehydrogenase (G6PD) on the long arm of the X chromosome, which possess differing sensitivities to heat inactivation. Early in the developing embryo, one X chromosome inherited from mother or one from father are in active state. Normal ratio of active isoforms of G6PD in cells of any tissue is 1:1, as 50% of cells exhibit G6PD-A and other 50% of cells demonstrate G6PD-B.

- **X-inactivation (also called lyonization):** X-inactivation (also called lyonization) is a process by which one of the two X chromosomes is inactivated in every cell and appears as a '**Barr body**' attached to the nuclear membrane in females during development.
  - Such X chromosome inactivation silences expression of almost all the genes on that chromosome related to differential methylation of cytosine in the DNA.
  - As a result, the tissues are having mosaic cells that express either A or B isoenzyme of G6PD. Only one isoform is present in tumor, which is monoclonal. G6PD isoenzyme monoclonality analysis is based on females heterozygous for a particular X-linked polymorphism.
- **G6PD isoenzyme (A or B) in uterus during pregnancy and leiomyoma:** During pregnancy, some uterine smooth muscle cells express G6PD-A isoenzyme, while rest of smooth muscle cells display G6PD-B isoenzyme indicating polyclonal origin. Uterine leiomyoma arising from a single cell shows either G6PD-A isoenzyme or G6PD-B isoenzyme. This finding demonstrates the monoclonal origin of the uterine leiomyoma. However, polyclonal tumor can have both G6PD-A isoenzyme and G6PD-B isoenzyme phenotype.

G6PD Isoenzyme (A or B) Analysis to Assess Monoclonal Properties in Human Cancers

The majority of human cancers analyzed by this technique have demonstrated monoclonal property.

- Monoclonality is demonstrated in many human cancers (e.g. breast carcinoma, colon carcinoma and cervical carcinoma).
- G6PD enzyme analysis is useful in the diagnosis of chronic myelogenous leukemia (CML). Cytogenetic analysis in CML is obtained from white blood cells (WBCs), which contain nuclei, while red blood cells (RBCs) and platelets lack nuclei.
- Some cases of colon carcinoma, breast carcinoma and hepatocellular carcinoma demonstrate double G6PD enzyme phenotypes.

G6PD Isoenzyme (A or B) Analysis to Assess Polyclonal Properties in Some Human Neoplasms

Polyclonal composition of these tumors is presumably related to the different time course and mechanism of tumorigenesis.

- Similarly, a polyclonal origin of colorectal adenomas in Gardner's syndrome (familial adenomatous polyposis syndrome) has been proposed based on assessment of G6PD mosaicism.
- These divergent results might be due to use of recombinant techniques and contaminating non-neoplastic cells, removed from the benign tumors by histologic examination of cryostat sections.

Restriction Fragment Length Polymorphism Technique to Assess Monoclonal Properties of Tumors

In molecular biology, restriction fragment length polymorphism (RFLP) technique detects variations in homologous DNA sequences, known as polymorphism. RPFL technique detects the locations of genes within DNA sequence, which can distinguish individuals, populations, or species. Molecular probes used for X-linked polymorphic genes now make it possible to detect clonal markers in tumor cells in same manner analogous to the G6PD isoenzyme studies. Active and inactive copies of polymorphic X-chromosome genes can be differentiated through differences in patterns of methylation of the genes.

- Diagnostic approach has widened the scope of clonality analysis to include all females in whom suitable X-linked polymorphism is present. Both normal and tumor DNA are first disgusted with appropriate restriction endonuclease to differentiate the paternal and maternal copies of the gene through X-linked RFLP. A second endonuclease sensitive to methylation of cysteine residues in its recognition

sequence differentiates active from inactive copies of the gene through changes in the DNA methylation pattern.

- X-linked gene polymorphism useful for studies include RFLPs of the hypoxanthine phosphoribosyltransferase gene and the phosphoglycerate kinase gene. Molecular probes for X-linked polymorphic genes are used to analyze clonality in acute leukemia, chronic leukemia, uterine leiomyoma, Wilms' tumor and parathyroid adenomas.

Lymphocyte Analysis Technique to Assess Monoclonal Properties of Tumors

Lymphocyte analysis is done to study monoclonality of B cell- or T cell-derived neoplasms. B cell-derived neoplasms are studied by immunoglobulin light chain analysis. On the contrary, immunoglobulin and TCR gene analysis are done to analyze monoclonality of T cell-derived neoplasms.

Immunoglobulin Light Chain Analysis Technique to Assess Monoclonal Properties of Tumors

Clonality of B lymphocyte is determined by immunoglobulin (Ig) light chain phenotype. immunoglobulin (Ig) is composed of heavy chains and light chains. Each B cell expresses light chain that is either κ or λ.

- Normal ratio of κ to λ light chain is 3:1. Ratio of κ and λ light chain increases to >6:1 or reverses to 1:3 in lymphoma after treatment. This ratio of κ and λ light chain is maintained in cell hyperplasia, which is polyclonal.
- One of the standard methods for analyzing monoclonal property of B cell neoplasms is the demonstration of immunoglobulin molecules in serum and immunoglobulin light chain rearrangement on the surface of atypical B cell in chronic lymphocytic leukemia (**CLL**) and some cases of non-Hodgkin's lymphomas.
- There is no analogous system of surface markers on T cells indicating monoclonality in T-cell derived neoplasms. However, monoclonal antibodies, which are specific for family of related variable regions, which use the same variable region genes can act as indicators of the monoclonality of some T cell populations.

Immunoglobulin and TCR Gene Analysis Technique to Assess Monoclonal Properties of Tumors

The more recent use of molecular probes to detect immunoglobulin and TCR gene rearrangements overcomes many of the limitations of immunoglobulin light chain analysis. This technique is applied to all lymphoid malignancies. Immunoglobulin and TCR gene rearrangements give rise to DNA markers unique to each individual lymphoid cell and its progeny. Monoclonal population of cells have the same genetic rearrangement, which can be detected by Southern blotting technique. In a polyclonal lymphoid proliferation, these rearrangements are not detectable by Southern blotting technique.

- **Immunoglobulin and TCR rearrangements analysis:** Immunoglobulin and TCR rearrangements also provide information about the cell-lineage of lymphoid neoplasms, whereas TCR rearrangements are regularly demonstrated in T cell-derived leukemias/non-Hodgkin's lymphomas. Using these techniques, it is possible to prove monoclonality of B cell origin of hairy cell leukemia and T cell-derived mycosis fungoides.
- **Southern blotting technique:** Southern blotting technique is unable to detect gene rearrangements at much below the level of 5%. Now polymerase chain reaction (PCR) technique overcomes the limitation of Southern blotting technique, and is used to detect clonal markers at much lower level by amplification of immunoglobulin and TCR gene rearrangement in the study of 'minimal residual disease' in T cell-derived leukemias/non-Hodgkin's lymphoma. It is worth mentioning that immunoglobulin gene rearrangements are not consistently stable clonal markers since these are subject to cause variability as a result of ongoing somatic gene mutations. Clonal property of lymphoid cell proliferation is given in Table 6.21.

Human Androgen Receptor Gene Analysis Technique to Assess Monoclonal Properties of Tumors

Human androgen receptor gene (HUMARA) analysis to assess monoclonality has been used to determine the clonal origin of malignant tumors. HUMARA analysis

Table 6.21 Clonal property of lymphoid cell proliferation

Cell Type	Benign Lymphoid Cells	Malignant Lymphoid Cells
B cells	Ig light chain heterogeneity	Ig κ and λ only
Plasma cells	Ig light chain heterogeneity on electrophoresis	Monoclonal Ig spike on electrophoresis
T cells	Heterogeneous variable regions	Homogenous variable regions

is based on using X chromosome inactivation analysis on the different methylation patterns of active and inactive alleles of gene called HUMARA mapped on Xq13 chromosome in multiple population. Recently, a polymerase chain reaction-based androgen receptor gene analysis method has been developed.

Somatic Mutations Analysis Technique to Assess Monoclonal Properties of Tumors

Somatic mutation refers to an alteration that occurs after conception in any of the cells of the body except the germ cells (sperm and ovum) and therefore are not transmitted to children. These somatic alterations in DNA can cause cancer or other disorders in some persons. Somatic mutations can be analyzed by cytogenetic and molecular studies.

Cytogenetic Analysis Technique to Assess Monoclonal Properties of Tumors

Many human cancers demonstrate consistent, nonrandom chromosome abnormalities, which demonstrate as clonal markers. Philadelphia chromosome analysis in chronic myclogenous leukemia (CML) detects clonal markers and provides information relevant for diagnosis, classification and prognosis. Limitation of cytogenetic analysis is that cells can be studied in mitosis only. This limitation is overcome in study of clonal chromosomal abnormalities in acute leukemias by a new technique using the detection of lineage-specific antigens in dividing leukemic cells by monoclonal antibodies.

Chromosomal Losses Detected by Molecular Probes Technique to Assess Monoclonal Properties of Tumors

The loss of chromosomal material is one of the most important alterations in malignant tumors. Homologous DNA sequence analysis depends on the ability to distinguish the two chromosomal homologues by the detection by restriction fragment length polymorphism (RFLP) technique.

- The DNA analysis is informative if constitutional DNA displays heterozygosity for a particular type of restriction fragment length polymorphism (RFLP). The loss of DNA sequences in tumor DNA indicates chromosomal loss in malignant tumors. The use of molecular probes to detect polymorphisms due to variable number of tandem repeats (VNTRs) makes this method feasible in majority of cases on account of their high heterozygosity rates.
- Molecular probes are small DNA or RNA segments that recognize complementary sequences in DNA or RNA molecules that allow detection and isolation of specific sequences. Comparative genomic *in situ* hybridization (CGS) uses a fluorescent reporter attached to a DNA probe to detect the presence of specific DNA sequences in chromosomes by molecular hybridization. Labeled genomic test DNA prepared from tumor specimens is mixed differently labeled control DNA prepared from normal cells with chromosome complements and analyzed.

Chromosomal Translocation Detected by Breakpoint Cluster Region Probes Technique to Assess Monoclonal Properties of Tumors

Chromosomal translocations can be detected to assess monoclonal property in certain human malignant tumors by DNA analysis. DNA probe is used, which hybridizes to the region of the breakpoint on one of the chromosomes involved in the translocation. Breakpoints must be clustered within a defined DNA sequence.

- Analysis of 'Philadelphia chromosome' illustrates variable breakpoints on chromosome 9 and clustered breakpoints on chromosome 22 in majority of cases over a region of 5.8 kilobases, known as 'breakpoint cluster region'. DNA probes are used, which hybridize to the 'breakpoint cluster region' on chromosome 22 detect most cases of Philadelphia chromosome positive chronic myelogenous leukemia.
- The prognosis of chronic myelogenous leukemia (CML) has changed radically due to the development of novel drugs able to target the enhanced tyrosine kinase activity of the BCR-ABL.
- Other chromosomal translocations can be detected directly by 'Southern blotting technique', which include t(11;14) in some B cell lymphomas t(14;18) in follicular lymphoma and t(8;14) in Burkitt's lymphoma. These chromosomal translocations present at low concentration in cells are detected by polymerase chain reaction (PCR), provided the breakpoints on the chromosomes are clustered within defined regions.

Point Mutations in Genes Analysis Technique to Assess Monoclonal Properties of Tumors

The RAS gene family consists of three small G proteins such as K-RAS, H-RAS and N-RAS, that play a central role in cell signaling relating to the cell proliferation, survival, adhesion, and motility. Mutations in RAS proteins are one of the most common genetic alterations observed in malignant tumors.

- *In vivo*, oncogenic RAS mutations have been shown to occur at exons 12, 13 and 16, resulting in any 1 of 19 possible point mutations in a given tumor for a specific RAS isoform. RAS mutations can be detected by polymerase chain reaction (PCR)/restriction fragment length polymorphism (RPFL) analysis.
- K-RAS mutation demonstrated at exon 12 in adenoma progressing to colon carcinoma has been associated

with a poor survival. Oncogenic N-RAS mutations at exon 12 in hematopoietic pluripotent stem cells cause RAS-associated myelofibrosis.

DNA Fingerprinting Analysis Technique to Assess Monoclonal Properties of Tumors

DNA fingerprinting is a technique used to detect lots of minisatellites (highly variable nucleotide DNA) in the human genome from a sample of DNA unique in an individual especially lacking an obvious cytogenetic abnormality. Comparative DNA fingerprint analysis of a patient's malignant tumor and constitutional DNA represents a new method for analyzing clonal markers. The feasibility of this technique depends on the properties of the minisatellite DNA probes, which detect a large number of variable number of tandem repeats (VNTRs) scattered throughout the human genome. Use of three minisatellite DNA probes have demonstrated differences between the tumor DNA and constitutional DNA (peripheral blood or mucosa) in gastrointestinal tumors.

Viral Integration Analysis to Assess Monoclonal Properties of Tumors

Molecular analysis of tumors containing viruses offers a novel diagnostic technique of clonality. Hepatitis B virus (HBV) infection is one of the leading causes of cirrhosis, hepatocellular failure and hepatocellular carcinoma. Polymerase chain reaction (PCR) technique is used to detect hepatitis B virus (HBV) integration in peripheral blood mononuclear cells in chronic hepatitis B infection. The monoclonality of cell populations within the hematopoietic system can be analyzed with specific probes by Southern blot hybridization of DNA technique.

TELOMERASE ACTIVITY IN TUMORS

Telomerase enzyme adds repetitive nucleotide sequences to maintain the length of the telomeres by preventing gene loss at the ends of chromosomes after multiple cell divisions. With each round of somatic cell replication, the telomere shortens. The length of telomeres acts as a molecular clock, which governs the life span of replicating germ cells to preserve species. Somatic cells do not normally express telomerase. Telomerase enzyme activity is increased in certain human malignancies. Activation of telomerase activity in malignancy does not lose genetic material after multiple cell divisions.

TUMOR ANGIOGENESIS

Tumor angiogenesis denotes formation of new blood vessels. It is a requirement for the continued growth and survival of malignant tumors, whether primary or metastatic. Newly formed blood vessels are essential for the inflow of nutrients and oxygen into the malignant tumor. In the absence of new vessels, malignant tumors do not grow larger than 1–2 mm in diameter. Hypoxia is most important driving force for angiogenesis by inducing hypoxia inducible factor-α (HIF-α) transcriptional factor. Tumor angiogenesis is a balancing act between proangiogenic and antiangiogenic factors.

Proangiogenic Factors in Tumor Angiogenesis

Proangiogenic factors synthesized by malignant tumors and inflammatory cells include: VEGF, FGF, TGF-β, HGV, PDGF, angiogenin, angiotensin II, angiopoietin, platelet activating factor (PAF), TNF-α, G-CSF, placental growth factor, proliferin, leptin, IL-6 and erythropoietin. Vascular endothelial growth factor (VEGF) is important angiogenic factor in the proliferation of blood vessels in a growing malignant tumor, in which new blood vessels become torturous, irregular, and leaky blood vessels. Eventually, the tumor outgrows its blood supply, and induces tumor necrosis resulting in slower tumor growth.

Anti-angiogenic Factors in Tumor Angiogenesis

Various anti-angiogenic factors such as angiostatin, endostatin, vasculostatin, and thrombospondin 1 are also synthesized, which inhibit development of cancers. Extracellular matrix (ECM) derived anti-angiogenic factors include endostatin, thrombospondin (I, II), chondromodulin I anastellin, fibulin, tumstatin and canstatin. Non-matrix derived anti-angiogenic factors include angiostatin, vasculostatin, antithrombin III, vasostatin, platelet factor 4, interferon-α and interleukins (IL-1, IL-12, IL-18).

TUMOR INVASION AND METASTASIS

Histologic features and rate of growth alone cannot distinguish between benign and malignant tumors. Invasion and metastasis are the most reliable feature of malignancy that distinguishes malignant tumors from benign tumors.

- Cancer stem cells invade basement membrane and surrounding tissues, and metastasize via lymphatic route, hematogenous route and transcoelomic route to distant organs of the body. Metastasis consists of multiple steps (i.e. invasion, intravasation and transport of CSCs, and extravasation), which are collectively called '**metastatic cascade**'. Invasion and metastatic cascade of malignant tumor is shown in Fig. 6.53.
- Approximately 30% of newly diagnosed solid malignant tumors present with clinically evident metastases. **Occult (hidden) metastases** are seen in 20% of patients at the time of diagnosis. Approximately 30% of newly diagnosed solid malignant tumors

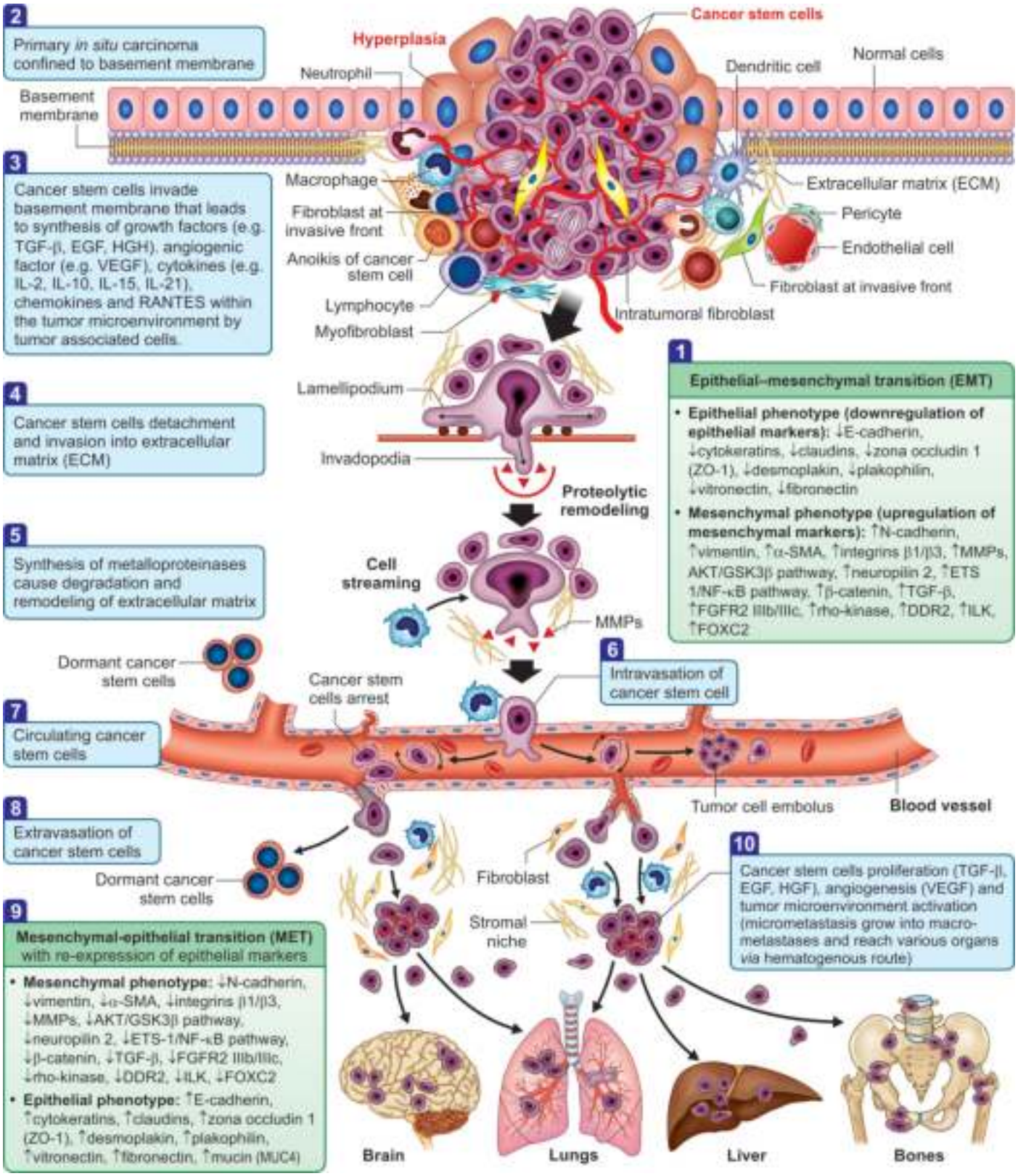


Fig. 6.53: Invasion and metastatic cascade of malignant tumor. Detachment of cancer stem cells from an epithelial-derived primary malignant tumor and its migration to reach a blood vessel or lymphatic vessel for dissemination to a secondary site is very complex process that includes alterations in the expression of multiple genes involved in the cell adhesion, chemoattractant and growth factors.

present with clinically evident metastases. The majority of deaths occur due to metastasis, which is the important hallmark of cancer, and associated with poor prognosis.

- Invasion and metastasis are triggered by genetic and epigenetic factors that are induced by environmental stimuli (e.g. aging and circadian disruption); adhesive signals from extracellular matrix (ECM) components (e.g. collagen); ECM mechanical pressures, including compression and tension; cell–cell interactions; soluble signals (e.g. growth factors, cytokines) and the intratumoral microbiota.

NORMAL PROCESSES THAT REGULATE CELLS AND INHIBIT CARCINOGENESIS

Cellular processes, such as transcription, DNA replication, and DNA repair involve interactions between multiple proteins: (a) extracellular signals (i.e. growth factors, and their cellular receptors), and (b) intracellular regulatory genes (i.e. proto-oncogenes and tumor suppressor genes). Normal cell growth requires a balance between the activity of proto-oncogenes and tumor suppressor genes, which produce proteins that act as 'on' and 'off'' switches of cell cycle.

- Mutations in regulatory genes result in loss of control over normal cell growth termed 'autonomy' resulting in transformation of normal cell to CSC, which differ from normal cell in terms of cell size, differentiation, function and ability to metastasize to distant tissues and organ system.
- Oncogenesis is the process through which healthy cell is transformed to CSC that occurs by a series of cellular changes and genetic alterations including activation of oncogenes (mutated proto-oncogene) or inactivation/biallelic loss of tumor suppressor genes, that lead to cell division in an uncontrolled manner. Several critical cellular processes provide protection and prevent tumor development, which are closely related, and intertwined, cell cycle regulation, DNA repair and telomerase activity.

Pathology Pearls: Regenerating Capacity of Cells

Cell proliferation is defined as an increase in cell number leading to cell growth and cell division. In cellular biology, regenerative capacity of cells allows them to be classified into three groups: labile, stable and permanent cells.

- Labile cells never go to G0 phase (quiescent phase) of cell cycle and continuously multiply and divide with a short G1 phase to reproduce new stem cells and replace functional cells that are lost in the body throughout life (e.g. hematopoietic stem cells in bone marrow, skin, hair follicles, gut epithelium, epithelia of ducts and germ cells). It is worth mentioning that labile cells are affected by chemotherapy.
- Stable cells remain in quiescent state but after stimulation, they may enter G1 phase of cell cycle to divide and regenerate when needed (e.g. hepatocytes, proximal convoluted cells of kidney, smooth muscle cells and periosteal cells).
- Permanent cells are terminally differentiated cells, which have lost the capacity to regenerate and enter cell cycle to divide (e.g. neurons skeletal muscle, cardiac muscle, red blood cells and cells in lens of eye). Permanent cells remain in G0 phase of cell cycle.

CELL CYCLE AND CELL DIVISION

Cell cycle refers to series of events that takes place in a cell as it grows and divides. Cell cycle (i.e. cell division) is divided into major two events: interphase and mitosis (M) phase. Interphase consists of G1 phase (centrosome duplication), S phase (chromosome replication), and G2 phase (assembly of mitotic spindles composed of microtubules). Mitosis phase refers to cell division to form two identical daughter cells. Quiescent cells are in the G0 phase (resting or inactive state) that have not entered the cell cycle (i.e. outside cell cycle), where cell has stopped division.

- During interphase of cell cycle, the cell grows and prepares for cell division. Cell division occurs during mitosis (M) phase, which consists of cell mitosis and cytokinesis. Cell division (mitosis and cytokinesis) consists of equal division of chromosomes and cytoplasmic components (termed cytokinesis), i.e. cell membrane, cytoplasm and organelles between two daughter cells with identical genomes. Mitotic activity remains restricted to somatic stem cells in the following circumstances: cell growth, and cell replacement during tissue repair and regeneration. G0, G1, G2, S and M phases of cell cycle are shown in Fig. 6.54 and given in Table 6.22.
- Meiosis occurs where a germ cell is divided to produce haploid cells containing half the number of chromosomes of the parent germ cell (gametes, i.e. spermatozoa in males and egg in females).

CELL CYCLE AND CELL DIVISION: PHASES

Cell cycle is divided into four phases: G1 (centrosome duplication), S (chromosome replication), G2 (assembly of mitotic spindles composed of microtubules)

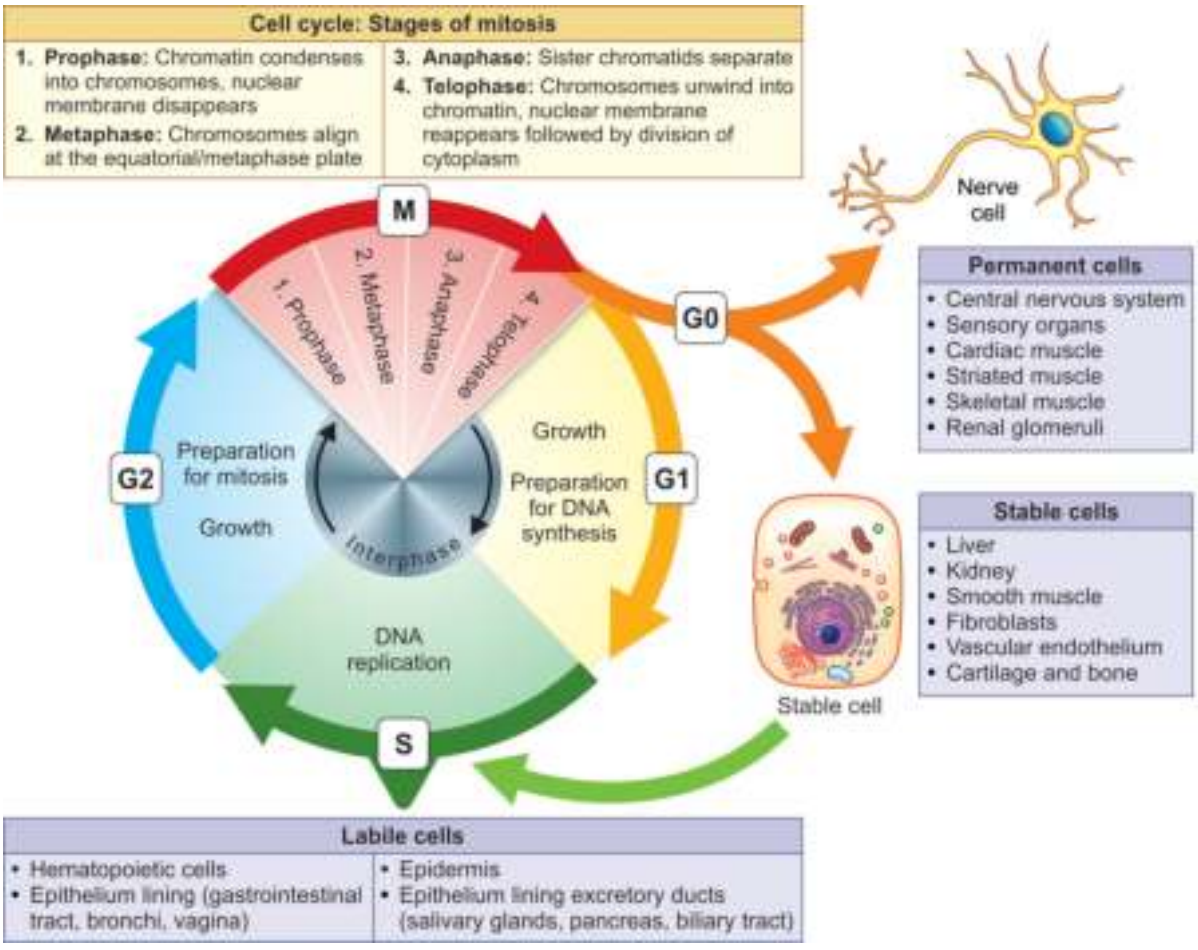


Fig. 6.54: G0, G1, G2, S and M phases of cell cycle. Location of the G1 is the restriction point. G1/S and G2/M are checkpoints of cell cycle. Cells from labile tissues such as bone marrow, epidermis, epithelial lining gastrointestinal tract, bronchi and vagina may cycle continuously.

Table 6.22 G0, G1, G2, S and M phases of cell cycle

Cell Cycle Stage (Phase)	Major Features
Resting phase	
G0 phase	Stable, nondividing period of variable length
Interphase	
G1 phase	Growth and development of the cell; G1/S checkpoint
S phase	Synthesis of DNA
G2 phase	Preparation for cell division; G2/M checkpoint
M phase	
Prophase	Chromosomes condense and formation of mitotic spindle
Prometaphase	Nuclear envelope disintegrates, and spindle microtubules anchor to kinetophores
Metaphase	Chromosomes align on the metaphase plate; spindle-assembly checkpoint
Anaphase	Sister chromatids separate, becoming individual chromosomes that migrate toward spindle poles
Telophase	Chromosomes arrive at spindle poles; the nuclear envelope re-forms and the condensed chromosomes relax leading to create two daughter cells with identical genomes

Note: Meiosis consists of two distinct processes; meiosis I and meiosis II. Meiosis includes the reduction division, in which homologous chromosomes separate and chromosome number is reduced by half. In meiosis II (equational division), sister chromatids separate.

and M (cell division to form two daughter cells). After completion of cell cycle, cell either starts the process of cell division from G phase or exists through G0 phase.

G0 Phase (Resting or Quiescent Phase)

G0 phase is a form of the resting state, or quiescent state outside the cell cycle, in which cell resides until it receives appropriate growth signals from growth factors (mitogens). Growth factors stimulate cell to enter and progress through the cell cycle. Terminally differentiated cells also remain in resting phase, or quiescent phase. Significance of G0 phase is that these cells cannot divide and undergo replication. On contrary, CSCs continue to divide as a result of deregulated cell cycle. The CSCs cannot be put into G0 phase until the environment becomes favorable for cell division.

Interphase: First Stage of Cell Cycle

Interphase is also known as **resting phase** of the cell cycle, in which the cell prepares for cell division (mitosis) by increasing cell growth. Interphase occupies around 95% of time of the overall cell cycle. The interphase is divided into three phases: G1, S and G2. Interphase aneuploidy results in increased cell proliferation.

G1 (Pre-synthetic) Phase of Cell Cycle

G1 phase is a period of cell growth and preparation for DNA synthesis (S phase). During the G1 phase (G1/S transition), the cell continues to grow as a result of synthesis of cytoplasmic organelles, RNA, and proteins, but lacks DNA replication. Growth factor (mitogen) is only required in the early G1 phase of cell cycle, while late G1 phase of cell cycle remains mitogen-independent.

- During early G1 phase, growth factors (EGF, HGF, PDGF, IGF1, IGF2, TGF-α, insulin) bind to cognate tyrosine kinase receptors on cell surface, which provide the stimulatory signal to proceed forward leading to cell growth as a result of synthesis of cytoplasmic organelles, RNA, and proteins. Cell differentiation, maturation and stimulation of cell division occurs via activation of proto-oncogenes. Once the cell progresses past the restriction point (R point), mitogens are no longer required for cell cycle progression. Length of G1 phase determines how slow or rapid, cell division takes place.
- **Restriction point (R point)** is the '**point of no return**' where the cell is committed to undergo progression to the next phase (i.e. G1 phase to S phase), that has been temporarily mapped at 2–3 hours prior to the onset of DNA synthesis.
- Phosphorylation of RB protein restricts cell cycle in mid-G1 phase by inhibiting E2F-mediated S phase transcription factors. At G1/S transition controlled by cyclin-dependent kinase 2 (CDK2), the cell checks whether internal or external conditions such as nutrients, molecular signals and DNA integrity are sufficient for cell division. DNA damage may result in DNA repair system pathways or apoptosis (programmed cell death).

S (Synthetic) Phase of Cell Cycle

Synthesis (S) phase of cell cycle refers to synthesis of DNA. During S phase of cell cycle, cell undergoes DNA replication with production of identical replicates of DNA. Synthetic phase also induces duplication of microtubule-organizing structure called '**centrosome**' that helps in separation of DNA during mitosis (M) phase. G1/S transition allows checking the DNA integrity before cell DNA is replicated.

- In early synthetic phase, S phase genes are activated as a result of cyclin A replacing cyclin E to form cyclin A-CDK2 complexes in early synthetic phase. In late synthetic phase, cyclin A-CDK1 complex is formed. Upon completion of synthetic phase, cyclin A-CDK2 phosphorylates E2F-DP heterodimers, thus terminating E2F's activity.
- After completion of synthetic phase, cyclin B replaces cyclin A, forming cyclin B-CDK1 complexes, which move the cell into the second gap phase of the cell cycle G2 phase and into mitosis.

G2 (Pre-mitotic) Phase of Cell Cycle

G2 phase (pre-mitotic phase) refers to preparation of cell for cell division. After synthetic phase of DNA replication, cell enters S phase that ensures everything (i.e. DNA replication, RNA, proteins and other macromolecules required for mitotic division) is ready to enter the mitosis (M) phase for cell division. G2 phase features synthesis of tubulin, which is required for formation of mitotic spindle essential for cell division.

- TP53 gene product (p53 protein) is an important regulator of cell cycle at G1/S and G2/M transition. TP53 gene product (p53 protein) can stop the cell cycle for DNA repair or induce apoptosis, if the DNA damage is beyond repair.

Mitosis: Second Stage of Cell Cycle

Mitosis is the process by which a cell replicates its chromosomes and then segregates them, with division of nucleus and followed by cytokinesis (division of cytoplasm) resulting in production of two new identical daughter cells. Mitosis is subdivided into four phases in strict sequential order and cytokinesis: prophase, metaphase, anaphase and telophase. Before cell division, a cell must double its mass and content that occurs during the growth phase, called interphase. Before cell division, a cell must double its mass and content that occurs during the growth phase, called interphase.

Prophase

Prophase is the first phase of mitosis, in which chromosomes coil and shorten, and centrioles move apart and dissolution of nuclear membrane. Each chromosome is made up of a pair of strands called chromatids, which are connected by a spindle of fibers called 'centromere'.

Metaphase

During metaphase, the centromere divides and pulls the chromosome apart, which line up along the metaphase or equatorial plate. The centromeres align themselves in the middle of the spindle.

Anaphase

During anaphase, the centromere and sister chromatids begin to separate and pull the newly replicated chromosomes toward opposite poles of the cell and cleavage furrow begins to develop. Centromere defect and chromosome misaggregation result in abnormal mitotic tripolar and quadrangular figures in malignant tumors.

Telophase

Telophase is the terminal phase of mitosis and characterized by cytokinesis, reconstruction of nucleus and nuclear envelope disappearance of mitotic spindle fibers, and unwinding of 46 chromosomes into chromatin. Mitotic cell division leads to production of two identical new daughter cells. Mitosis occurs actively in the bone marrow and epidermis to replace cells, which have limited life span. Mitotic spindle defects lead to aberrant cellular divisions. Acquisition of successive chromosome mutations initiates carcinogenesis.

CELL CYCLE CHECKPOINTS

Checkpoints control transitions occur between various phases of cell cycle. This process is regulated by cyclins, cyclin-dependent kinases (CDKs) and tumor suppressor genes.

- M phase is the shortest phase of cell cycle, which includes mitosis (prophase, prometaphase, metaphase, anaphase, telophase) and cytokinesis (cytoplasm splits into two). G1 and G0 phases of cell cycle are of variable duration.
- Growth factors bind to cognate surface tyrosine kinase receptors on the cell; and then transmembrane proteins relay information to the cell by signal transduction. Growth factors stimulate cell division. On the contrary, growth-inhibiting factors inhibit cell division.
- Normal cells divide only when growth factor and growth-inhibiting factor in balanced proportions favor cell division. CSCs divide without constraint.
- Cancer is primarily caused by mutations in growth factor genes and growth factor inhibiting tumor suppressor genes, and signaling pathways, that inhibit the normal sequence of events associated with apoptosis. Genomic instability (failures in checkpoints and apoptosis system) results in cell proliferation and aberrant chromosomes division.

Cell Cycle Checkpoints: Phases

Cell cycle checkpoints (i.e. transition between phases of the cell cycle) monitor each phase of mitosis to maintain genomic integrity. Checkpoint is a stage in the eukaryotic cell cycle at which the cell examines internal and external stress to DNA and decides whether or not to move forward with cell division. Positive cell cycle regulator molecules allow cell cycle to advance to the next stage. Negative cell cycle regulator molecules monitor cellular conditions, and halt the cell cycle until specific requirements are made.

- Important internal checkpoints of cell cycle include: (a) G1 checkpoint at G1/S transition to analyze integrity of the DNA, (b) G2 checkpoint at G2/M transition to analyze proper chromosome duplication, and (c) M checkpoint (spindle checkpoint) at the transition from metaphase to anaphase to analyze chromosome attachment to spindle at metaphase. G1, G2 and M checkpoints of cell cycle are shown in Fig. 6.55.

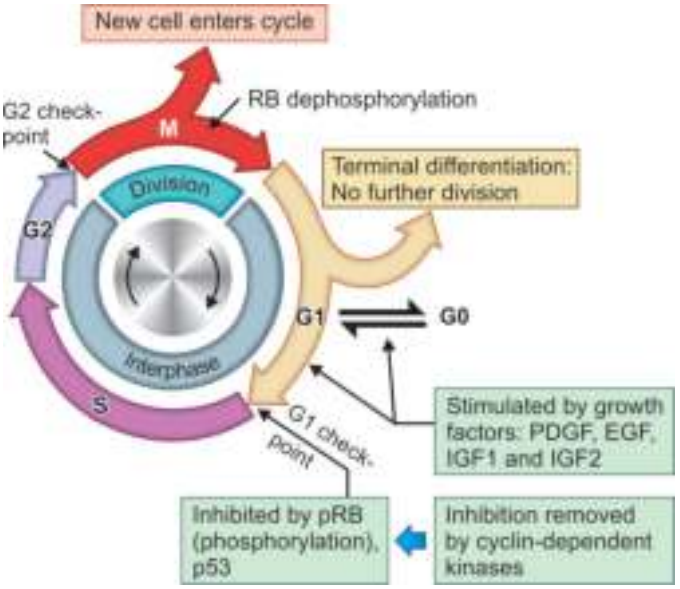


Fig. 6.55: G1, G2 and M checkpoints of cell cycle. The cell cycle consists of two phases: interphase (G1, S and G2) and mitotic phase. G1 phase is the first growth period of the cell cycle during interphase, in which the cell grows and cytoplasmic organelles are replicated. DNA replication is the biological process of producing two identical replicates of DNA during synthetic phase (S phase) of cell cycle. G2 phase allows replicated DNA to be checked before mitosis at the G2/M transition. DNA replication and duplication occur during the four phases of mitosis during mitotic phase.

- RB gene (pRB), TP53 gene (p53 protein) and p21 protein act the G1 checkpoint. It has been studied that RB protein is responsible for a major G1/S transition, blocking S phase entry and cell growth.
- Tumor suppressor TP53 gene product (p53 protein) is a multifunctional protein that plays a critical role in growth arrest, recruitment of enzymes to repair damaged DNA and apoptosis. The growth arrest inhibits the progression of cell cycle and thus prevents replication of damaged DNA. Failure to repair the cellular DNA damage, TP53 (p53 protein) can trigger apoptosis to prevent duplication of damaged chromosomes. Increased concentration of TP53 (p53) protein level triggers production of p21 protein that binds and inhibits the activity of the cyclin–CDK complex, and thus prevents cell cycle progression.

G1 Checkpoint at G1/S Transition to Analyze Integrity of the DNA

G1 checkpoint (restriction checkpoint) determines whether all conditions are favorable for cell division to proceed. The G1/S transition is checkpoint, also called 'restriction checkpoint' (R checkpoint). At restriction checkpoint phosphorylation of RB by cyclin-dependent kinases (CDKs) causes the release of transcriptional activator E2F that forms E2F/DP1/RB complex (positive regulator of cell cycle) in the nucleus.

- G1 checkpoint is the main decision-making point for a cell to choose whether or not to divide. Once the cell passes the G1 checkpoint, cell enters synthetic phase (S phase), and the cell becomes irreversibly committed to divide by barring unexpected replication errors in DNA.
- G1 checkpoint, the cell checks whether internal or external conditions such as nutrients, molecular signals and DNA integrity are right for cell division. If the cell does not get the go-ahead cues, it needs at the G1/S transition, cell may leave the cell cycle and enter a resting state called G0 phase.
- Some cells stay permanently in G0 phase, while other cells resume cell division if conditions improve. Failure of DNA repair leads to either apoptosis or accumulation of mutations responsible for transformation of normal cell to CSC. Defect in the G1 checkpoint is the most important cause of human cancers.

G2 Checkpoint at G2/M Transition to Analyze Proper Chromosome Duplication

The cell has an additional G2 checkpoint before M phase of cell cycle, that ensures smooth cell division with healthy DNA to maintain genomic integrity. At this stage, the cell checks DNA integrity, whether there are any replication errors in DNA. However, most important role of G2 checkpoint is to ensure that all the chromosomes have been replicated, and the replicated DNA is not damaged. G2 checkpoint is most important checkpoint in cells exposed to ionizing radiation.

- Chromosomal abnormality occurs due to defects in G2 checkpoint that initiates carcinogenesis. If the G2 checkpoint detects errors in DNA, the cell cycle halts, and the cell tries to repair DNA damage. If the cell is unable to repair DNA damage, it may undergo apoptosis (programmed cell death).
- Excessive DNA damage activates BAX pro-apoptotic gene that inhibits BCL-2 antiapoptotic gene resulting in release of 'cytochrome c' from the mitochondria and apoptosis of the cell. Apoptosis ensures that damaged DNA is not passed to the daughter cells during cell division resulting in inhibition of transformation of normal cell to CSC.

M (Mitosis) Checkpoint (Spindle Checkpoint) at the End of Metaphase Stage of Karyokinesis

M (mitosis) checkpoint, also known as the '**spindle checkpoint**', occurs near the end of the metaphase stage of karyokinesis to examine whether all the sister chromatids are correctly attached to the spindle microtubules. Because the separation of the sister chromatids during anaphase is an irreversible step, the cell cycle does not proceed until all the chromosomes are firmly attached to at least two spindle fibers from opposite poles of the cells. The cell does not scan the metaphase plate to confirm that all the chromosomes are there, instead, the cell looks for 'straggler (stray) chromosomes' that are in the wrong place. If a chromosome is misplaced, the cell mitosis halts, and allows time for the spindle to capture the stray chromosome.

CELL CYCLE REGULATORS

Cell cycle regulators control transition from G1/S transition in normal tissues. Cell cycle is controlled by regulatory molecules that either promote the process of cell cycle or inhibit progression of cell cycle.

- Positive cell cycle regulators drive cell cycle progression, which includes cyclins (A, B, D, E) and cyclin-dependent kinases (CDKs) and other molecules.
- Negative regulators of cell cycle include as pRB, TP53 (p53 protein), INK family of CDK inhibitory proteins (p16, p15, p18, p19) and CIP/KIP (CDK interacting protein/kinase inhibitory proteins, i.e. p27, p21, p57) which inhibit cyclin-dependent kinases thus block cell cycle progression within G1 phase. Hence, these negative regulators of cell cycle provide protection against development of cancers.

- Mutation in cell cycle regulators results in uncontrolled cell division via disruption of signaling pathways, which initiate carcinogenesis. If negative regulatory proteins are not produced or become nonfunctional, then these resulting in uncontrolled cell division initiation of carcinogenesis. Aberrant expression of cell cycle regulatory proteins analyzed by immunohistochemistry technique is valuable in diagnosis of some tumors as well as predicting prognosis in a number of tumors.
- Positive and negative regulators of cell cycle are given in Table 6.23. Cell cycle of positive and negative regulators are shown in Fig. 6.56. Regulation of the cell cycle is shown in Fig. 6.57.

Cell Cycle: Positive Regulators

The cell cycle starts when a growth factor acts on a quiescent cell to provoke the cell to divide. Cell is triggered to go from G0 to G1 by binding of growth factors such as EGF, HGF, PDGF, IGF1, IGF2, TGF-α to cognate tyrosine kinase receptors on cell to promote cell differentiation, maturation and stimulation of cell division via activation of proto-oncogenes. Once, the cell progresses past the restriction point, mitogens are no longer required for cell cycle progression in late phase of G1 (mitogen-independent). Growth factors stimulate the production of two types of cell cycle regulators: (a) positive regulators of the cell cycle, and (b) negative regulators of the cell cycle. Cyclins and cyclin-dependent kinases (CDKs) are key positive regulators of the cell cycle.

Cyclin Family of Proteins: Positive Regulators of Cell Cycle

Cyclin family of proteins regulate the progression of a cell through the cell cycle by tightly binding and activating CDK enzymes. To be fully active, cyclin/cyclin-dependent kinase complex must be phosphorylated in specific locations to coordinate cell cycle progression.

- Binding of S cyclin to CDK results in DNA replication. M cyclin participates in chromosome condensation and breakdown of nuclear envelope during mitosis phase of cell cycle.
- Concentration of cyclin proteins fluctuate throughout three major cell cycle checkpoints in a predictable cycle. There is sharp decline of cyclin levels following each checkpoint (i.e. transition between phases of the cell cycle), as cyclin is degraded by cytoplasmic enzymes.

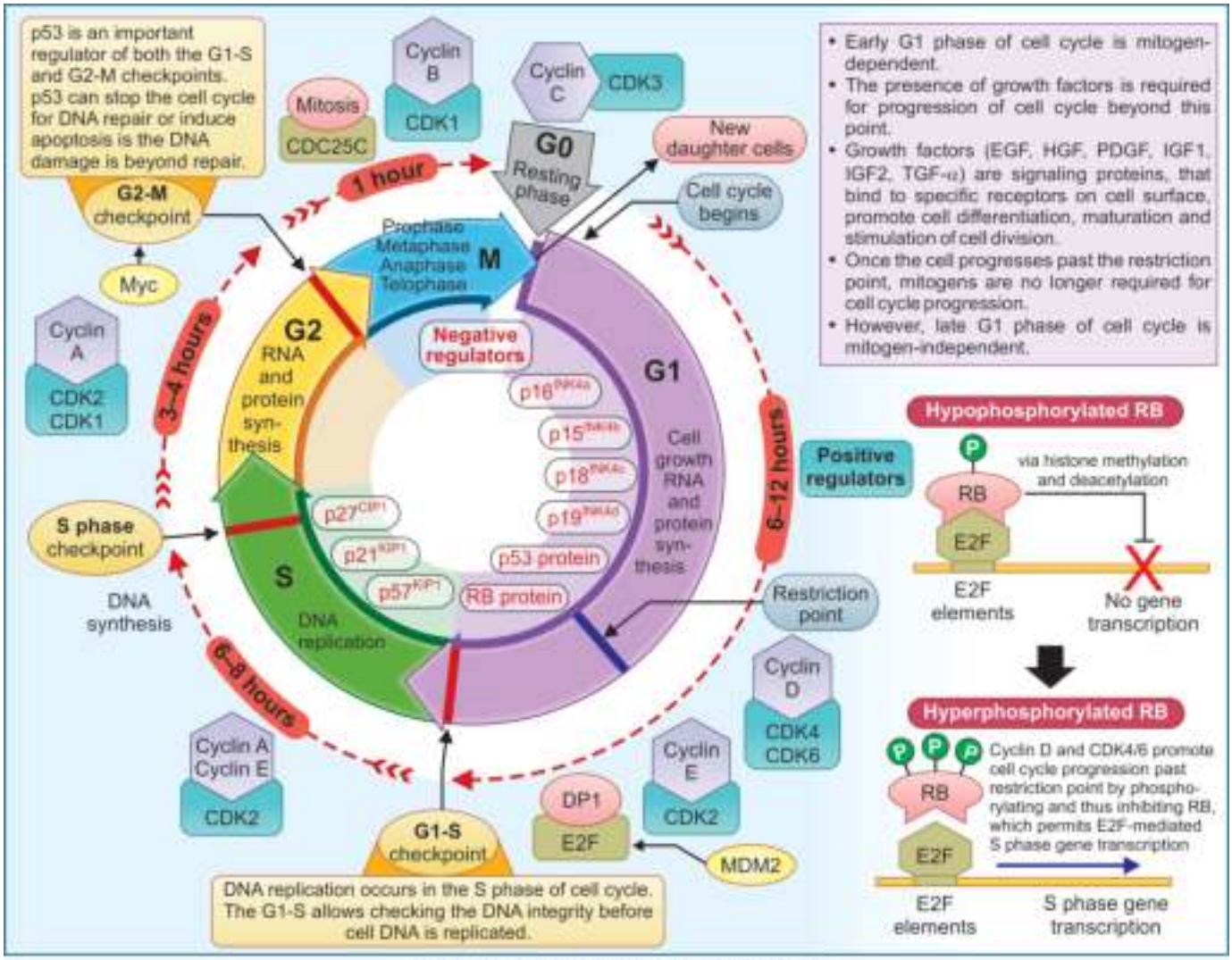
Cyclin-dependent Family of Serine/Threonine Kinases (CDKs): Positive Regulators of Cell Cycle

Cyclin-dependent kinases family of serine/threonine kinases interact with cyclins, and participate in regulation of cell cycle through regulation of cell cycle checkpoints, transcription response to extracellular and intracellular signals as well as communication, metabolism and apoptosis. The CDK activities are regulated by their association with partner with cyclins and without their corresponding cyclin subunit, the CDK is less active that fails to induce functional response.

- Binding of cyclin to CDK forms cyclin–CDK complex, that can phosphorylate specific target molecule. The attached phosphate group acts like a switch, making the target protein more or less active. Binding of cyclin to CDK results in breakdown of nuclear membrane at G1/S transition. Binding of cyclin-D to CDK results in phosphorylation of retinoblastoma protein (pRB) at G1/S transition. CDK-cyclin E complex in G1 phase and CDK-cyclin A complex in S phase mediate the G2/M transition. Binding of CDK1 to cyclin B results in G2/M transition.
- Dysregulation of CDKs is a hallmark of cancers, and inhibition of specific member is considered an attractive target in cancer therapy. Dual CDK4/CDK6 inhibitors are used for the treatment of

Table 6.23 Positive and negative regulators of cell cycle

Cell Cycle Phase	Positive Cell Cycle Regulators	Negative Cell Cycle Regulators
G0	Cyclin C+ CDK3	CDK inhibitor (no role)
G1 (6–12 hours)	▪ Cyclin D (D1, D2, D3) ▪ Cyclin E + CDKs (4,2,6)	▪ RB gene (pRB) ▪ TP53 gene (p53 protein) ▪ INK family of CDK inhibitory proteins (p16, p15, p18, p19)
S phase (6–8 hours)	▪ Cyclin A + CDK2 ▪ Cyclin E + CDK2	CIP/KIP (CDK interacting protein/kinase inhibitory proteins, i.e. p27, p21, p57)
G2 phase (3–4 hours)	Cyclin A + CDKs (2,1)	CDK inhibitor (no role)
M phase (1 hour)	Cyclin B + CDK1	CDK inhibitor (no role)



Cell cycle positive and negative regulators

Cell cycle	Positive regulators			Negative regulators	
Phase	Cyclins	Cyclin-dependent kinases (CDKs)	Other positive regulators include	Cyclin-dependent kinase inhibitors	Other negative regulators include
G0 phase	**Cyclin C**	**CDK3**	–	–	–
G1 phase (6–12 hours)	**Cyclin D (D1, D2, D3) Cyclin E**	**CDK4, CDK2, CDK6**	**MDM2**	**INK family of proteins** ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$) **RB protein, p53 protein**	**BRCA1, BRCA2, MSH2, MLH1, PMS1, PMS2, ATM proteins**
S phase (6–8 hours)	**Cyclin A Cyclin E**	**CDK2**	–	**CIP/KIP family of proteins** ($p27^{CIP1}$, $p21^{KIP1}$, $p57^{KIP1}$)	–
G2 phase (3–4 hours)	**Cyclin A**	**CDK2, CDK1**	**MYC**	**p53 protein**	–
M phase (1 hour)	**Cyclin B**	**CDK1**	–	–	–

Concept: *Cell cycle is tightly controlled by positive and negative regulators. Dysregulation of cell cycle is a hallmark of all malignancies. Metastatic breast disease is treated by cyclin-dependent kinase inhibitor (e.g. palbociclib, CDK4/CDK6 inhibitor) that can cross blood–brain barrier.*

Fig. 6.56: Cell cycle of positive and negative regulators. Positive cell regulators such as cyclins and cyclin-dependent kinase (CDK) enzymes regulate DNA replication and apoptosis. Cell cycle negative regulators are tumor suppressors such as RB (pRb), TP53 (p53 protein), INK family of proteins and CIP/KIP family of proteins, which restrict the ability to a cell to progress from G1 to S phase in the cell cycle.

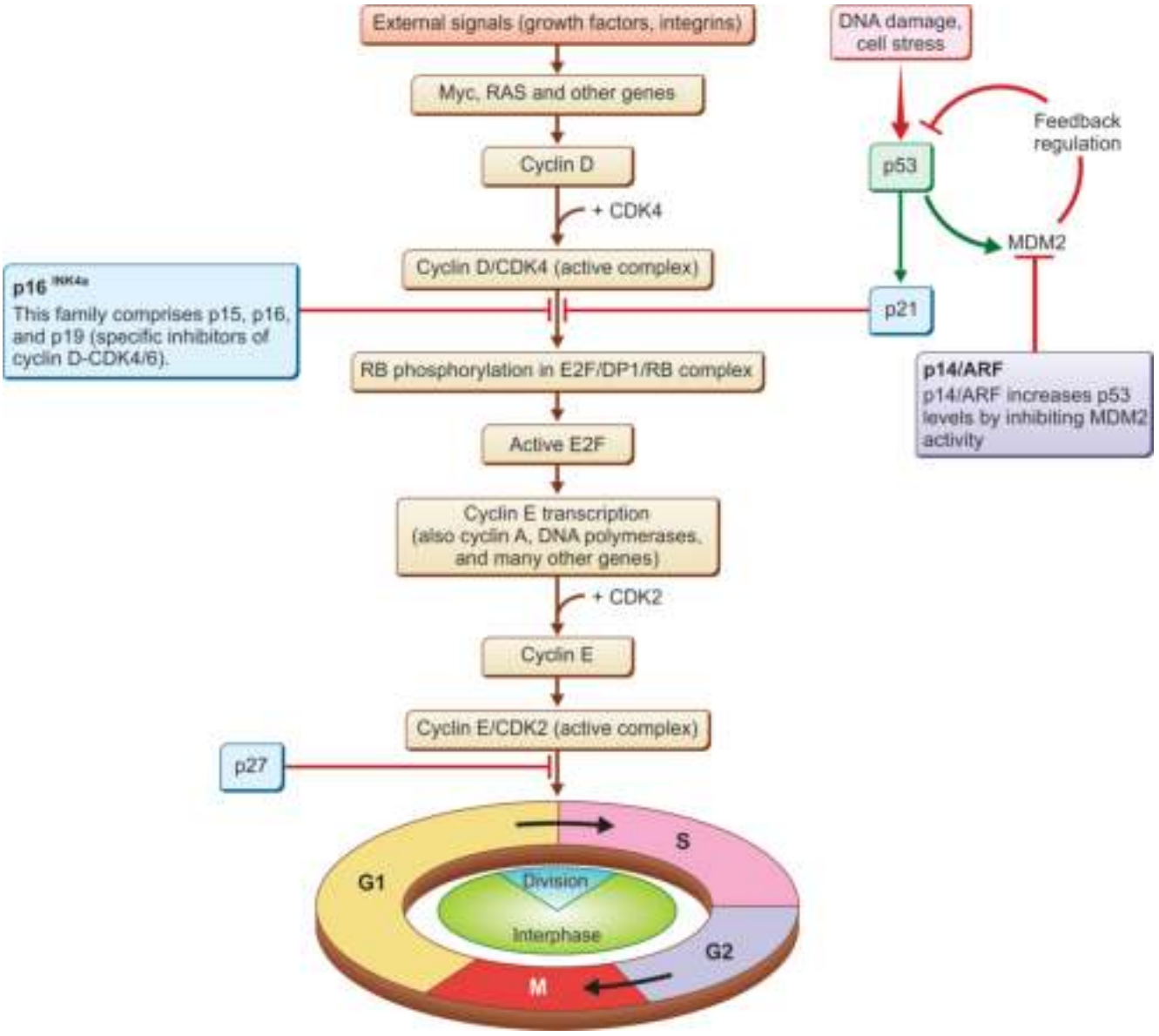


Fig. 6.57: Regulation of the cell cycle. Cells are triggered to go from G1 to G0 by growth factors and cytokines via activation of proto-oncogene. A significant time in the movement of cells from G1 to S phase is the restriction point R. An important regulatory event in this process is the phosphorylation of RB by cyclin-dependent kinases (CDKs), which causes the release of transcriptional activator E2F that forms E2F/DP1/RB complex in the nucleus. CDKs are suppressed by cyclin-dependent kinase inhibitors (CKIs) that are regulated by p53 protein. Tumor suppressor proteins block cell cycle progression within G1.

estrogen-positive (ER+)/human epidermal growth factor 2 negative (HER2/neu negative) breast cancer. Dysregulation of cyclins and cyclin-dependent kinase associated human cancers is given in Table 6.24.

Maturation/Mitosis Promoting Factor (MPF): Positive Regulators of Cell Cycle

Maturation/mitosis promoting factor is the universal master regulator of mitosis (M phase) of cell cycle

Table 6.24 Dysregulation of cyclins and cyclin-dependent kinase associated human cancers

Cyclin 1 Dysregulation and Human Cancers		
▪ Breast carcinoma	▪ Esophageal carcinoma	▪ Mantle cell lymphoma
▪ Multiple myeloma	▪ Hepatocellular carcinoma	
Cyclin-dependent Kinase 4 (CDK4) Dysregulation and Human Cancers		
▪ Glioblastoma multiforme	▪ Melanoma	▪ Sarcoma

from G2 phase by phosphorylating several different proteins involved in breakdown of nuclear envelope and chromosome condensation needed for mitosis. MPF is activated at the end of G2 phase, which removes an inhibitory phosphate group added earlier.

E3 Ubiquitin Ligase Anaphase-promoting Complex/ Cyclosome: Positive Regulators of Cell Cycle

E3 ubiquitin ligase anaphase-promoting complex/ cyclosome regulates ordered cell cycle, that ensures accurate chromosome segregation during mitosis, thus contributing to maintenance of genomic stability.

- Anaphase-promoting complex/cyclosome is a multi-subunit of E3 ubiquitin ligase, that causes degradation of cell cycle regulatory proteins and thus pushes the cell out of mitosis allowing the new daughter cells to enter G1 phase of the cell cycle.
- Dysregulation of E3 ubiquitin ligase anaphase-promoting complex/cyclosome can trigger the abnormal accumulation of its substrates, thus, leading to genomic instability and human cancers.

E2F Transcription Factor/DP1 Protein Complex: Positive/Negative Regulators of Cell Cycle

E2F regulates the expression of number of cellular genes involved in DNA replication and progression through G1/S transition of cell cycle either by activating or repressing their transcription. It is worth mentioning E2F can function as tumor promoter and tumor suppressor. The activity of E2F is dependent on its binding partners, i.e. dimerization protein 1 (DP-1) and RB tumor suppressor protein (pRB). During cell cycle progression, cyclin-dependent kinases (CDKs) phosphorylate RB tumor suppressor protein releasing E2F, which is then available to promote the expression of genes involved in S phase entry, DNA synthesis and mitosis.

MDM2 (Murine Double Minute 2) Protein: Positive Regulator of Cell Cycle

MDM2 (murine double minute 2) protein is an important negative regulator of p53 tumor suppressor protein. MDM2 protein functions as an E3 ubiquitin ligase to degrade unphosphorylated p53 tumor suppressor protein.

- MDM2 protein also binds to another ARF transcription factor that facilitates sequestration of MDM2 protein in the nucleolus thus promotes activation of p53 tumor suppressor protein and thus causes cell cycle arrest.
- Dysregulation of MDM2 proto-oncogene has significant impact on the p53 tumor suppressor functions and in turn leading to tumorigenesis. Considering the key role of MDM2 in the development of human cancers, a better understanding of the regulation of MDM2 will help to develop targeted chemotherapeutic agents against MDM2 protein with an aim to activate p53 tumor suppressor protein in the cells.

Myc Proteins: Positive Regulators of Cell Cycle

Myc proteins (c-Myc, L-Myc, N-Myc, S-Myc family of transcription factors) stimulate cell cycle progression and cellular proliferation through the regulation of genes related to cell cycle control. Myc protein induces positive regulators of cell cycle such as cyclins, cyclin-dependent kinases (CDKs) and E2F transcription factors involved in cell cycle progression at G2/S transition.

Cell Cycle: Negative Regulators

In cell cycle regulation, active positive regulatory molecules such as cyclin/CDK complexes induce cell cycle progression. Negative cell cycle regulators inhibit cell cycle progression and hence provide protection against tumor development, which include retinoblastoma protein (pRB), p53, INK family of proteins (p15, p16, p18, p19), CIP/KIP family of proteins (p21, p27), and p57, which inhibit CDKs thus block cell cycle progression within G1. If negative regulatory proteins are not produced or become non-functional then these induce uncontrolled cell proliferation leading to development of malignant tumor.

Retinoblastoma Tumor Suppressor Protein

Retinoblastoma (RB) gene is a tumor suppressor gene mapped on long arm of chromosome 13p14 that codes for nuclear pRB protein, master brake on cell cycle. Normal people have two alleles of the RB tumor suppressor gene that inhibits the cell cycle progression at G1/S transition. Normal people have two alleles of the RB tumor suppressor gene that inhibits the cell cycle progression at G1/S transition. Role of RB gene as a cell cycle regulator is shown in Fig. 6.58.

- Two opposing enzymatic reactions regulate the activity of retinoblastoma tumor suppressor protein (pRB). Phosphorylated RB releases E2F transcription factor, which binds to DNA and '**turns on**' gene expression, thus inducing cell cycle progression, whereas dephosphorylated pRB is unable to bind to DNA and '**turns off**' gene and thus inhibits cell division at G1/S transition.
- Mutation of RB gene (deletion and nonsense) causes loss of regulation of cell cycle activation through sequestration of transcriptional factors. Germline and somatic mutations of RB tumor suppressor gene are linked to human cancers.

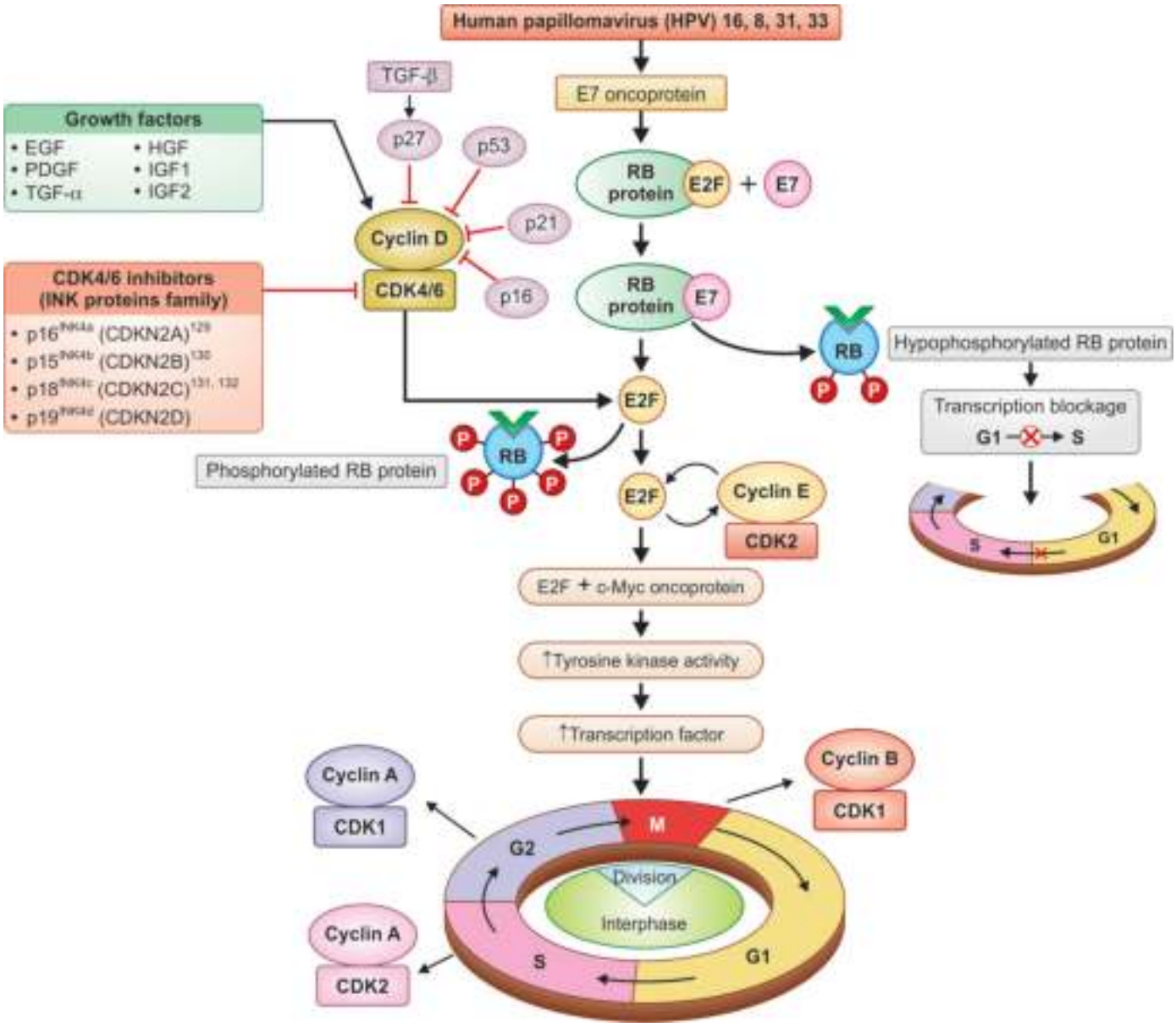


Fig. 6.58: Role of RB gene as a cell-cycle regulator. Various growth factors promote the formation of the cyclin D to CDK4 complex. This complex transforms active hyperphosphorylated RB to hypophosphorylated inactive state. RB inactivation allows the cell to pass the G1 checkpoint at G1/S transition. Virtually all cancers show dysregulation of the cell cycle.

- **Germline mutations in RB gene:** According to the '**Knudson two-hit hypothesis**', both alleles of the RB1 gene must be lost for development of cancer. Germline mutations in both alleles of the RB gene predispose to retinoblastoma and osteosarcoma.
- **Somatic mutation in RB gene:** Somatic mutation in RB gene can cause retinoblastoma (unilateral and unifocal in 1–2 years of age group) including other cancers such as osteosarcoma, breast carcinoma, colon carcinoma, prostatic carcinoma, urinary bladder carcinoma, glioblastoma, pancreatic carcinoma and small cell lung carcinoma.

Pathology Pearls: RB Gene Germline and Somatic Mutations Associated with Familial and Sporadic Cancers

RB Germline Mutations and Familial Tumors

- Retinoblastoma (B/L and multifocal before the age of one year)
- Osteosarcoma

RB Somatic Mutations and Sporadic Tumors

- Retinoblastoma (unilateral and unifocal)
- Osteosarcoma
- Breast carcinoma
- Colon carcinoma
- Prostatic carcinoma
- Urinary bladder carcinoma
- Lung carcinoma

TP53 (p53 Protein) Tumor Suppressor Gene in the Cell Cycle

TP53 tumor suppressor gene is mapped on chromosome on 17p13, that encodes nuclear transcription factor p53 multifunctional protein. TP53 is **guardian** of **genome**. The p53 protein level is low in healthy cells. DNA damage and other stress signals may trigger the increase in level of p53 protein in the cells.

- TP53 tumor suppressor gene product (p53) performs many functions: (a) inhibits G1/S and G2/M transitions of cell cycle, (b) promotes apoptosis interacting with numerous proapoptotic and antiapoptotic proteins, (c) repairs damaged DNA by recruitment of enzymes, (d) activates autophagy, and (e) regulates many metabolic pathways of glucose, lipid and amino acid.
- MDM2 (murine double minute 2) protein is an important negative regulator of p53 tumor suppressor protein, which functions as an E3 ubiquitin ligase to degrade unphosphorylated p53 tumor suppressor protein. Phosphorylated p53 protein is not degraded by MDM2. MDM2 protein also binds to another ARF transcription factor that facilitates sequestration of MDM2 protein in the nucleolus, thus promotes activation of p53 tumor suppressor protein and thus causes cell cycle arrest.
- TP53 gene mutation has been demonstrated in familial and sporadic cancers in more than 50% of patients.
  - **Germline mutations** in **TP53 gene:** Germline mutations in TP53 gene can result in unrestrained cell division and cause Li-Fraumeni syndrome (e.g. breast carcinoma, brain tumors, soft tissue sarcoma, leukemia, and adrenocortical carcinoma). Similar to retinoblastoma, the two-hit hypothesis also applies to Li-Fraumeni syndrome as well. One allele of TP53 is lost in the germline and second allele is lost somatically.
  - **Somatic mutations** in **TP53 gene:** Somatic mutations in TP53 gene are demonstrated in 50% of sporadic cancers such as breast carcinoma (20–40%), colorectal carcinoma, brain cancers (several types), urinary bladder carcinoma, small cell lung carcinoma, cholangiocarcinoma, hepatoblastoma and squamous cell carcinoma in head and neck region.

Pathology Pearls: TP53 Gene Mutation Associated with Familial and Sporadic Cancers

Familial Tumors (Li-Fraumeni Syndrome Associated with Cancers in Children and Young Adults)

- Breast carcinoma
- Osteosarcoma
- Brain tumors
- Soft tissue sarcoma
- Leukemias
- Adrenocortical carcinoma

Sporadic Tumors

- Breast carcinoma (20–40%)
- Colorectal carcinoma
- Brain cancers (several types)
- Urinary bladder carcinoma
- Small cell lung carcinoma
- Cholangiocarcinoma
- Hepatoblastoma
- Squamous cell carcinoma of head and neck region

Cyclin-dependent Kinase (CDK) Inhibitor Proteins

CDKN1A is tumor suppressor gene mapped on chromosome 6p21.2 that encodes two families of cyclin-dependent kinase inhibitors: CIP/KIP family of proteins (p21, p27 and p57) and INK family of proteins (p15, p16, p18 and p19). Cyclin-dependent kinases (CDKs) are positive regulators of cell cycle. CIP/KIP and INK family of proteins inhibit the activity of cyclin-dependent kinases (CDKs).

- **CIP/KIP family of proteins:** CIP/KIP family of proteins include p21, p27 and p57, which possess a wider spectrum of inhibitory activity of cyclin/CDK complexes (cyclin/CDK4/CDK6, cyclin E/CDK2, and cyclin A/CDK2) at G1 phase and S phase of cell cycle.
  - **The p21 protein:** The p21 protein plays a significant role in modulating DNA repair process, which inhibits activity of cyclin-dependent kinases (CDKs, e.g. CDK2, CDK1, CDK4/6) and thus halts cell cycle progression at G1/S transition, and allows DNA repair to proceed while inhibiting apoptosis. Cytoplasmic expression of p21 can be significantly correlated with lymph node metastasis, distant metastasis, advanced TNM stage and overall poor survival.
  - **The p27 protein:** The p27 protein is found in cells and tissues throughout the body. Within cells, p27 protein is located primarily in the nucleus, where it plays an important role in controlling cell growth and division. TGF-β induces p27 protein production that mediates G1 phase cell cycle arrest. Loss or reduced expression of p27 protein is associated with poor prognosis in cancers of breast, prostate, lung, stomach and colorectal region.
  - **The p57 protein:** The p57 is a tumor suppressor multifunctional protein, that inhibits cyclin to CDK complexes and halts cell cycle at G1 phase. Due to its role in cell cycle control, p57 is involved in the many cellular processes such as embryogenesis and tissue differentiation. Loss-of-function of p57 protein is linked to development of **Wilms' tumor** and **hepatoblastoma** during childhood in cases of **Beckwith-Wiedemann syndrome** (BWS). Loss-of-function of p57 protein is also linked to complete hydatidiform mole.

- **INK family of proteins:** INK family of proteins include p15, p16, p18 and p19, which possess a wider spectrum of inhibitory activity of cyclin to CDK complexes (cyclin D to CDK4/6 complex).
  - **The p16 protein:** The p16 is a tumor suppressor protein that plays an important role in regulating the cell cycle. The p16 inhibits cell cycle progression at G1/S transition by inactivating CDKs 4 and 6, that phosphorylates RB tumor suppressor protein, which regulates the cell cycle.
  - **The p15 protein:** The p15 protein is present in the cytoplasm and nucleus and interacts strongly with cyclin-dependent kinases (CDK4 and CDK6) and inhibits their activities leading to cell cycle arrest. The p15 protein is a critical tumor suppressor in the absence of p16.
  - **The p18 protein:** The p18 protein interacts strongly with CDK6, and weakly with CDK4. It inhibits cell growth and cell cycle progression at G1/S transition with a correlated dependence on endogenous RB tumor suppressor protein.
  - **The p19 protein:** The p19 protein inhibits cell growth and cell cycle progression at G1/S transition. The $p19^{INK4D}$ protein is also involved in the cellular senescence mechanism contributing to heterochromatin formation.

DNA REPAIR PROTECTS CELLULAR GENOMES FROM GENOTOXIC STRESSES

Deoxyribonucleic acid (DNA) is an organic chemical that stores information and instructions for protein synthesis. These instructions are stored inside cells and distributed among 46 chromosomes, which are made of numerous short segments of DNA, called genes. DNA damage denotes alterations in the chemical structure of DNA such as a break in DNA strand, nucleotide base pair missing from the backbone of DNA, and a chemical alteration in the nucleotide base pair. DNA damage can be recognized by DNA repair enzymes and correctly get repaired.

- **Causes of DNA damage:** DNA damage can be caused by errors during DNA replication, alkylating agents, reactive oxygen species (ROS) during normal cellular respiration and other biochemical pathways, and physical mutagens (ultraviolet rays, X-rays and γ-radiation). DNA damage leads to genome instability, increased cancer risk, accelerated aging process and neurodegenerative disorders. Damage to DNA can occur by cellular metabolic or hydrolytic processes (deamination and depurination).
- **DNA damage at various levels:** Most forms of localized DNA damage occur at various regions, which include single-strand breaks, double-strand breaks, DNA adducts, nucleotide base insertions or deletions, and nucleotide base pair mismatches. Dysfunction of the mismatch repair system is linked to human cancers. Comet assay or single-cell gel electrophoresis assay is a convenient diagnostic tool for measuring universal DNA damage in individual's cells.

DNA REPAIR PATHWAYS

DNA repair mechanisms depend on the presence of two strands of DNA because nucleotide base pairs in the damaged region are removed and replaced by mismatch nucleotide base pair repair, nucleotide base-excision repair and nucleotide-excision repair. Poly-ADP-ribosyl-polymerase (PARP) plays a central role in nucleotide-excision repair (NER) and base-excision repair (BER) and enables repair of DNA damage caused by alkylating agents and chemotherapeutic agents. PARP is also involved in many other cellular processes including transcription and modulation of chromatin structure. DNA repair mechanisms occur by using a common four-step pathway.

- **Detection of damaged DNA:** Damaged region of the DNA is recognized.
- **Excision of damaged DNA:** Endonuclease gives a small cut to the sugar-phosphate backbone on either one or both sides of the DNA damage to remove one or more nucleotides.
- **Polymerization of damaged DNA:** DNA polymerase enzyme adds nucleotides to the newly exposed 3'OH group by using the other strand of DNA as a template and replacing damaged nucleotide base pairs as well as some undamaged nucleotides in base-excision, mismatch and nucleotide repair mechanisms.
- **Ligation and sealing of damaged DNA:** DNA ligase seals and fills in the small gaps produced by the excision and removal of the damaged nucleotides base pairs in the sugar-phosphate backbone in all three base-excision, mismatch and nucleotide base repair mechanisms.

Pathology Pearls: Microsatellite Instability—Indicators of Defective Mismatch Repair

- Microsatellite instability is an important indicator of defective mismatch repair. Microsatellite instability is short repeated sequences of nucleotide base pairs in genomic DNA.
- Microsatellite mutations are usually recognized and repaired by mismatch repair enzymes. Mediators of mismatch repair system include various mismatch gene products.
- DNA single nucleotide base pair mismatch is detected by MSH2 and MSH6 proteins, which recruit a group of mismatch repair enzymes, which correct the defective sequence.
  - If the mismatch pairing occurs as a result of small insertion or deletion, a second group of mismatch repair enzymes (i.e. MSH2 and MSH3) detect the defective sequence and restore the correct sequence.

- Failure to repair microsatellite mutations in germline or somatic cells is linked to human cancers.

- Human beings, who possess mutations in mismatch-repair genes often exhibit upregulated somatic mutation rates and are frequently susceptible to colon cancer.

Pathology Hallmarks: DNA Repair Mechanisms

- Base excision repair (BER) mechanism
- Direct reversal of DNA damage mechanism
- Repair of DNA protein crosslinks mechanism
- Nucleotide excision repair (NER) mechanism
- Mismatch excision repair (MMR) mechanism
- Double DNA break repair mechanism
- Homologous recombination mechanism
- Nonhomologous end-joining mechanism
- DNA replication fork repair mechanism

Direct DNA Damage Reversal

Direct DNA damage reversal system acts directly on damaged single nucleotide base pair chain, that restores the DNA genome to its normal original state in a single-reaction step. But only a few damaged nucleotides can be repaired directly. Pyrimidine dimers are repaired by a light-dependent direct system called **photoreactivation**.

Mismatch Repair Mechanism

DNA mismatch repair is a mechanism of repairing errors in DNA during DNA replication by the removal of mismatched nucleotide base pairs via mismatch repair (MMR) proteins. DNA replication is extremely accurate in healthy cells.

- DNA mismatch repair (MMR) recognizes and repairs erroneous insertion, deletion and misincorporation of nucleotide base pairs that can arise during DNA replication and recombination, and repair some forms of DNA damage.
- DNA mismatch repair plays an important role in maintaining genomic stability and cellular homeostasis. Most of the errors that initially arise during DNA replication are corrected and never becomes permanent mutations.
  - Some of these errors in nucleotide base pairs are corrected during proofreading by polymerases. During proofreading, some of the incorrect nucleotide base pairs are inserted to the newly synthesized strand of DNA.
  - Mismatched nucleotide base pairs and other DNA errors are corrected by mismatch repair mechanism by inhibiting DNA replication at the G2/M checkpoint, correct errors and then allow DNA replication to continue.
  - Exonucleases remove nucleotide base pairs on the newly synthesized strand of DNA between the GATC sequence and the mismatch.
  - DNA polymerase replaces the nucleotide base pairs, correcting the mismatch, and DNA ligase seals the nick in the sugar-phosphate backbone.
- Examples of mismatch gene products include MLH1 gene (3p21), MSH2 gene (2p15), MSH3 gene (5q11.12), MSH4 gene (1p31.1), MSH5 gene (6p21.33), MSH6 gene (2p16), MLH3 gene (14q24.3), PMS1 gene (2p32.2), PMS2 gene (7p22.1), and HFM1 gene (1p22.2). Defective mismatch repair may be inherited (germline) or acquired (somatic). Acquired defects in mismatch repair may reflect either mutations that develop overtime in somatic cells or epigenetic silencing of component of mismatch repair system.

Excision Repair Mechanism

Excision repair mechanism involves excision of a segment of the polynucleotide containing a damage site followed by synthesis of the correct nucleotide sequence by DNA polymerase enzyme and then ligation. Excision repair occurs by two mechanisms: base excision repair and nucleotide excision repair.

Nucleotide Base Excision Repair Mechanism

Nucleotide base excision repair refers to repair of damage to a single-nucleotide base caused by oxidation, alkylation, hydrolysis or deamination. The damaged nucleotide base pair is removed by DNA glycosylase enzyme, synthesis by DNA polymerase enzymes, and sealing by DNA ligase enzyme.

Nucleotide Excision Repair Mechanism

Damage to the DNA distorts the configuration of the molecule. An enzyme complex recognizes the distortion of DNA resulting from damage. DNA strand becomes separate. Single-strand binding proteins stabilize the single strand. An enzyme cleaves the damaged strand on both sides of the damage. Part of the damaged strand is removed, and the gap is filled in by DNA polymerase and sealed by gyrase.

Recombination Repair Mechanisms of Single- and Double-Strand Breaks of DNA

Ionization, certain chemical agents and ROS can produce both single-strand breaks (SSBs) and double strand breaks (DSBs) in the DNA backbone. Breaks in single-strand of the DNA molecule are repaired using the same enzyme systems that are used in base excision repair. The damaged nucleotide base pair is removed by DNA glycosylase enzyme, synthesis by DNA polymerase enzymes, and sealing by DNA ligase enzyme. DSB inhibits DNA replication and may lead to chromosomal rearrangements such as deletions, inversions, duplication, and translocations. Two major pathways for repairing DSBs include homologous recombination and nonhomologous end joining.

Homologous Recombination Repair Mechanism

Homologous recombination (HR) repairs a broken DNA molecule by using the identical or nearly identical genetic information contained in another DNA molecule, usually a sister chromatid or homologous chromosome.

- The process homologous recombination is responsible for crossing over of chromosomes that results in creation of new combinations of DNA sequences in each chromosome.
- The breast cancer tumor suppressor BRCA1 and BRCA2 genes are essential for the maintenance of genomic integrity in human cells through their role in DNA repair by homologous recombination. Inherited (germline) mutations of these genes predispose women to breast and ovarian cancers.

Nonhomologous End-joining Mechanism

Nonhomologous end joining (NHEJ) repairs double-strand breaks without using a homologous template. This pathway is most often used when the cell is in G1 phase of cell cycle and a sister chromatid is not available for repair through homologous recombination.

- Nonhomologous end joining uses proteins that recognize the broken ends of DNA molecule, bind to the ends and then join together. NHEJ is more prone to errors such as deletions, insertions and translocations than homologous recombination.
- Errors in nonhomologous end joining may be associated in translocation in various cancers such as Burkitt's lymphoma, Philadelphia chromosome in chronic myelogenous leukemia (CML) and B cell non-Hodgkin's lymphoma.

TELOMERES AND SENESCENCE

Healthy cells can divide a limited of times before becoming senescent. Cell senescence process is regulated by the shortening of telomeres after each cell cycle division.

- Once the telomeres shorten to certain threshold, DNA repair gene products of TP53 gene and RB gene, the abnormal telomere length induce cell cycle arrest, thereby stopping further DNA replication of the senescent cell. If p53 protein is lost, a special type of DNA repair occurs, called **nonhomologous end joining**. The ends of the random chromosomes are joined together, forming dicentric chromosomes (with two centromeres).
- Continuation of mitosis would result in mitotic catastrophe with breakage of the chromosomes because of the pulling apart of aberrantly located centromeres and resulting in cell death.
- In tumor cells, telomerase enzyme present in self-regenerating stem cells allows lengthening of telomerases after each cell division. The tumor cell evades senescence and can continue replicating despite accumulation of DNA damage.

APOPTOSIS INHIBITS TUMOR GROWTH

Apoptosis is way of eliminating a cell without eliciting a major host inflammatory and/or immune response. Apoptosis is essential for development, tumor suppression, immune function and maintenance of homeostasis. The accumulation of DNA damage usually triggers the intrinsic pathway to induce apoptosis of cells.

- The loss of apoptotic control allows CSCs to survive longer and gives more time for the accumulations of gene mutations, which induce tumorigenesis, angiogenesis, invasion and metastasis to distant organ(s).
- BCL-2, an antiapoptotic protein, is usually upregulated in human cancers to protect against apoptosis. TP53 gene product is usually downregulated in cancers to evade apoptosis, even in the face of irreparable DNA damage.

EPIDEMIOLOGY OF CANCER

Many human cancers are related to lifestyle factors and specific environmental carcinogenic agents, which can act either as an initiator or promoter or both in the process of carcinogenesis. Cancer arises from the transformation of normal cell to cancer stem cell in a multi-step process due to interaction between a person's genetic factors and environmental carcinogenic agents such as physical carcinogens (e.g. ultraviolet radiation and ionizing radiation), chemical carcinogens (e.g. asbestos exposure, cigarette smoke, alcohol consumption, food contaminant aflatoxin B1 produced by *Aspergillus flavus* in stored agriculture crops such as maize, peanuts, tree nuts and cotton seed in warm and humid regions), and biological carcinogens (e.g. bacteria, viruses and parasites).

- Cancer begins as a single mutated transformed CSC. Approximately, 30 cell divisions form 1–2 mm tumor size before the earliest clinical symptoms appear in the host. Each cell division (doubling time) results in increased gene mutations. Cancers that do not produce symptoms until late in the course of disease, which would have undergone unrestricted cell proliferation, and hence, additional gene mutations

occur. Cancers that are detected late tend to invade surrounding tissues, metastasize to distant organ(s) associated with poor prognosis.

- Goal of cancer screening is to detect severe dysplasia (carcinoma *in situ*) before it becomes invasive carcinoma, before patients have clinical symptoms. Papanicolaou smear (named after George Nicholas Papanicolaou) detects cervical intraepithelial neoplasia III (CIN-III) before it becomes invasive cervical carcinoma. Mammography detects ductal carcinoma *in situ* of breast (DCIS) before it becomes palpable invasive ductal carcinoma, that invades surrounding tissues and metastasizes to distant organ(s). Prostate-specific antigen (PSA) and digital rectal examination can detect prostatic adenoma before it transforms to prostatic adenocarcinoma.

GLOBAL IMPACT OF CANCER

Cancer is a leading cause of death worldwide due to breast carcinoma, lung carcinoma, colon carcinoma and prostatic carcinoma. About 33% of cancer patients population have fatal outcome due to tobacco smoking, tobacco chewing, high-body index, alcohol consumption, low-fiber vegetable consumption, low-intake of fruits and lack of physical activity. About 33% patients develop malignancy due to human papillomavirus (HPV 16, 18), hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. Many cancers can be cured, if detected early and treated effectively by surgery, radiotherapy and chemotherapy. As malignant tumors obtain nutrients from the host, they can alter normal cellular biological processes and cause cachexia.

CANCER EPIDEMIOLOGY AND PREVENTION STRATEGIES

Cancer epidemiology is the study of the distribution, determinants and frequency in specific populations. Its main objective is to define etiologic factors to formulate preventive strategies for controlling the cancer disease. The main epidemiological parameters used in the study of human cancers are sex predilection, age, ethnicity, geography, style factors and specific environmental carcinogenic agents, which can act either as an initiator or promoter or both in the process of carcinogenesis. Epidemiologic assessment provides the clinician with a quantification of cancer risk, outlines the basis for screening modalities for high-risk populations and determines the efficacy of a preventive intervention.

Incidence of Cancers in Men and Women

Incidence of cancer varies from country to country and region to region across world. Many cancers are related to specific environmental and lifestyle factors that predispose a person to develop cancer. Incidence of cancers in women and men is shown in Fig. 6.59.

- In Western countries (USA and Europe), men are most often affected by prostatic carcinoma (32%), lung carcinoma (16%), colorectal carcinoma (12%), urinary bladder carcinoma (9%) and melanoma (4%). On the other hand, women develop breast carcinoma (32%), colorectal carcinoma (13%), lung carcinoma (12%), urinary bladder carcinoma (4%) and ovarian carcinoma (4%).
- In Indian population, men most often develop cancers of oral cavity, pharynx and lung, and women are most often affected by cervical carcinoma and breast carcinoma. Incidence of cervical carcinoma is declining due to early detection by exfoliative cytology stained by Papanicolaou stain. Incidence of oropharyngeal carcinoma is more prevalent in North Indian population, while gastric carcinoma in South Indian population.

Age Group and Cancers

The incidence of many human cancers increases with age. Few cancers are found in children, and the incidence of cancer correlates directly to increasing age, which indicates that numerous cumulative events are essential for the initial gene mutation to continue, eventually forming a malignant tumor. Children ≥15 years suffer from lethal cancers like acute lymphoblastic leukemia (ALL), Burkitt's lymphoma, Wilms' tumor, hepatoblastoma, cerebellar astrocytoma, retinoblastoma, neuroblastoma, testicular germ cell tumors, botryoid rhabdomyosarcoma and osteosarcoma. Most frequent types of cancers in children from birth to childhood are given in Table 6.25.

Sex Predilection and Cancer

Certain cancers are more prevalent in women, and others in men. Sex hormones influence development of breast carcinoma, endometrial carcinoma and prostatic carcinoma. Researchers believe that sex hormones sensitize the cell to initial precipitating factor, thus promoting carcinogenesis.

- **Male predominance** has been observed in prostatic carcinoma, lung carcinoma, colorectal carcinoma, esophageal carcinoma, renal cell carcinoma, urinary bladder carcinoma, squamous cell carcinoma of skin, pancreatic carcinoma, and hematolymphoid malignancies (leukemias/lymphomas).
- **Female predominance** has been observed in breast carcinoma, cervical carcinoma, endometrial carcinoma, leiomyosarcoma of uterus, lung carcinoma, colorectal carcinoma, cutaneous squamous cell carcinoma, pancreatic carcinoma and lymphomas.

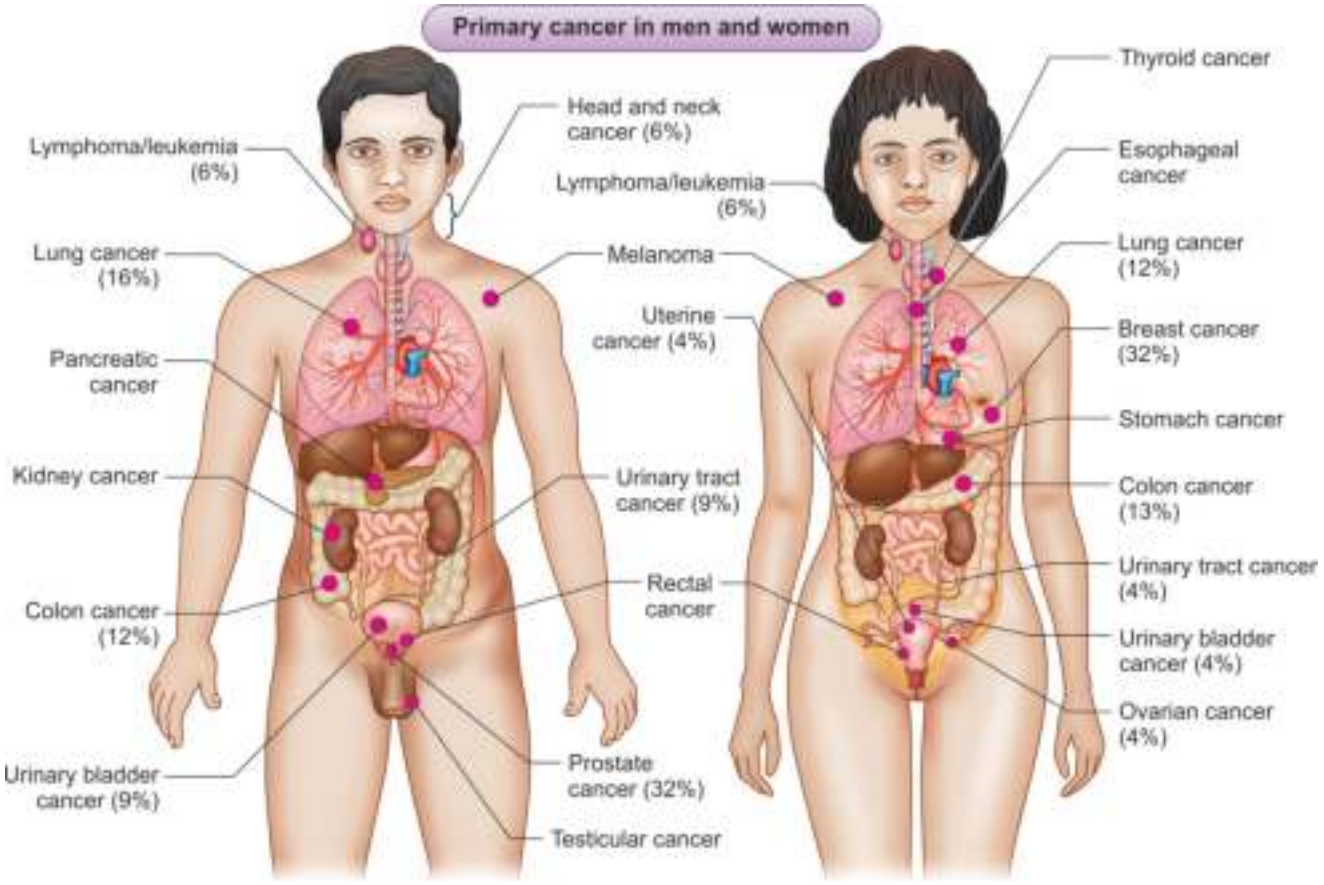


Fig. 6.59: Incidence of cancers in women and men. In men, excluding skin cancers, other cancers account for 75% cases. In women, excluding skin cancers, other cancers account for 80% cases. Most common locations for cancer in men include skin, prostate, lung and colon. Most common locations for cancer in women include skin, breast, lung, colon and uterus. Most common cancers in young children include acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), neuroblastoma, retinoblastoma and botryoid rhabdomyosarcoma.

Table 6.25 Most frequent types of cancers in children from birth to childhood

Nomenclature of Tumors	Malignancies	
Hematolymphoid malignancies	▪ Acute myelogenous leukemia ▪ Acute lymphoblastic leukemia	▪ Hodgkin's disease ▪ Burkitt's lymphoma
Primitive neuroectodermal tumors (PNETs)	▪ Astrocytoma ▪ Primitive neuroectodermal tumors ▪ Brain stem gliomas	▪ Cerebellar astrocytoma ▪ Ependymomas
Germ cell tumors	▪ Yolk sac tumor of the testis	
Bone tumors	▪ Osteosarcoma	▪ Ewing sarcoma
Soft tissue tumors	▪ Alveolar rhabdomyosarcoma	▪ Embryonal rhabdomyosarcoma (e.g. botryoid rhabdomyosarcoma)
Blastomas	▪ Retinoblastoma ▪ Neuroblastoma	▪ Nephroblastoma (Wilms' tumor) ▪ Hepatoblastoma

Geographic and Racial/Ethnic Disparities in Epidemiology and Genomics in Cancer

Geographic and racial/ethnic disparities in rates of incidence, mortality, survival, prevention, early detection and genomics of many cancers are well documented and often associated with socioeconomic status in particular population and subpopulation resulting from exposure to environmental carcinogens.

Geographic and racial/ethnic differences in cancer epidemiology are given in Table 6.26.

- System level and health care professional-related issues likely to contribute to specific racial and ethnic health disparities have been reviewed to provide possible suggestions for future strategies to reduce the disproportionate burden of many specific cancers.
- Although, carcinogenesis is a multifactorial process driven by environmental exposures to carcinogens, genetic variations, and accumulation of somatic genetic events, which appear to have racial and ethnic disparities, that in turn impact the observed epidemiological differences in rates, incidence, mortality and survival.
- Incidence of breast cancer is higher in Japanese women, as compared to American women. Incidence of gastric carcinoma is higher in Japan and Iceland persons, as compared with the USA. Incidence of hematologic malignancy is higher in sub-Saharan African persons, as compared with persons in USA. Prostatic carcinoma is more prevalent in the Black population, than White population in USA. Skin cancer is more prevalent in patients with fair skin and light hair.
- The genomic diversity of oncogenes and tumor-suppressor genes across racial and ethnic groups poses unique but important challenges for therapeutic opportunities to provide personalized medical care. Molecular genomic profiling for specific alterations is essential to identify patients likely to benefit from targeted therapy.

Alcohol Consumption and Tobacco Products Linked to Cancer

Excessive alcohol consumption is linked to cirrhosis leading to hepatocellular carcinoma in some cases. Patient can also develop breast carcinoma and colorectal carcinoma. Heavy use of alcohol and cigarette smoking

Table 6.26 Geographic and racial/ethnic differences in cancer epidemiology

Cancers	Higher Cancer and Geographical Distribution
Malignant melanoma	Australia (Queensland)
Oral carcinoma	Indian population (consumption of oral betel nut and tobacco chewing), and France population (consumption of cider-based liquors)
Lip carcinoma	Canada
Breast carcinoma	United States of America and Northern Europe, persons with BRCA gene mutation (less prevalent in Japanese women)
Esophageal carcinoma	South Africa, belt from Turkey to Eastern China
Gastric carcinoma	Japan (consumption of smoked fish), Chile, persons with blood group A (incidence low in United States of America)
Nasopharyngeal carcinoma	China (Epstein-Barr virus), Hong Kong, Singapore
Hepatocellular carcinoma	Sub-Sharan African nations (incidence low in United States of America)
Lung carcinoma	United States (Louisiana African American), Eastern Europe (male)
Cervical carcinoma	Brazil, Eastern Europe
Ovarian carcinoma	New Zealand (Polynesian)
Colon carcinoma	Australia, New Zealand (male)
Pancreatic carcinoma	United States (Los Angeles—Korean Americans)
Burkitt's lymphoma	African population (Epstein-Barr virus)
Kaposi sarcoma	African population (human herpesvirus 8)
Hepatocellular carcinoma	South East Asia (hepatitis B virus and aflatoxin produced by *Aspergillus flavus*), China (Shanghai)
Cutaneous squamous cell carcinoma	Northern Australia, South Western of United States, persons with fair skin, light hair and blue eyes, xeroderma pigmentosum, Kashmiri population carries a basket with burning charcoal (*Khangri*) held close to abdomen to keep them warm during winter season, and develops skin carcinoma on the abdominal region (low incidence in dark skin persons)
Penile carcinoma	United States of America, Brazil, persons with balanitis (incidence low in Muslims and Jews, who undergo circumcision of prepuce. It may be probably smegma that acts as a solvent for environment carcinogens)
Prostatic carcinoma	United States of America Black population (incidence low in White population in USA)
Choriocarcinoma	Asia pacific region near the Western Pacific Ocean

synergistically increase the incidence of cancers of the mouth, larynx, pharynx, and esophagus. It is likely that alcohol acts as a solvent for the carcinogenic agents in tobacco smoke, thus enhancing their absorption. Cigarette smoke comprises neutral and acidic fractions. Neutral fraction contains polycyclic hydrocarbons, which bind to nuclear DNA and exert mutagenic effect. Acidic fraction of tobacco smoke contains a number of tumor-promoting agents. Tobacco smoking increases risk of lung carcinoma.

Dietary Factors and Lifestyle Factors Linked to Cancers

High consumption of smoked foods (salted fish or meats), and dietary nitrites are linked to gastric carcinoma. Consumption of red meat increases risk of lung carcinoma (i.e. squamous cell lung carcinoma, lung adenocarcinoma), esophageal carcinoma, colorectal carcinoma, prostatic carcinoma, and ER therapy-induced breast carcinoma. Aflatoxin B1 synthesized by *Aspergillus flavus* in stored food grains increases risk of hepatocellular carcinoma. High body mass index (BMI) increases risk for various cancers. Exercise and soy food consumption decrease risk of breast carcinoma.

Sexual Factors Linked to Cancer

Cervical carcinoma is one of the most common cancers affecting women worldwide. It is readily detected and treated at precursor stage, i.e. 'carcinoma *in situ*'. Cervical carcinoma associated with high-risk human papillomavirus (HPV 16, 18) infection transmitted via sexual route develop primarily at the '**transformation zone**', region, where metaplastic squamous epithelial cells are detected in otherwise columnar epithelium lined endocervical glands. HPV 16, 18 infection can result in cervical carcinoma 5–30 years after the initial infection.

- Woman is at high-risk of HPV infection linked to cervical carcinoma, who has sex without the use of condoms with multiple sexual partners. Enabling woman to successfully negotiates condom use with her sexual partners may therefore decrease the risk for cervical carcinoma.
- Early age at first sexual intercourse increases the risk for cervical carcinoma as damage might be caused to the cervix at a time, when it is developing. The risk of getting HPV infection and cervical carcinoma in woman is more, who has sexual course around 15 years of age.

Reproductive Hormones Linked to Cancers

Steroid hormones, i.e. estrogen, progesterone, and testosterone have been implicated as promoters of breast carcinoma, endometrial carcinoma, ovarian carcinoma, or prostatic carcinoma. Researchers believe that the hormone sensitizes the cells to the initial precipitating factor, thus promoting carcinogenesis. Breast carcinoma is more common in women than men, probably due to the greater mammary epithelial volume and to the promoting effects of circulating estrogens.

Environmental Pollutants Linked to Cancer

Many outdoor air pollutants such as arsenic, benzene, hydrocarbons, polyvinyl chloride, and other industrial emissions as well as vehicle exhaust have carcinogenic properties. Chloromethyl ether is a potent carcinogen causing small cell lung carcinoma. Combustion of coal and other fossils releases polycyclic aromatic hydrocarbons (benzopyrene), which increase risk for lung carcinoma.

Occupational Workers Exposed to Carcinogens Linked to Cancers

Exposure to various carcinogens, occupational workers may develop cancers. Occupational workers involved in the production of aniline dyes, textiles, rubber and paint, exposure to β-naphthylamine increases risk for urothelial carcinoma.

- Mesothelioma (pleura, peritoneum), lung adenocarcinoma and gastrointestinal cancers are more prevalent in pipe fitter and ship builder occupational workers exposed to asbestos mineral dust.
- Nickel exposure in mine workers is associated with cancers in occupational works of nasal cavity and lungs. Arsenic exposure may cause squamous cell carcinoma of skin, lung carcinoma, and liver angiosarcoma. Beryllium exposure may cause bronchogenic carcinoma. Cadmium used in batteries and metal paintings is implicated in prostatic carcinoma. Chromium used in paints, pigments and preservatives is linked to lung carcinoma. Occupational workers exposure to carcinogens and associated cancers are given in Table 6.27.

Radiation Exposure Linked to Cancers

Exposure to ultraviolet solar radiation, causes genetic mutation in TP53 gene. Ultraviolet radiation is a direct cause of melanoma, basal cell carcinoma of skin and squamous cell carcinoma of skin. Ionizing radiation may cause lung carcinoma. Japanese are at high risk of lung carcinoma (squamous cell lung carcinoma, adenocarcinoma and small cell lung carcinoma) after atom bomb explosion in Hiroshima and Nagasaki. Prolonged exposure to X-ray radiation is linked to acute leukemia, thyroid carcinoma, breast carcinoma, gastric carcinoma, colon carcinoma, urinary bladder carcinoma, and multiple myeloma. Low doses of X-ray radiation can cause DNA mutations and chromosomal alterations which play important role in carcinogenesis.

Table 6.27 Occupational workers exposure to carcinogens and associated cancers

Heavy Metals and Inorganic Chemical Agents	Occurrence and Type of use	Associated Cancers
Arsenic compounds	Component of alloys, preparation of electrical and semiconductor device, medications, fungicides and herbicides	Squamous cell carcinoma of skin, basal cell carcinoma of skin, lung carcinoma and hepatic angiosarcoma
Asbestos	Used as material in building construction	Lung carcinoma, esophageal carcinoma, gastric carcinoma, and mesothelioma (pleura and peritoneum)
Benzene	Used in printing, paint, rubber, dry cleaning, detergents, adhesives	Acute myelogenous leukemia (AML)
Uranium	Mine workers	Lung carcinoma
Nickel compounds	Used in nickel plating ceramics, ferrous alloys, batteries and stainless steel welding	Lung carcinoma and oropharyngeal carcinoma
Chromium compounds	Component of metal alloys, paints, pigments and preservatives	Lung carcinoma
Beryllium compounds	Used in missile fuel, nuclear reactors, aerospace application and light weight alloys	Lung carcinoma
Cadmium compounds	Used in batteries and metal paintings	Lung carcinoma and prostatic carcinoma
Vinyl chloride	Used to make polyvinyl chloride (PVC) pipes, plastic kitchenware, wire coatings, pesticides, dyes, drugs and synthetic polymers	Hepatic angiosarcoma

Genetic Factors Linked to Cancers

Genotype of an individual plays a role in determining susceptibility to malignant disease as a result of chromosomal alterations related to various genetic syndromes or single gene abnormalities.

- Three chromosomal disorders are prone to develop cancers. Down's syndrome is prone to develop acute lymphoblastic leukemia (ALL) or acute myelogenous leukemia (AML). There is increased risk for breast carcinoma in males with Klinefelter's syndrome. **Gonadal dysgenesis** with male genotype is more likely to develop tumors of gonads.
- Person with xeroderma pigmentosum is prone to develop squamous cell carcinoma of skin and basal cell carcinoma of skin. Familial adenomatous polyposis coli predisposes to colorectal carcinoma. Women with BRCA1 gene mutation have high-risk for breast carcinoma.

IMMUNODEFICIENCY STATES IN CANCER EPIDEMIOLOGY

Transformation of normal cell to CSC and their subsequent successful clonal expansion and progression to development of clinically apparent solid malignant tumors and hematologic malignancies is a complex multifactorial process due to genetic, immunologic, microbial and environmental constituents.

- Immune system is composed of highly specialized cells, tissues, organs, and soluble factors that interact in a complex way to ensure immune defense. Patients, who are deficient in T cell immunity are at high-risk to develop malignancies especially caused by oncogenic viruses.
- Primary immunodeficiency disorders are characterized by impaired humoral and/or cell-mediated immunity in persons, which include common variable immunodeficiency (CVID), hyper-IgE syndrome (Job syndrome), familial hemophagocytic lymphohistiocytosis, selective IgA deficiency, DNA repair disorders (e.g. ataxia-telangiectasia, Wiskott-Aldrich syndrome, Nijmegen breakage syndrome), severe congenital neutropenia (SCN), and severe combined immunodeficiency disease (SCID). The patients suffering from primary immunodeficiency disorders are linked malignancies.

Common Variable Immunodeficiency Disease

The hallmark of CVID disease is hypogammaglobulinemia due to impaired B cell differentiation. Patient presents with recurrent sinopulmonary recurrent infections, autoimmune disorders and granulomatous disease, who have increased risk of developing B cell non-Hodgkin's lymphomas in extranodal sites (especially in brain), gastric carcinoma, breast carcinoma, urinary bladder carcinoma, and cervical carcinoma. Defective immune system fails to eliminate transformed cancer stem cells. Immune dysregulation and oncogenic Epstein-Barr virus (EBV) infection probably are linked to B cell non-Hodgkin's lymphoma.

Hyper-IgE Syndrome (Job Syndrome)

Hyper-IgE syndrome (Job syndrome) is characterized by occurrence of recurrent boils, eczema, and pneumonitis in those patients, who have very high level of serum IgE. A mutation in the signal transducer and the activator of the transcription 3 (STAT3) gene has been detected in the majority of cases. These patients are at high risk for B cell non-Hodgkin's lymphoma due to abnormalities in STAT3/IL-21-dependent differentiation of B cells into plasma cells with possible involvement of CD4+ helper T cells in the lymphoid follicles. Defective B cells play important role in the carcinogenesis, invasion and metastases of many cancers such as lung carcinoma and melanoma. Viral infection in these patients is associated with increased risk for cancer.

Familial Hemophagocytic Lymphohistiocytosis

Familial hemophagocytic lymphohistiocytosis (FHL) occurs due to defects in natural killer cells, and CD8+ cytotoxic T cells due to mutation in PRF1 gene resulting in a multisystem inflammatory disease with persistent fever and hepatosplenomegaly, pancytopenia and metabolic alterations. Mutations in PRF1 gene leads to reduced ability of immune cells to perform essential immunosurveillance roles against development of B cell non-Hodgkin's lymphoma in these patients.

Selective IgA Deficiency

Selective IgA deficiency is asymptomatic in many patients, who present with gastrointestinal system and respiratory system infections, atopy, autoimmune disorders, and later in life developing abdominal lymphoid and gastrointestinal malignancies. Recently, primary cutaneous marginal lymphoma with subsequent development of nodal marginal lymphoma has been reported in patients with selective IgA deficiency.

Ataxia-Telangiectasia

Ataxia-telangiectasia is an autosomal recessive disorder with DNA repair defect and characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia and dysarthria. Cells have reduced ability to activate cell cycle checkpoints following exposure to γ-radiation due to mutation in ATM gene. Normally, ATM protein acts as sensor of double-stranded DNA breakage. Mutations in ATM gene predisposes to leukemia, lymphoma and breast carcinoma, thought to be related to excessive production of DNA translocation.

Nijmegen Breakage Syndrome

Nijmegen breakage syndrome (NBS) is an autosomal recessive disorder with DNA repair defect closely related to ataxia-telangiectasia seen across world. The defective NBS gene is a component within the same pathway as ATM gene. Both NBS and ATM gene products are part of a multi-subunit complex involved in correcting γ-radiation induced chromosomal aberrations and repair of double-strand DNA breaks.

- Patients with Nijmegen breakage syndrome have severe immunodeficiency of both humoral (i.e. agammaglobulinemia, IgA, IgG2 deficiency) and cellular immunity (lymphopenia, decreased CD4+ helper T cells). Physical examination reveals microcephaly, short stature and bird-like face.
- Nijmegen breakage syndrome is linked to immune dysfunction and high-risk of developing non-Hodgkin's lymphoma (NHL), acute lymphoblastic leukemia (ALL), breast carcinoma and prostatic carcinoma.

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome is an X-linked primary immunodeficiency disorder with DNA repair defect due to mutation in Wiskott-Aldrich syndrome (WAS) gene located on short arm of X chromosome encoding WASp only expressed in hematopoietic cells. Patient presents with recurrent infections, microthrombocytopenia, eczema and increased incidence of autoimmune disorders, acute myelogenous leukemia (AML) and myelodysplastic syndrome (MDS).

Severe Congenital Neutropenia

Severe congenital neutropenia (SCN) occurs due to the arrest of myelopoiesis maturation at the promyelocyte/myelocyte stage, resulting in neutropenia with systemic neutrophil blood counts $<0.5 \times 10^9$/L. There is increased risk of developing myelodysplastic syndrome (MDS), acute myelogenous leukemia (AML), chronic myelomonocytic leukemia (CMML).

Molecular/Cytogenetic Mutations

Mutation in elastase 2 gene has been detected in 50–60% of severe congenital neutropenia cases. In addition, other gene mutations have been detected in severe congenital neutropenia, which include HAX1 gene encoding hematopoietic cell-specific Lyn substrate 1 (HCLS1), glucose-6-phosphatase, catalytic 3 (G6PC3), GATA2 gene and granulocyte colony-stimulating factor 3 receptor (G-CSF3R) gene.

- Heterozygous mutations in GATA2 gene causes disorders of hematopoiesis, lymphatics, and immune system.
- Normally, GATA2 is a zinc finger transcription factor, which is essential for differentiation of hematopoietic precursor cells, and regulation of phagocytosis by alveolar macrophages. Upregulation of GATA2 gene in alveolar macrophages increases phagocytic activity up to threefold.

Clinical Features

Patient with severe congenital neutropenia presents with **recurrent severe infections** due to HPV, Epstein-Barr virus (EBV), mycobacteria, invasive fungi infection and persistent neutropenia. Daily administration of granulocytic colony-stimulating factor (**G-CSF**) results in elevation of blood neutrophil counts to prevent recurrent severe infections. Patient can also develop human papillomavirus (HPV) positive malignant tumors (cervical carcinoma, vaginal carcinoma, penile carcinoma, anal carcinoma and oropharynx) and Epstein-Barr virus (**EBV**) positive tumors such as Burkitt's lymphoma, immunoblastic lymphoma, nasopharyngeal carcinoma, and gastric carcinoma.

ACQUIRED PREMALIGNANT DISEASES

Acquired premalignant diseases are associated with an increased risk for cancer resulting from persistent focal cell regeneration, atypical hyperplasia, metaplasia and dysplasia in different anatomic sites. Premalignant diseases linked to increased risk of development of cancer are given in Table 6.28.

- Acquired premalignant disease is a pathologic state in different anatomic sites, i.e. skin, gastrointestinal tract, hepatobiliary system, respiratory system, female genital system, skeletal system, urinary system, and thyroid gland, that progresses directly to cancer disease without a known intermediate step that can help in providing detail about the dynamic carcinogenesis process, before development of clinical cancer disease.
- Although the cell-of-origin of cancer is difficult to pinpoint cancer clones, which harbor information about their clonal ancestries. In an effort to detect premalignant cells before they evolve into life-threatening cancer disease, clinicians currently diagnose premalignant diseases at frequencies that substantially exceed those of clinical cancers.
- Cancer risk prediction relies on our ability to distinguish between premalignant diseases and cancer malignant tumors show marked heterogeneity in their cellular morphology and clonal architecture, e.g. adenoma-carcinoma paradigm in colorectal carcinoma progression, and Barrett's metaplasia-dysplasia-carcinoma sequence.
- It is difficult to obtain tissue sampling, detect and diagnose precursor lesions in ovary and pancreas. Premalignant diseases do not invariably progress to malignant phenotype, because some premalignant lesions are detected on screening procedure, which are treated, thereby decreasing risk of developing malignant tumor. This is one of the rationales behind the screening programmes.

Acquired Precursor Lesions Linked to Cancer

Term 'acquired precursor lesions' encompasses several entities that are linked to cancer, which include persistent regeneration of cells, atypical epithelial hyperplasia, metaplasia and dysplasia in various anatomic sites.

Table 6.28 Premalignant diseases linked to increased risk of development of cancer

Conditions	Premalignant Conditions	Human Cancers
Persistent focal regeneration of epithelial cells and fibrosis	Cirrhosis	Hepatocellular carcinoma
Atypical hyperplasia of epithelial cells	▪ Atypical hyperplasia of epithelial cells lining fistulous tract in anal region ▪ Atypical endometrial hyperplasia ▪ Atypical ductal/lobular hyperplasia	▪ Squamous cell carcinoma of skin ▪ Endometrial carcinoma ▪ Ductal/lobular carcinoma of breast
Metaplasia of epithelial cells	Barrett's esophagus	Esophageal adenocarcinoma
Dysplastic change in epithelial cells	▪ Cervical intraepithelial neoplasia III (CIN-III) ▪ Dysplastic nevus ▪ Bronchial dysplasia ▪ Pancreatic intraepithelial neoplasia	▪ Squamous cell carcinoma of cervix ▪ Malignant melanoma ▪ Bronchogenic carcinoma ▪ Pancreatic adenocarcinoma
Chronic inflammation	▪ Atrophic gastritis ▪ Ulcerative colitis ▪ *Opisthorchis viverrini*-induced cholangitis ▪ Cholelithiasis ▪ *Helicobacter pylori*-induced chronic gastritis ▪ HBV and HCV-induced hepatitis ▪ Discharging sinuses of skin	▪ Gastric adenocarcinoma ▪ Colonic adenocarcinoma ▪ Cholangiocarcinoma ▪ Gallbladder adenocarcinoma ▪ Gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma ▪ Hepatocellular carcinoma ▪ Squamous cell carcinoma of skin

Persistent Regeneration of Cells in Organ(s) Linked to Cancer

In Crohn's disease, a fistula develops when characteristic inflammation occurs in the gastrointestinal tract so that an ulcer forms, that spreads through the intestinal wall and then bores a hole through muscle and skin near the anus. Persistent regeneration of cells results in development of squamous cell carcinoma in the margins of a chronic skin fistula or in delayed skin wound healing. Persistent regeneration of hepatocytes in cirrhosis can result in hepatocellular carcinoma.

Atypical Epithelial Hyperplasia Linked to Various Cancers in Females

Atypical ductal hyperplasia is a relatively common lesion demonstrated in 5–20% of breast biopsies. It is classified as a high-risk precursor lesion due to its association with progression to ductal carcinoma *in situ* and invasive ductal carcinoma of breast.

- Endometrial atypical hyperplasia thickens the epithelial lining of uterus with resultant increase in gland to stroma ratio, causing dysfunctional bleeding. Excessive circulating estrogens (exogenous administration or ovarian granulosa cell tumor and thecoma) may induce endometrial atypical hyperplasia (precursor lesion) leading to development of endometrial carcinoma. Histologic examination of endometrial atypical hyperplasia reveals closely packed endometrial glands of variable sizes, irregular luminal contours with glands to stroma >3:1 ratio.
- Excess diethylstilbestrol intake by pregnant mother can cause vaginal adenosis (precursor lesion) in the daughter, who has great risk of developing vaginal adenocarcinoma.

Metaplasia in Organ(s) Linked to Cancers

Many acquired precursor lesions arising in the settings of chronic inflammation can progress to malignancies of esophagus, stomach and lung. Acquired precursor lesions can be recognized by the presence of glandular metaplasia of distal esophagus in Barrett's esophagus in response to gastroesophageal reflux disease (GERD), *Helicobacter pylori*-induced gastric glandular metaplasia due to chronic atrophic gastritis, squamous metaplasia in bronchi in tobacco smokers, and squamous metaplasia in the urinary bladder due to *Schistosoma haematobium* infection.

Dysplasia in Organ(s) Linked to Cancers

Dysplasia is a term used to describe the presence of cytologically abnormal cells confined within their original basement membrane, which can be mild, moderate or severe, depending on how abnormal the cells look under a light microscope and how much of the tissue or organ is affected. Squamous severe dysplasia/carcinoma *in situ* of oropharynx, larynx, and uterine cervix may undergo squamous cell carcinoma. Bronchogenic carcinoma develops in the dysplastic bronchial mucosa in chronic cigarette smokers. Patients with dysplastic nevus may develop malignant melanoma.

Benign Tumors Undergoing Malignant Transformation

Colorectal adenomatous polyps (e.g. tubular adenoma, villous adenoma or tubulovillous adenoma), and familial adenomatous polyposis (**FAP**) are linked to **colorectal carcinoma**. Mortality can be prevented by performing colonoscopy or virtual colonoscopy for several years to eliminate adenomatous polyps in order to reduce risk of colorectal carcinoma and its consequences.

Chronic Inflammation Linked to Cancers

In chronic inflammation, chemical mediators exert pleiotropic effects in the development of cancer. Chronic inflammation in the microenvironment surrounding premalignant (precursor) lesion favors carcinogenesis, transformation of normal cell to cancer stem cell, tumor growth, invasion and metastasis to distant organ(s). Microenvironment surrounding precursor lesion is composed of immune cells, myoepithelial cells, fibroblasts, blood vessels, and extracellular matrix (ECM). Chronic inflammation can stimulate immune system that might limit carcinogenesis. Chronic inflammation and their related cancers in various anatomic sites are given in Table 6.29.

Table 6.29 Chronic inflammation and their related cancers in various anatomic sites

Premalignant Conditions or Lesions	Development of Cancer
Skin disorder linked malignancies	
Acinic (solar) keratosis	Squamous cell carcinoma of skin
Chronic irritation at sinus orifice	Squamous cell carcinoma of skin
Third degree burns	Squamous cell carcinoma of skin
Dysplastic nevus	Malignant melanoma of skin
Xeroderma pigmentosum	Squamous cell carcinoma and basal cell carcinoma of skin

Contd...

Table 6.29 Chronic inflammation and their related cancers in various anatomic sites (*Contd...*)

Premalignant Conditions or Lesions	Development of Cancer
Head and neck including oral cavity disorder linked malignancies	
Leukoplakia oral cavity	Squamous cell carcinoma of oral cavity
Proliferative verrucous leukoplakia	Squamous cell carcinoma of oral cavity
Oral submucosal fibrosis	Squamous cell carcinoma of oral cavity
Lichen planus oral cavity	Squamous cell carcinoma of oral cavity
Thyroid gland disorder linked malignancies	
Autoimmune disorders (Hashimoto's thyroiditis or Sjögren syndrome)	Mucosa-associated lymphoid tissue (MALT) lymphoma
Hashimoto's thyroiditis	Papillary thyroid carcinoma
Gastrointestinal tract disorder linked malignancies	
Leukoplakia oral cavity (caused by ingestion of tobacco products)	Squamous cell carcinoma of oral cavity
Dysplasia of oropharynx	Squamous cell carcinoma of oropharynx
High-grade dysplasia (chronic atrophic gastritis)	Gastric adenocarcinoma
Barrett's esophagus (glandular metaplasia of esophagus induced by reflux esophagitis: high-grade dysplasia)	Esophageal adenocarcinoma
Plummer-Vinson syndrome	Esophageal carcinoma
Helicobacter pylori-induced chronic atrophic gastritis	Gastric adenocarcinoma (distal-half region), mucosa-associated lymphoid tissue (MALT) lymphoma and carcinoid tumors
Inflammatory bowel disease by gut pathogens (ulcerative colitis/ Crohn's disease: adenoma—high-grade dysplasia)	Colorectal carcinoma and bile ductal adenocarcinoma
Villous and tubulovillus adenoma	Colon carcinoma
Exocrine pancreas disorder linked malignancies	
Pancreatitis (induced by chronic alcoholism)	Pancreatic adenocarcinoma
Pancreatic intraepithelial neoplasia (alcoholism and germline mutation in trypsinogen gene)	Pancreatic adenocarcinoma
Hepatobiliary system disorder linked malignancies	
Persistent regeneration and repair forming nodule in liver	Hepatocellular carcinoma
Cirrhosis	Hepatocellular carcinoma
Hepatotropic viruses (HBV, HCV) infection	Hepatocellular carcinoma
Chronic cholecystitis with cholelithiasis (bile duct bacteria and cholelithiasis)	Gallbladder carcinoma
Opisthorchis cholangitis (liver fluke)	Cholangiocarcinoma and colon carcinoma
Respiratory system disorder linked malignancies	
Squamous dysplasia of larynx and bronchi	Squamous cell carcinoma of larynx and squamous cell lung carcinoma
Chronic bronchitis induced by tobacco smoke	Lung carcinoma
Atypical ductal hyperplasia	Lung carcinoma
Asbestosis	Mesothelioma and lung carcinoma
Female genital system disorder linked malignancies	
Vaginal adenosis in daughter (diethylstilbestrol intake by pregnant mother)	Vaginal adenocarcinoma (in the daughter)
Lichen sclerosus of vulva	Vulvar squamous cell carcinoma
Severe dysplasia, i.e. cervical intraepithelial neoplasia III (CIN-III) induced by human papillomavirus (HPV 16, 18)	Cervical carcinoma
Endometrial hyperplasia (estrogen induced)	Endometrial adenocarcinoma
Endometriosis in ovary	Ovarian endometrioid carcinoma
Hydatidiform mole	Choriocarcinoma

Contd...

Table 6.29 Chronic inflammation and their related cancers in various anatomic sites (*Contd...*)

Premalignant Conditions or Lesions	Development of Cancer
Female breast disorder linked malignancies	
Atypical ductal hyperplasia of breast	Breast carcinoma
Sclerosing adenosis of breast	Breast carcinoma
Small duct papilloma of breast	Breast carcinoma
Skeletal system infection linked malignancies	
Chronic bacterial osteomyelitis discharging sinuses	Squamous cell carcinoma in regions of draining sinuses
Urinary bladder disorder linked malignancies	
Chronic cystitis (*Schistosoma haematobium* infection)	Urinary bladder carcinoma
Hematological disorder linked malignancies	
Myelodysplastic syndrome (MDS)	Acute leukemia
Monoclonal gammopathy of unknown significance (MGUS)	Multiple myeloma
Autoimmune disorders (Sjögren syndrome)	MALT lymphoma

Metaplastic and hyperplastic cells become dysplastic before progressing to cancer

- **Initiation of tumorigenesis:** In the initial phase of tumorigenesis, chemical mediators of chronic inflammation such as cytokines (e.g. IL-1, IL-6, TNF, PGSH-2), reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) derived from immune cells in precursor lesion microenvironment induce epigenetic alterations, which silence tumor suppressor genes. Self-sufficiency in growth signals and insensitivity to tumor suppressor gene signals induce genomic instability in precursor lesion parenchymal cells. Limitless replicative potential and evading apoptosis contribute to sustained proliferation and survival of premalignant cells.
  - Chronic inflammation induces production of transcription factors such as nuclear factor kappa-B (**NF-κB**), STAT-3, and hypoxia-inducible factor 1 (HIF-1) and accumulation of tumor-promoting factors modulated by chemical mediators in the microenvironment surrounding precursor lesions. NF-κB and STAT-3 interact at multiple levels leading to carcinogenesis.
  - Nuclear factor kappa-B (NF-κB) is a transcription factor, that regulates inflammation-related genes, such as inducible nitric oxide synthase (**iNOS**) and cyclooxygenase-2 (**COX-2**) leading to production of proinflammatory cytokines in the premalignant tissue environment resulting in proliferation and survival of precursor cells. Nuclear factor kappa-B (**NF-κB**) also induces production of chemokines that attract additional immune cells/inflammatory cells to sustain tissue microenvironment-associated inflammation.
- **Promotion of tumorigenesis:** During tumor promotion, immune cells secrete cytokines and chemokines, that act to stimulate proliferation of transformed cells and their survival. The '**angiogenic switch**' is essential for an adequate supply of nutrients, oxygen and survival factors to transformed cells. Angiogenesis induces alterations in the cells essential for invasion and metastasis. Angiogenesis, localized immunosuppression, and favorable microenvironment lead to proliferation and survival of transformed cells, and accumulation of additional gene mutations and epigenetic modifications. Eventually, chronic inflammation also promotes metastatic dissemination to organ(s).
- **Progression of tumorigenesis:** During tumor progression and metastasis, CSCs and immune cells of tumor microenvironment secrete cytokines and chemokines leading to an increase in cell survival, motility and invasiveness. Epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) are crucial processes involved in tumor invasiveness and metastasis to distant organs(s).

Cervical Intraepithelial Neoplasia III (CIN-III) Linked to Cervical Carcinoma

Human papillomavirus (HPV 16 or HPV 18) is the most common viral infection of the reproductive tract transmitted through sexual contact, that plays important role in cervical carcinogenesis. Protein products (E6 and E7) of the HPV viral genome inhibit the function of two important suppressors; retinoblastoma protein (pRB) and TP53 (p53) protein.

- Cervical intraepithelial neoplasia (CIN) has a proliferating epithelium, which has three grades: (a) CIN-I (mild dysplasia), (b) CIN-II (moderate dysplasia), and (c) CIN-III (severe dysplasia/carcinoma *in situ*). Morphologic features of cervical epithelial neoplasia III (CIN-III) include abnormal pleomorphic hyperchromatic nuclei, loss of nuclear polarity and differentiation, and numerous mitotic figures indicating extensive cell proliferation. Screening of cervical intraepithelial neoplasia and its treatment can prevent development of cervical carcinoma.
- With reference to the development of cervical carcinoma, cervical intraepithelial neoplasia precursor lesions can be detected by cytologic sampling (cervical smears/scrapes or liquid-based cytology stained with Papanicolaou stain), and treated effectively, preventing progression to invasive cervical carcinoma—as a part of cervical screening programmes.
- The basis of successful cervical carcinoma prevention programmes is excision or destruction of the transforming zone of the the cervix, where almost all cervical carcinomas arise after histologic confirmation of precursor lesion.

Barrett's Esophagus Linked to Esophageal Adenocarcinoma

Development of esophageal adenocarcinoma in Barrett's esophagus occurs as a result of metaplastic change in lining epithelium of distal esophagus. Metaplasia is provoked by gastroesophageal reflux disease (GERD), i.e. chronic acid reflux from the stomach can become a precursor lesion to an ulcerated esophageal adenocarcinoma. In Barrett's esophagus, the squamous cells that normally line the residual squamous mucosa of esophagus are replaced by secretory cells that migrate from the lining of the stomach.

Leukoplakia and Erythroplakia of the Oral Mucosa Linked to Oropharyngeal Carcinoma

Leukoplakia (dysplastic white patch in mouth) and erythroplakia (dysplastic red region in the oropharynx that bleeds easily) are the two most common potentially malignant disorders of the oral mucosa in persons due to tobacco smoking and consumption of chewing tobacco. The acquired precursor lesions can also develop in badly fitting dentures that constantly rub gums or the inside of mouth or tongue. The prognosis and overall survival of patients with oral carcinoma is dependent on the early detection of precursor lesion that might identify high-risk patients before development of metastatic disease.

INTERACTIONS BETWEEN GENETIC AND ENVIRONMENTAL FACTORS LINKED TO INHERITED CANCER SYNDROMES

Mounting evidence supports the concept that inherited cancer syndromes are largely attributable to environmental factors or acquired pre-malignant diseases. Most inherited cancer syndromes are associated with a 'germline mutation' in tumor suppressor genes, which have entered the zygote via the egg or spermatozoa and is consequently present in every cell of the human body.

- Every person has two copies of tumor suppressor genes, i.e. one from each parent (father and mother). Most individuals are born with two normal copies of each gene. When a person inherits a copy of the faulty gene from the mother or father, cell contains one functional copy and one mutated copy of tumor suppressor gene, and the cell still works.
- According to 'Knudson' two-hit hypothesis, two-hit mutations within the genome are essential for a malignant phenotype to develop. Since one-hit mutation is already present in every cell from germline mutation in tumor suppressor gene since birth, and only one additional hit to the remaining functional copy (allele) of the tumor suppressor genes is necessary to develop malignant phenotype. On the contrary, two-hit mutations in two copies of tumor suppressor gene occurs within a single somatic cell after birth. Cancers have a tendency to demonstrate karyotypic abnormalities such as chromosomal translocation, deletions and gene amplifications.
- The inherited cancer syndromes fall into two categories: autosomal dominant and autosomal recessive disorders.
- Most inherited cancer syndromes follow autosomal-dominant inheritance, in which the patient's first-degree relatives (parents, children and siblings) have a 50% risk of carrying the causative gene mutation themselves. In these cases, one faulty allele of gene has to be present for an individual to have a predisposition to cancer.
- Individuals with one normal allele and one faulty allele of the gene are known as **heterozygous**. The detrimental effects of recessive cancer gene are less common within population as compared to their dominant cancer gene counterparts.
- Homozygous inheritance of these recessive cancer genes does not cause cancer directly, but rather, the loss of functional protein that gene encodes causing genomic instability, which provides an environment ideal for oncogenic mutations and predisposes the affected person to cancer.

Table 6.30 Comparison of inherited and acquired (sporadic) cancers

Characteristics	Inherited Cancers with Germline Mutations	Acquired (Sporadic) Cancers
Incidence of cancers (%)	Incidence—10%	Incidence—90%
Gene mutation	Born with germline gene mutations (first hit) in all the cells; and second hit in gene copy in hereditary tumor	During lifetime acquired gene mutation and both hits in sporadic tumor but none in the blood
Family tree inheritance pattern	Present	Absent
Human cancers	Multiple cancers in same individual	Solitary sporadic cancer
Age group affected with cancer	Younger persons	Elderly persons

Table 6.31 Inherited cancer syndromes

Inherited Cancer Syndromes	Comments
Mendelian hereditary cancer syndrome	▪ Autosomal dominant disorder (adult) ▪ Autosomal recessive disorder (children)
Defective DNA repair-linked hereditary cancer syndrome	▪ Autosomal dominant disorder ▪ Autosomal recessive disorder
Familial cancers	▪ Relative at cancer risk (breast, ovary, prostate) ▪ Transmission unclear ▪ Genetic testing

- Extensive clinical history of screening of early development of cancers is required in the management of these patients developing colorectal cancer, breast carcinoma, Wilms' tumor, or renal cell carcinoma. Molecular genetic testing is done to detect inherited cancer-causing gene in CSCs as well as tissues of family members. Comparison of inherited and acquired (sporadic) cancers is given in Table 6.30. Inherited cancer syndromes are given in Table 6.31.

Pathology Pearls: Medical Genetics

- All diseases have a genetic component, which do not require a medical genetic consultation.
- **Germline mutations** affect egg cells or spermatozoa and are transmitted via mitosis to the daughter cells.
- **Somatic mutations** occur in individual body cells, but not in germ cells, and therefore, somatic mutations are not transmitted to progeny.
- **Autosomal dominant inheritance** occurs when a single copy of a gene mutation on an autosomal chromosome is sufficient to cause disease that can be passed down through families to their 50% of children.
- **Autosomal recessive inheritance** has a risk of mutations for 25% of siblings.
- **Penetrance** is defined as the proportion of mutation carriers who harbor any manifestations of a disease.
- **Single gene mutation** in inherited cancer syndrome has high but incomplete penetration.
- **Mutation carriers** are at high risk of developing malignancies. Some mutation carriers do not develop malignancy.
- All persons with a gene mutation will not develop the same manifestations. For example, BRCA1 gene mutation in mother patient develops ovarian carcinoma and daughter develops breast carcinoma. Von Hippel-Lindau (**vHL**) syndrome has variety of systemic manifestations, but all patients will not have same organ involvement.
- **Down syndrome** child has increased risk of developing acute lymphoblastic leukemia (**ALL**) and acute myeloblastic leukemia (**AML**). **Klinefelter syndrome** patient presents with male sterility, who has increased risk for developing breast carcinoma. **Gonadal dysgenesis** with male genotype is more likely to develop tumors of gonads.

Clinical Pearls: Genomic Instability Linked to Inherited Cancer Syndromes

- Usually, multiple ≥2 family members suffer from same type of cancer or cancers are related to hereditary cancer syndromes.
- Multiple generations of related affected family members include mother, daughter and aunt.
- Family members develop cancer at younger age than sporadic cancers developing in elderly persons. Rarely, males may also develop cancer (breast cancer) related to hereditary cancer syndrome.
- Multiple primary cancers can occur in the same individual (i.e. breast and ovarian cancers in the same women).
- Physical findings that may suggest a hereditary syndrome, e.g. Peutz-Jeghers syndrome is characterized by excessive melanin pigment deposits on the skin around oral cavity, and gastrointestinal hamartomatous polyps.

Autosomal Dominant Inheritance Linked to Inherited Cancer Syndromes

Most inherited cancer syndromes follow autosomal dominant inheritance in which the patient's first-degree relatives (parents, children and siblings) have a 50% risk of carrying the causative mutations themselves. These cancers are caused by '**germline mutation**' which has entered the zygote via the egg or spermatozoa and is consequently present in every cell of the human body later. Autosomal dominant inherited cancer syndromes are given in Table 6.32.

BRCA1 and BRCA2 Gene Mutations and Associated Inherited Breast and Ovarian Cancers

BRCA1 and BRCA2 genes are highly penetrant breast cancer susceptibility genes linked to high-risk of

Table 6.32 Autosomal dominant inherited cancer syndromes

Inherited Cancer syndrome(s)	Mutated Tumor Suppressor Gene(s)	Associated Tumors
Inherited cancer syndromes involving breast and ovaries		
Breast and ovarian cancer syndrome	BRCA1 gene (17q21) and BRCA2 gene (13q12) encode DNA repair proteins and regulation of cell division	Breast carcinoma, ovarian carcinoma, prostatic carcinoma, fallopian tube carcinoma
Site-specific breast and ovarian cancer syndrome	BRCA2 gene (13q12) (encoding DNA repair proteins)	Breast carcinoma, ovarian carcinoma, prostatic carcinoma, pancreatic carcinoma
Breast carcinoma	PALB2 gene (partner and localization of BRCA1 gene)	Breast carcinoma, pancreatic carcinoma, ovarian carcinoma
Inherited cancer syndromes involving nervous system		
Hereditary retinoblastoma	RB gene (13q14.2) encodes pRB that regulate cell cycle pathway	Retinoblastoma (most often bilateral), osteosarcoma
Inherited cancer syndromes involving gastrointestinal tract		
Familial adenomatous polyposis (FAP) with >100 polyps in colon	APC gene (5q21)	Colon carcinoma, Gardner's syndrome (multiple adenomatous polyposis + desmoid tumors), Turcot syndrome (multiple polyps + primary CNS tumors (medulloblastoma, astrocytoma and ependymoma)
Hereditary nonpolyposis colorectal cancer (HNPCC)	DNA mismatch repair and cell cycle regulation genes such as MLH1 gene (3p21), MSH2 gene (2p15), MSH3 gene (5q11.12), MSH6 gene (2p16), PMS1 gene (2p32), PMS2 gene (7p22)	Colon carcinoma, endometrial carcinoma, gastric carcinoma, urothelial carcinoma
Juvenile polyposis coli (multiple polyps in colorectal region)	DPC4/SMAD4 gene, BMPR1A gene	Colorectal carcinoma, other gastrointestinal cancers
Hereditary diffuse gastric cancer	CDH1 gene encodes proteins regulating cell adhesion pathway	Gastric carcinoma
Hereditary gastrointestinal stromal tumors	KIT gene (4q12)	Gastrointestinal stromal tumors (GISTs)
Peutz-Jeghers syndrome	LKB1/STK11 gene (19p13.3)	Hamartomatous polyps in stomach and intestine, adenocarcinoma gut
Oligodontia-colorectal cancer syndrome	AXIN2 gene (17q23-24)	▪ Gastrointestinal polyposis ▪ Early-onset colorectal cancer and/or breast cancer ▪ Medulloblastoma
Inherited cancer syndromes involving skeletal system		
Hereditary multiple osteochondromas (previously called hereditary multiple exostoses)	▪ EXT1 gene (8q24.1) ▪ EXT2 gene (11p11–13) ▪ EXT3 gene (19p)	Osteochondroma can transform to osteochondrosarcoma

Contd...

Table 6.32 Autosomal dominant inherited cancer syndromes (*Contd...*)

Inherited Cancer syndrome(s)	Mutated Tumor Suppressor Gene(s)	Associated Tumors
Hyperparathyroidism jaw-tumor syndrome	HRPT2 gene also called CDC73 (1q25–q31)	Parathyroid adenoma, parathyroid carcinoma, ossifying fibroma of the maxilla and mandible, renal tumors, uterus tumors
Inherited cancer syndromes involving kidney		
Hereditary papillary renal cell carcinoma (HPRCC)	MET gene (4q12)	Hereditary papillary renal cell carcinoma
Familial Wilms' tumor	WT (11p13) regulate cell cycle and transcription	Wilms' tumor (nephroblastoma)
Von Hippel-Lindau (vHL) disease	vHL gene (3p25) encodes multifunctional protein involved in hypoxia response	Hemangioblastoma (brain, spinal cord and retina), pheochromocytoma, multiple cysts in kidney, pancreatic endocrine tumors, reproductive tract, renal cell carcinoma arising in renal cyst
WAGR, Denys-Drash syndrome, Frasier syndrome and non-syndromic hereditary Wilms' tumor	WT1 gene (11p13)	Wilms' tumor (nephroblastoma)
Hereditary leiomyomatosis and renal cell carcinoma (HLRCC)	FH (fumarate hydratase) gene (1q43)	Leiomyoma (skin, uterus), renal cell carcinoma
Inherited cancer syndromes involving endocrine system		
Hereditary paraganglioma-pheochromocytoma syndrome	SDHA gene (5p15), SDHB gene (1p35–36.1), SDHC gene (1q21–23), SDHD (11q23) gene	Multiple bilateral paragangliomas arising from neuroendocrine tissues along the spine from the base of the skull to the pelvis; pheochromocytoma (paraganglioma) confined to adrenal gland
Multiple endocrine neoplasia 1 (MEN1)	MEN1 gene (11q13) encodes 'Menin' protein	Pancreatic islet neoplasms (glucagonoma and VIPoma), pituitary adenomas (e.g. prolactinoma and growth hormone producing adenoma, parathyroid hyperplasia/adenoma)
Multiple endocrine neoplasia 2 (MEN2A, MEN2B and familial medullary thyroid carcinoma)	RET gene (10q11.2) encodes RET receptor tyrosine kinase	Medullary carcinoma of thyroid, pheochromocytoma (germline mutation), parathyroid hyperplasia
Inherited cancer syndromes involving skin		
Familial melanoma	CDKN2A gene (p16/INK4A, p14/ARF) on 8p21.3, CDK4 gene (4q12), TERT gene (5p15.32), POT1 gene (7q31.33)	Cutaneous malignant melanoma
Xeroderma pigmentosum	Xp group of genes	Squamous cell carcinoma of skin, malignant melanoma
Familial atypical multiple mole melanoma	CDNK2A (p16/INK4A, p14/ARF) on 8p21.3	Cutaneous melanoma, pancreatic carcinoma
Basal cell nevus syndrome (also called Gorlin-Goltz syndrome)	PTCH1 gene (9q22.3), PTCH2 gene (1p34.1), SUFU gene (10q24.32)	Multiple nevoid basal cell carcinomas
Inherited cancer syndromes involving multiple sites		
Li-Fraumeni syndrome	TP53 gene (17p13.1) encodes p53 protein that regulate cell cycle pathway	Breast carcinoma, osteosarcoma, soft tissue sarcoma, brain tumors, acute leukemia
Carney complex type 1	PRKARIA gene (17q24.2)	Skin myxoma, cardiac myxoma, breast myxoma, testicular neoplasms, thyroid carcinoma

Contd...

Table 6.32 Autosomal dominant inherited cancer syndromes (*Contd...*)

Inherited Cancer syndrome(s)	Mutated Tumor Suppressor Gene(s)	Associated Tumors
Cowden syndrome part of PTEN hamartomatous syndrome (features overlap with Bannayan-Riley-Ruvalcaba syndrome)	PTEN gene (10q23) encodes protein (P13K antagonist)	Colorectal carcinoma, breast carcinoma, thyroid carcinoma, squamous cell carcinoma of skin, endometrial carcinoma, renal cell carcinoma, melanoma
Werner syndrome	WRN	Soft tissue sarcoma, thyroid carcinoma, melanoma of skin, osteosarcoma
Ataxia-telangiectasia	ATM	Lymphoma, leukemia, breast carcinoma
Brooke-Spiegler syndrome (familial cylindromatosis)	CYLD gene (16q12)	Skin adnexal tumors, salivary gland benign/malignant tumors, colon polyps, colon carcinoma
Birt-Hogg-Dube syndrome	FLCN/BHD gene (17p11.2)	▪ Diverse renal tumors (chromophobe oncocytoma hybrid renal cell carcinoma) ▪ Skin fibrofolliculoma ▪ Pulmonary cysts
Inherited cancer syndromes involving skin and nervous system		
Neurofibromatosis type 1 (can be associated with Noonan syndrome and Watson syndrome)	NF1 gene (15q13) encodes neurofibromin is a negative regulator of RAS protein	Malignant peripheral nerve sheath tumor (neurofibrosarcoma), astrocytoma, malignant melanoma, neurofibroma, optic nerve glioma
Neurofibromatosis type 2	NF2 gene (22q12.2) encodes cytoskeleton protein	Bilateral vestibular schwannoma, meningioma
Tuberous sclerosis and lymphangioleiomyomatosis (LAM)	▪ TSC1 gene (9q34.13) encodes hamartin ▪ TSC2 gene (16p13.3) encodes tuberin (both TSC1 and TCS2 form mTOR-inhibiting complex, which is disrupted by mutation)	CNS cortical hamartomas, skin subungual keratomas, renal angiomyolipoma

hereditary breast cancer (female or male) and ovarian cancer (HBOC) in women in an autosomal dominant inheritance pattern including cancers of fallopian tube and peritoneum, and to a lesser extent other cancers of prostate, pancreas and melanoma.

- Mammography, breast self-examination, regular clinical examinations and annual mammography and magnetic resonance imaging (MRI) help in early detection of breast cancer.
- Annual transvaginal ultrasonography and blood CA-125 levels may be considered for ovarian cancer screening. Molecular genetic testing of BRCA1 and/or BRCA2 genes is performed to for breast cancer screening in women. Women with BRCA1 and/or BRCA2 gene mutations could consider prophylactic bilateral mastectomy or oophorectomy as a primary surgical management due to high rate of ipsilateral and contralateral breast cancer.

Phosphatase and Tensin Homolog Linked to Hamartomatous Syndrome

Patients with phosphatase and tensin homolog (**PTEN**), a tumor suppressor gene mutation (10q23) has increased risk of breast carcinoma, thyroid carcinoma, endometrial carcinoma, colorectal carcinoma, renal cell carcinoma and melanoma.

Li-Fraumeni Syndrome

Germline mutation in TP53 gene (aberrant p53) in Li-Fraumeni syndrome (LFS) is linked to diverse spectrum of childhood and adult-onset malignancies, i.e. breast carcinoma, osteosarcoma, soft tissue sarcoma, brain tumors, acute leukemia, adrenocortical carcinoma, gastrointestinal cancers, lung carcinoma, renal cell carcinoma, thyroid carcinoma, ovarian carcinoma, testicular carcinoma, prostatic carcinoma, and skin cancer.

- It is important to note that not every person with mutation in TP53 gene will necessarily develop cancer, but the risks are substantially higher than the general population.
- The lifetime of cancer risk in persons with Li-Fraumeni syndrome is ≥70% for men and ≥90% for women. Diagnosis of Li-Fraumeni syndrome depends on clinical manifestations and/or genetic testing for mutation in the TP53 gene. Molecular genetic testing of germline mutation in TP53 tumor suppressor gene is performed for early detection of cancer in these patients.

Hereditary Retinoblastoma

Hereditary retinoblastoma is an autosomal dominant disorder caused by germline mutation in RB1 tumor suppressor gene mapped on chromosome 13q14.2 with

subsequent somatic inactivation of the other allele of RB1 gene. Tumor has incomplete penetrance in 90% of cases. Patient presents with multifocal and/or bilateral retinoblastoma with an anticipation of the mean age of diagnosis within the first year of life of child. Patient presents with white pupillary reflexes (leukocoria) in one or both eyes or strabismus, glaucoma, inflammation and poor visual tracking. Molecular genetic testing of germline mutation in RB tumor suppressor gene is performed for early detection of retinoblastoma in these patients.

Familial Adenomatous Polyposis

Familial adenomatous polyposis (FAP) is an autosomal dominant disorder caused by germline mutation in APC tumor suppressor gene mapped on chromosome 5q21 that encodes aberrant APC protein in cells of colon. Both copies of the APC gene are mutated in 80% of sporadic colorectal tumors.

- Patients with FAP develop numerous potentially precancerous tubular adenomas (>100–1000 polyps) in the rectosigmoid region during second decade of life (50%) and by 35 years (90%) of life.
- Patients can later develop adenomatous polyps in the periampullary region of duodenum, which can progress to colorectal cancers within 10–20 years.
- About 10% have a family history of polyps and/or colorectal carcinoma at younger age. Nonspecific symptoms such as unexplained rectal bleeding (hematochezia), diarrhea and abdominal pain in young patients may be suggestive of familial adenomatous polyposis.
- Preventive surgery is the standard treatment of FAP. Until the colon is surgically excised, these noncancerous polyps will become malignant. Surgical specimen of familial adenomatous polyposis coli is shown in Fig. 6.60.

Fig. 6.60: Surgical specimen of FAP. FAP coli is an autosomal dominant disorder in which numerous adenomatous polyps (>100–1000 in number) form mainly in the epithelium of the large intestine.

Attenuated Familial Adenomatous Polyposis

Attenuated familial adenomatous polyposis (AFAP) is an autosomal dominant disorder due to germline mutation in APC tumor suppressor gene located on chromosome 5q21 that encodes aberrant protein in every cell in their colon. Patient develops multiple adenomatous polyposis (<100 polyps) in colon, upper gastrointestinal tract (fundus, duodenum), colorectal carcinoma (70% risk), hepatoblastoma, gastric carcinoma and breast carcinoma.

Polymerase Proofreading-associated Polyposis

Polymerase proofreading-associated polyposis (PRAP) is an autosomal dominant disorder caused due to germline mutations in POLE (12q24.3) and POLD1 (19q13.33) genes, that encode aberrant proteins leading to mismatch DNA repair. Germline mutations in POLE and POLD1 genes are linked to multiple adenomatous polyposis in colon (number of polyps unknown), colorectal carcinoma, ovarian carcinoma, endometrial carcinoma, pancreatic carcinoma and brain malignancies.

Hereditary Nonpolyposis Colorectal Cancer

Hereditary nonpolyposis colorectal cancer (HNPCC), also known as Lynch syndrome (described by Dr. Henry Lynch) is an autosomal dominant disorder, that is associated with high-risk HNPCC in the region proximal to splenic flexure in 70–85% of cases, which is most often preceded by serrated adenomas (pedunculated with broad-based polypoid pattern of growth), rather than tubular adenomas as seen in the traditional–APC carcinoma pathway within three years due to germline mutations versus 8–10 years for sporadic adenoma-carcinoma sequence.

- **Molecular genetic testing** in hereditary nonpolyposis colorectal cancer reveals mutation in one of the mismatch repair genes such as MLH1 gene (3p21), MSH2 gene (2p15), MSH3 gene (5q11–12), MSH6 gene (2p16), PMS1 gene (2p32) and PMS2 gene (7p22). Immunohistochemical analysis can help to pinpoint the affected mismatch repair gene.
- Hereditary nonpolyposis colorectal cancer (HNPCC) associated additional malignancies include colon carcinoma (most common), endometrial carcinoma (40–60%), ovarian carcinoma (12–15%), gastric carcinoma, pancreatic carcinoma, hepatobiliary carcinoma, urothelial carcinoma of renal pelvis and ureter, brain tumors and Muir-Torre syndrome (e.g. sebaceous adenomas, carcinoma and multiple keratoacanthoma) and visceral malignancy due to alterations in microsatellite nucleotide sequences.
- Hereditary nonpolyposis colorectal cancer is treated by surgical interventions such as subtotal colectomy

Table 6.33 Cardinal features of hereditary nonpolyposis colon carcinoma (HNPCC) are also called Lynch syndrome

Parameters	Features
Mode of inheritance	Autosomal dominant inheritance
Colorectal carcinoma (mean age)	45 years
Progression period from adenoma to colorectal carcinoma	About three years due to germline mutations (versus 8–10 years for sporadic adenoma-carcinoma)
Colorectal carcinoma site	70–85% proximal to splenic flexure
Risk of development of additional colorectal carcinoma since subtotal colectomy	25–30% develop colorectal carcinoma within 10 years since subtotal colectomy
Development of additional extracolonic malignant tumors	Endometrial carcinoma (40–60%), ovarian carcinoma (12–15%), gastric carcinoma, pancreatic carcinoma, hepatobiliary carcinoma, renal pelvis and ureter carcinoma, brain tumors (Turcot syndrome), skin tumors, i.e. Muir-Torre syndrome (e.g. sebaceous adenomas, carcinoma and multiple keratoacanthoma)
Histologic features	Poorly-differentiated colorectal carcinoma with excess of mucinous and signet ring cell type carcinoma; excess of tumor infiltrating lymphocytes
Conformation of diagnosis	Identification of germline mutation in one of the mismatch repair genes, e.g. (MSH2 (2p15), MLH1 (3p21), PMS1 (2p32) and PMS2 (7p22)

with ileorectal anastomosis or total colectomy with ileoanal pull-through (pouch procedure). Cardinal features of hereditary nonpolyposis colon carcinoma (HNPCC), also called Lynch syndrome, are given in Table 6.33.

Juvenile Polyposis Syndrome

Juvenile polyposis syndrome (JPS) is an autosomal dominant disorder due to germline mutations in two different genes (SMAD4 and BMPR1A), characterized by multiple benign polyps on lining epithelium of gastrointestinal tract anywhere from stomach to the rectum before 20 years of age even in multiple family members of same family. There is risk of developing gastrointestinal carcinomas. Patient presents with gastrointestinal bleeding, abdominal pain and diarrhea. About 15% of patients may have abnormalities such as intestinal malrotation, cardiac or brain abnormalities, cleft palate, polydactyly and urogenital abnormalities.

Hereditary Diffuse Gastric Carcinoma

Hereditary diffuse gastric carcinoma (HDGC) is an autosomal dominant disorder caused due to germline mutation in one copy (allele) of CHD1 gene, that shows a diffuse gastric carcinoma without a solid growth.

- Patient presents with abdominal pain in gastric region, nausea, vomiting, dysphagia, anorexia and weight loss as well as symptoms related to organs involved (e.g. hepatomegaly, pathological bone fractures, ascites).
- Prophylactic gastrectomy is performed to prevent developing and spreading of HDGC. When surgery is not possible, chemotherapy is administered to treat in such HDGC cases.

Oligodontia-Colorectal Cancer Syndrome

Oligodontia-colorectal cancer syndrome is an autosomal dominant disorder due to nonsense germline mutation in AXIN2 gene located on chromosome 17q23–24, that encodes aberrant AXIN2 related protein.

- Patient presents with congenital severe permanent tooth agenesis of six or more permanent teeth (excluding the third molars), and risk of developing gastrointestinal polyposis to early-onset colorectal cancer and/or breast cancer and medulloblastoma.
- There may be associated ectodermal dysplasia (sparse hair and/or eyebrows). Molecular genetic testing of family members is done to detect carrier before the age of 16–18 years.

Brooke-Spiegler Syndrome (Familial Cylindromatosis)

Birt-Hogg-Dubé (BHD) syndrome is an autosomal dominant disorder due to germline mutation in FLCN/BHD tumor suppressor gene located on chromosome 17p11.2, that encodes aberrant '**folliculin**' **protein**. Patient presents with multiple benign skin tumors over face, neck and upper chest, associated with increased risk of benign or malignant tumors of kidney, and cysts in the lungs that cause pneumothorax and lung collapse.

Hereditary Leiomyomatosis and Renal Cell Carcinoma

Hereditary leiomyomatosis and renal cell carcinoma (HLRCC) is an autosomal dominant disorder caused due to germline mutation in fumarate hydratase (FH) gene mapped on chromosome 1q43 encodes an aberrant FH protein that inactivates enzyme leading to alteration of tricarboxylic acid (TCA/Krebs) cycle.

- Patient develops cutaneous (e.g. on trunk, arms, legs and occasionally over face) and uterine leiomyomas and associated with increased risk of papillary renal cell carcinoma that presents with triad (low back pain, hematuria and palpable flank mass) and manifestations related to paraneoplastic syndrome and disseminated disease.
- Molecular genetic testing of family members is done to detect carrier from the age of 16–18 years of age.

Multiple Endocrine Neoplasia Type 2

Multiple endocrine neoplasia 2 (MEN 2) is an autosomal dominant disorder due to germline mutation in RET tumor suppressor gene located on 10q11.2, that encodes aberrant 'RET receptor tyrosine kinase'.

- There are two types of MEN2 syndromes: (a) MEN2A (Sipple syndrome) is characterized by medullary thyroid carcinoma, pheochromocytoma or parathyroid adenoma/hyperplasia (inducing primary hyperparathyroidism, and (b) MEN2B (Gorlin syndrome) is characterized by familial medullary thyroid carcinoma.
- Prophylactic thyroidectomy should be performed in patients with germline mutation in RET proto-oncogene. Medullary thyroid carcinoma is treated by surgical excision and tyrosine kinase inhibitor therapy. Annual analysis of serum calcitonin concentration is done to detect residual or recurrent medullary thyroid carcinoma after thyroidectomy.

Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder caused due to germline mutation in NF1 tumor suppressor gene located on chromosome 15q13, that encodes an aberrant 'neurofibromin' protein, which is normally a negative regulator of RAS protein.

- Patient presents with skin pigmentation (multiple café-au-lait spots), and growth of tumors along nerves in the skin (neurofibromas), brain (optic neuroma affecting vision) and nerves near the spinal cord or along nerves elsewhere in the body in adults.
- In some patients, neurofibroma can undergo transformation to malignant peripheral nerve sheath tumor. NF1 can be associated with other autosomal dominant disorders such as Noonan syndrome and Watson syndrome.
- Molecular genetic testing is performed to detect mutations in NF1 gene prior to development of clinical manifestations.

Neurofibromatosis Type 2

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder caused due to **germline mutation** in **NF2 tumor suppressor gene** mapped on chromosome 22q12.2, that encodes aberrant 'merlin' (also known as schwannomin) cytoskeleton protein.

- Patient develops benign bilateral tumors of auditory nerves (vestibular schwannomas/acoustic neuroma) and meningiomas (brain or spinal cord) and/or ependymomas.
- Molecular genetic testing can be done to analyze NF2 gene mutation to establish diagnosis. Surgery is the primary treatment for most peripheral nerve tumors.

Von Hippel-Lindau Disease

Von Hippel-Lindau (vHL) disease is an autosomal dominant disorder caused due to **germline mutation** in **vHL tumor suppressor gene** located on chromosome 3p25, that encodes aberrant vHL protein.

- Clinical hallmarks of vHL disease are development of tumor and/or cysts in multiple organs (e.g. brain, spinal cord, eyes, kidneys, pancreas, adrenal glands, inner ears, reproductive tract, liver and lung).
- Hemangioblastoma occurs in brain (cerebellum), spinal cord and retina that have subsequent potential for malignant change.
- Pheochromocytoma results in high blood pressure. Pheochromocytoma should be diagnosed by biochemical catecholamine analysis and CT/MRI scanning of abdomen.

Hereditary Papillary Renal Cell Carcinoma

Hereditary papillary renal cell carcinoma (HPRCC) is an autosomal dominant disorder due to germline mutation in MET gene mapped on chromosome 4q12, that encodes aberrant receptor for hepatocyte growth factor (HGF) protein affecting many cell types. Diagnosis of HPRCC is suspected on the basis of a family history of papillary renal cell carcinomas and can be confirmed by molecular genetic testing to detect germline mutation in the MET exons 15–21, which encodes aberrant cytoplasmic domain of the receptor.

Nevoid Basal Cell Carcinoma Syndrome (Gorlin-Goltz Syndrome)

Nevoid basal cell carcinoma syndrome (also called Gorlin-Goltz syndrome) is an autosomal dominant disorder due to germline mutations in **PTCH1** (9q22.3), **PTCH2** (1p34.1) and **SUFU** (10q24.32) tumor suppressor genes, which encode aberrant patched-receptor proteins. Patient can develop benign tumors (e.g. ovarian fibroma, myocardial fibroma, medulloblastoma, meningioma, rhabdomyoma and lymphomesenteric cysts), ocular defects (colobomas, cataracts, glaucoma) and cleft lip and palate.

Familial Paraganglioma-Pheochromocytoma Syndrome

Familial paraganglioma-pheochromocytoma syndrome is an autosomal dominant disorder due to germline mutation genes such as **SDHA gene** (5p15), **SDHB** (1p35–36.1), **SDHC** (1q21–23), and **SDHD** (11q23), which encode aberrant succinate dehydrogenase (**SDH**) protein complex, which links the tricarboxylic acid cycle with the electron transport chain.

- Familial paraganglioma-pheochromocytoma syndrome is characterized by the presence of tumors: (a) paraganglioma (sympathetic/parasympathetic) arising from neuroendocrine tissues symmetrically distributed along the spine from the base of the skull to the pelvis, and (b) pheochromocytoma.
- Molecular genetic testing is done to establish diagnosis by detecting mutations in SDHA, SDHB, SDHD, SDHAF2 and MAX genes.

WAGR Syndrome, Denys-Drash Syndrome, and Nonsyndromic Hereditary Wilms' Tumor

WAGR syndrome, Denys-Drash syndrome, and non-syndromic hereditary Wilms' tumor are autosomal dominant disorders caused due to germline mutations in WT1 gene located on chromosome 11p13, that encodes an aberrant zinc finger-containing protein.

- WAGR syndrome (also called as WAGR complex or Wilms tumor-aniridia syndrome) is occurs due to germline mutation in WT1 tumor suppressor gene along with PAX6 gene and characterized by development of Wilms' tumor, aniridia (absence of iris due to PXA6 gene mutation) and genitourinary anomalies. Most cases of WAGR syndrome are not inherited.
- Denys-Drash syndrome (DDS) consists of a triad of intersex, Wilms' tumor and nephrotic syndrome (diffuse mesangial sclerosis) associated with progressive renal failure.
- Nonsyndromic hereditary Wilms' tumor can be inherited (10%) or sporadic (90%) forms in children caused due to mutations in WT1 tumor suppressor gene mapped on chromosome 11p13. Nearly all cases of Wilms' tumor are diagnosed before the age of 10 years with two-thirds being found before age of 5 years.

Familial Melanoma

Familial melanoma is an autosomal dominant disorder caused due to germline mutation in CDK4 gene (4q12), CDKN2A gene (8p21.3), TERT gene (5p15.32) and POT1 gene (7q31.33) encode aberrant proteins linked to familial malignant melanoma. High and intermediate penetrance genes involved in familial melanoma susceptibility are given in Table 6.34.

Familial Atypical Multiple Mole Melanoma Syndrome

Familial atypical multiple mole melanoma syndrome (FAMMM syndrome) is an autosomal dominant disorder caused due to germline mutations in CDNK2A gene located on chromosome 8p21.3, that encodes aberrant $p16^{INK4A}$ and $p14^{ARF}$ proteins. Patient presents with multiple melanocytic nevi (often >50) with atypical appearance (different shades of tan, brown, black or red on back, chest, buttocks, breasts and scalp). There is a family history of melanoma in 80% of patients. Early diagnosis and treatment of melanoma is associated with better prognosis.

Table 6.34 High and intermediate penetrance genes involved in familial melanoma susceptibility

Gene	Encoded Protein	Function	Gene Mutation Prevalence in Families
High penetrance genes linked to familial melanoma susceptibility			
CDKN2A (cyclin-dependent kinase 2A) gene (8p21.3)	▪ p16 INK4A protein ▪ p14ARF protein	▪ Cell cycle regulator ▪ Cell cycle regulator	▪ 20–40% ▪ 1%
CDK4 (cyclin-dependent kinase 4) gene (4q12)	CDK protein	Cell cycle regulator	17%
TERT (telomere reverse transcriptase) gene (5p15.32)	Catalytic subunit of telomere protein	Telomere elongation	2%
POT1 (protection of telomere) gene (7q31.33)	POT1 protein	Telomere maintenance	14%
Intermediate penetrance genes linked to familial melanoma susceptibility			
MC1R (melanocortin 1 receptor) gene (16q24.3)	MC1R protein	Melanocyte synthesis and their proliferation	Not applicable
MITF (microphthalmia-associated transcription factor) gene (3p12–p14.1)	MITF protein	Melanocyte development and differentiation	Not applicable

Autosomal Recessive Inheritance Linked to Inherited Cancer Syndromes

Detrimental effects of autosomal recessive cancer gene are observed less often within the population as compared to their autosomal dominant counterparts. In autosomal recessive inheritance, two copies of the recessive allele of gene must be inherited, one from each parent (mother and father), in order for the mutation to have effect on individual's phenotype. Homozygous inheritance of these recessive cancer genes does not cause human cancer directly. However, loss of functional protein results in genomic instability in autosomal recessive homozygous inheritance. This genomic instability fosters an ideal environment for oncogenic mutations and predisposes the affected individual to cancer. Autosomal recessive inheritance linked to inherited cancer syndromes are given in Table 6.35.

NTHL1-Associated Polyposis Disorder

NTHL1-associated polyposis (NAP) is an autosomal recessive disorder caused due to **germline mutation NTHL1 gene** in mapped on chromosome on 16p13.3, that encodes aberrant DNA N-glycosylase of the endonuclease III family. DNA N-glycosylase catalyzes the first step in base excision repair (BER) of oxidative DNA damage. Patient presents with attenuated adenomatous polyposis and colorectal carcinoma(s) and extracolonic malignancies (e.g. endometrial carcinoma, duodenal carcinoma, breast carcinoma and pancreatic carcinoma).

Ataxia-Telangiectasia

Ataxia-telangiectasia is an autosomal recessive disorder caused due to **germline mutation** in **ATM gene** mapped on chromosome 11q22.3, that encodes aberrant ATM

Table 6.35 Autosomal recessive inheritance linked to inherited cancer syndromes

Inherited Cancer Syndrome	Gene and Locus	Associated Tumors
Attenuated polyposis known as MUTYH-associated polyposis (MAP)	Base pair excision repair (BER) MUTYH gene (1p34.1)	▪ Multiple adenomatous polyposis in colon ▪ Colon carcinoma
NTHL1-associated polyposis (NAP)	NTHL1 gene (16p13.3)	▪ Multiple adenomatous polyposis in colon ▪ Colorectal carcinoma
Polymerase proofreading-associated polyposis (PRAP)	▪ POLE (12q24.3) ▪ POLD1 (19q13.33)	▪ Multiple adenomatous polyposis in colon ▪ Colorectal carcinoma ▪ Ovarian carcinoma ▪ Endometrial carcinoma ▪ Pancreatic carcinoma ▪ Brain malignancies
Ataxia-telangiectasia	ATM gene (11q22.3)	▪ Leukemia ▪ Non-Hodgkin's lymphoma
Fanconi anemia	Fanconi anemia DNA repair genes	▪ Acute myelogenous leukemia ▪ Liver tumors ▪ Squamous cell carcinoma (oropharyngeal and anogenital regions)
Bloom syndrome	BLM gene (15q26)	▪ Leukemia ▪ Non-Hodgkin's lymphoma ▪ Gastrointestinal stromal tumors
Rothmund-Thompson syndrome	RECQLA gene (8q24.3)	Osteosarcoma (early onset)
Werner's syndrome	WRN gene (8p12)	▪ Meningioma ▪ Soft tissue sarcoma
Nijmegen breakage syndrome	NBSI gene (8q21)	▪ Leukemia ▪ Non-Hodgkin's lymphoma
Familial medulloblastoma	SUFU gene (10q24.32)	Medulloblastoma
Xeroderma pigmentosum	Xeroderma pigmentosum genes	▪ Squamous cell carcinoma of skin ▪ Basal cell carcinoma of skin

protein that is unable to repair damaged DNA and thus genomic instability.

- Patient presents with progressive cerebellar degeneration (ataxia, abnormal eye movements and postural instability), telangiectasia on ocular region and skin, immunodeficiency (sinopulmonary infection), radiation sensitivity and cancer susceptibility (e.g. leukemia and non-Hodgkin's lymphoma, urinary bladder carcinoma, breast carcinoma, melanoma, lung carcinoma and ovarian carcinoma).
- Diagnosis of ataxia-telangiectasia is made based on a detailed patient history, a thorough clinical evaluation, identification of clinical manifestations and a wide variety of specialized tests include blood tests, magnetic resonance imaging and karyotyping.

Fanconi Anemia

Fanconi anemia (FA) is an autosomal recessive disorder caused due to germline mutations in Fanconi anemia DNA repair genes that encodes aberrant proteins. Genomic instability is linked to bone marrow failure and Fanconi anemia-associated with acute myelogenous leukemia (AML), hepatocellular malignancy, and squamous cell carcinoma in oropharyngeal and anogenital regions. Fanconi anemia is most often diagnosed in children between 3 and 14 years of age.

Bloom Syndrome (Congenital Telangiectasia Erythema)

Bloom syndrome is an autosomal recessive disorder caused due to germline mutation in BLM gene mapped on chromosome 15q26, that encodes aberrant one of the five RECQ DNA helicases.

- Bloom syndrome is seen in persons of multiple ethnic backgrounds but highest prevalence within Ashkenazi Jewish population.
- Patient presents with short stature, prominent nose and ears, ultraviolet radiation sensitivity and occasionally mental retardation. Patient is at risk of development of leukemia, non-Hodgkin's lymphoma and gastrointestinal tumors.

Rothmund-Thomson Syndrome

Rothmund-Thomson syndrome is an autosomal recessive disorder due to **germline mutation** in **RECQLA gene** mapped on chromosome 8q24.3, that encodes aberrant RECQLA protein. Patient presents with poikiloderma, juvenile cataracts, growth retardation, chronic diarrhea, skeletal dysplasia, sparse scalp hair, hypogonadism (25%) and increased susceptibility to early onset of osteosarcoma and skin cancers (basal cell carcinoma of skin and squamous cell carcinoma of skin).

Nijmegen Breakage Syndrome

Nijmegen breakage syndrome (NBS) is an autosomal recessive disorder caused due to germline mutation in NBSI gene mapped on chromosome 8q21, that encodes aberrant NBS1 (nibrin) protein leading to chromosomal instability. Patient presents with radiosensitivity, cancer predisposition (e.g. leukemia and non-Hodgkin's lymphoma), progressive microcephaly (bird-like facial appearance), growth retardation and immunodeficiency.

Werner's Syndrome

Werner's syndrome is an autosomal recessive disorder due to germline mutations in WRN gene located on chromosome 8p12, that encodes aberrant Werner protein. Normal Werner (WRN) protein is involved in repairing damaged DNA. Patient presents with premature aging, graying and thinning of hair, wrinkling and ulceration of skin, atherosclerosis, osteoporosis and cataracts. In addition, patient exhibits an increased incidence of diabetes mellitus type 2, hypertension and susceptibility to development of benign and malignant neoplasms (meningiomas and soft tissue sarcoma).

Familial Medulloblastoma

Familial medulloblastoma is an autosomal recessive disorder caused due to germline mutation in SUFU gene located on chromosome 10q24.32, that encodes aberrant protein domain suppressor of fused protein (SUFU). Clinical neurologic manifestations of familial cerebral medulloblastoma depend on the extent of tumor.

Xeroderma Pigmentosum

Xeroderma pigmentosum is an autosomal recessive disorder due to germline mutations in xeroderma pigmentosum genes encode aberrant proteins. Patient presents with photosensitivity, hyperpigmentation of skin, premature skin aging and increased risk of malignancies (e.g. squamous cell carcinoma and basal cell carcinoma) due to ultraviolet radiation induced defect in DNA repair.

X-linked Inheritance Disorders Linked to Inherited Cancer Syndromes

X-linked recessive inheritance refers to genetic disorders passed from parent to the child associated with mutations in genes on the X chromosome.

- As males have only one chromosome, mutation in the copy of the gene on the single X chromosome causes the X-linked recessive genetic disorders. Females, who have two X chromosomes must have a mutation

on both X chromosomes in order to be affected with the genetic disorder.

- If only the father or the mother has the mutated X-linked genes, the daughters are usually not affected and are called '**carriers**' because one of their X chromosomes has the mutation but the other X chromosome is normal. Sons will be affected if they inherit the mutated X-linked gene from their mother. Father cannot pass X-linked genetic orders to their sons.
- Loss of X-linked gene expression has been observed in 20% of cancers, including breast carcinoma and ovarian carcinoma. Loss of heterozygosity (LOH) at the X chromosome is also associated with sporadic colorectal carcinoma, renal cell carcinoma, melanoma and neuroendocrine tumor.
- Recent whole genome-wide scan analysis has provided substantial information regarding single-hit inactivation of X-linked cancer-related tumor suppressor gene involved in development of cancers in breast, colorectal region and skin.
  - Single-hit inactivation of X-linked cancer-related tumor suppressor genes (FLNA, PFC, PRPS1, TAF, KLH4, MAGEE1) have been observed in breast carcinoma.
  - Single-hit inactivation of X-linked cancer-related tumor suppressor genes (FLNA, TBX22, KIAA2022, IRS4, PCDH11X, GPR112 and F8) has been found in colorectal carcinoma.
  - Single-hit inactivation of tumor suppressor genes (ZNF280C, PNMA3, IL3RA, NHS and FGD1) has been observed in melanoma.

Simpson-Golabi-Behmel Syndrome

Simpson-Golabi-Behmel syndrome is an X-linked recessive disorder due to germline mutations in GPC3 gene mapped on chromosome Xq26, that encodes aberrant glypican protein. There is increased risk for development of benign and malignant tumors (e.g. Wilms' tumor, neuroblastoma and hepatoblastoma) in 10% of cases.

Unknown Mode(s) of Inheritance Inherited Cancer Syndromes

Unknown mode(s) of inheritance inherited cancer syndromes include familial carcinoid syndrome, familial Hodgkin's disease, familial pancreatic carcinoma and familial testicular germ cell tumors.

Familial Carcinoid Syndrome

Carcinoid syndromes (neuroendocrine tumors) are capable of secreting vasoactive substances such as serotonin, histamine and bradykinin. Patients harbor sporadic slow-growing carcinoids (neuroendocrine tumors) in gastrointestinal tract (98%) and familial (1–2%). A newly discovered condition called familial small intestinal neuroendocrine tumor occurs due to mutation of IPMK gene.

Familial Hodgkin's Disease

Genetic susceptibility plays a role in the pathogenesis of familial Hodgkin's disease. Approximately 1% of patients with Hodgkin's disease have a family history of the disease. Siblings of an affected person are at higher risk for development of familial Hodgkin's disease in younger age.

Familial Pancreatic Carcinoma

Familial pancreatic carcinoma (FPC) is defined by the presence of pancreatic cancer in two or more first-degree relatives in younger age group. Tobacco smoking represents a significant risk factor for familial pancreatic carcinoma arising from the exocrine pancreas (95%) especially in head of pancreas [arise from the exocrine (95%)] or endocrine portions of the pancreas.

- Patient presents with pain in the upper abdomen, that radiates to the back, loss of appetite, significant weight loss and painless jaundice due to bile duct obstruction. As the clinical course is silent, and FPC goes undetected until advanced stages of the disease.
- In more than 80% of cases, familial pancreatic carcinoma is either locally advanced or disseminated disease at the time of diagnosis that can metastasize to regional lymph nodes and via hematogenous route to distant organs (liver, peritoneum and lungs).

Familial Testicular Germ Cell Tumors

Familial testicular germ cell tumors (FTGCT) occur in more than one blood relative of the same family. Testicular germ cells arise from the primordial germ cells that are blocked in maturation. This process is thought to be initiated during fetal development and can be caused by both genetic and environmental factors. A noninvasive stage (intratubular germ cell neoplasia unclassified or carcinoma *in situ*) precedes progression to testicular germ cell tumor. There is increased risk of testicular germ cell tumors in the setting of cryptorchidism, infertility and microlithiasis.

Familial Cancers in Various Organs

Certain common cancers of breast, ovary, prostate and colon may occur in more than one member of the same family, but are not thought to be hereditary. Multiple family members may be diagnosed with the same cancer, but usually the cancer occurs at later ages and

does not follow the same patterns that are observed in hereditary cancers.

- Familial cancers are not caused by mutation in specific gene. Instead, family cancers are caused by combination of mutations in several genes and other environmental factors such as diet and exercise. Family members may need earlier frequent cancer screening.
- Family cancers should be suspected on following criteria: clustering of cancer in one family, onset of adult type of carcinoma at an early age, multiple independent carcinomas arising in individual, bilateral cancer in paired organs, presence of a rare cancer and presence of the same or associated cancers that comprise a syndrome.

PREFERENTIAL ROUTES OF METASTASIS OF TUMORS

Preferential routes of metastasis vary with specific neoplasms. Carcinomas most often tend to metastasize via lymphatic route, while sarcomas tend to metastasize via hematogenous route to distant organ(s). Notable exceptions include renal cell carcinoma and hepatocellular carcinoma, which most often metastasize via hematogenous route to distant organ(s).

- Malignant epithelial tumors (carcinomas) most commonly metastasize to via lymphatic route to draining lymph nodes, and later via hematogenous route to distant organs (e.g. lungs, liver, bones, brain and adrenal glands). Malignant mesenchymal tumors (sarcomas) metastasize via hematogenous route to lungs, which rarely metastasize to skeletal muscles and spleen.
- Metastasize to liver often arises in patients with colon carcinoma. The portal vein drains blood from the colon into the liver, which provides a route of metastasis of colon carcinoma directly to the liver. Breast carcinoma often metastasizes to brain. Ovarian carcinoma most often involves peritoneum. Rarely metastasizing tumors include basal cell carcinoma of skin and gliomas.
- Tumor progression is characterized by the accumulation of successive molecular/cytogenetic alterations. For example, progression of changes in normal colonic epithelium to colon carcinoma to dissemination occur with parallel successive molecular/cytogenetic alterations in APC, K-RAS, DCC, TP53 and other genes. Individual cancer stem cells within a malignant tumor may possess varying metastatic potential. Malignant tumors disseminate by one of five pathways. Local invasion and metastasis of various cancers via various routes are shown in Fig. 6.61. Routes of metastasis of primary lung carcinoma are shown in Fig. 6.62.

LOCAL INVASION: FIRST STEP IN METASTASIS

Local invasion of malignant tumor is the first step in metastasis, which follows line of least resistance. The proliferating CSCs break through normal barrier, invade, permeate surrounding tissues, and exert mechanical pressure on the surrounding tissues. Lytic enzymes (collagenases) secreted by CSCs invade basement membrane. CSCs lack cell adhesion molecules, e.g. E-cadherin, hence become less adhesive, thus via increased amoeboid movements invade vasculature and metastasize to distant organs.

- Cancer stem cells (CSCs) may stimulate the production of new collagen fibers which are sometimes converted into excess dense fibrous tissue that contracts and fixes the growth into surrounding structures. Breast carcinoma causes nipple retraction and fixation of tumor to underlying skeletal muscle of involved breast.
- Cancer stem cells (CSCs) invade, compress nerve and cause loss-of-function. Brain astrocytoma spreads by direct extension into surrounding tissue.
- Lung carcinoma invades surrounding lung parenchyma via local spread and produces bronchopleural fistula. Cervical carcinoma invades surrounding tissues and produces vesicovaginal fistula. Pancreatic carcinoma invades celiac plexus resulting in severe pain. Ovarian carcinoma may involve peritoneum.

LYMPHATIC ROUTE OF METASTASIS

Lymphatic route is the commonest mechanism of dissemination of carcinomas and melanomas, but rarely of sarcomas. Cancer stem cells (CSCs) easily invade lymphatic channels of tissue spaces.

- Group of CSCs detach to form clusters, which are carried in the form of tumor emboli and trapped in the first draining lymph node, it encounters. Tumor emboli appear first in the subcapsular sinus, penetrate its efferent channels, proliferate, metastasize and destroy chain of lymph node structure. Invaded lymph nodes become enlarged, firm and white. Localized immune reaction to the CSCs may limit their further spread.
- Mesenteric lymph node invaded by CSCs of abdominal malignancies may invade cisterna chyli and eventually reach the venous blood circulation via

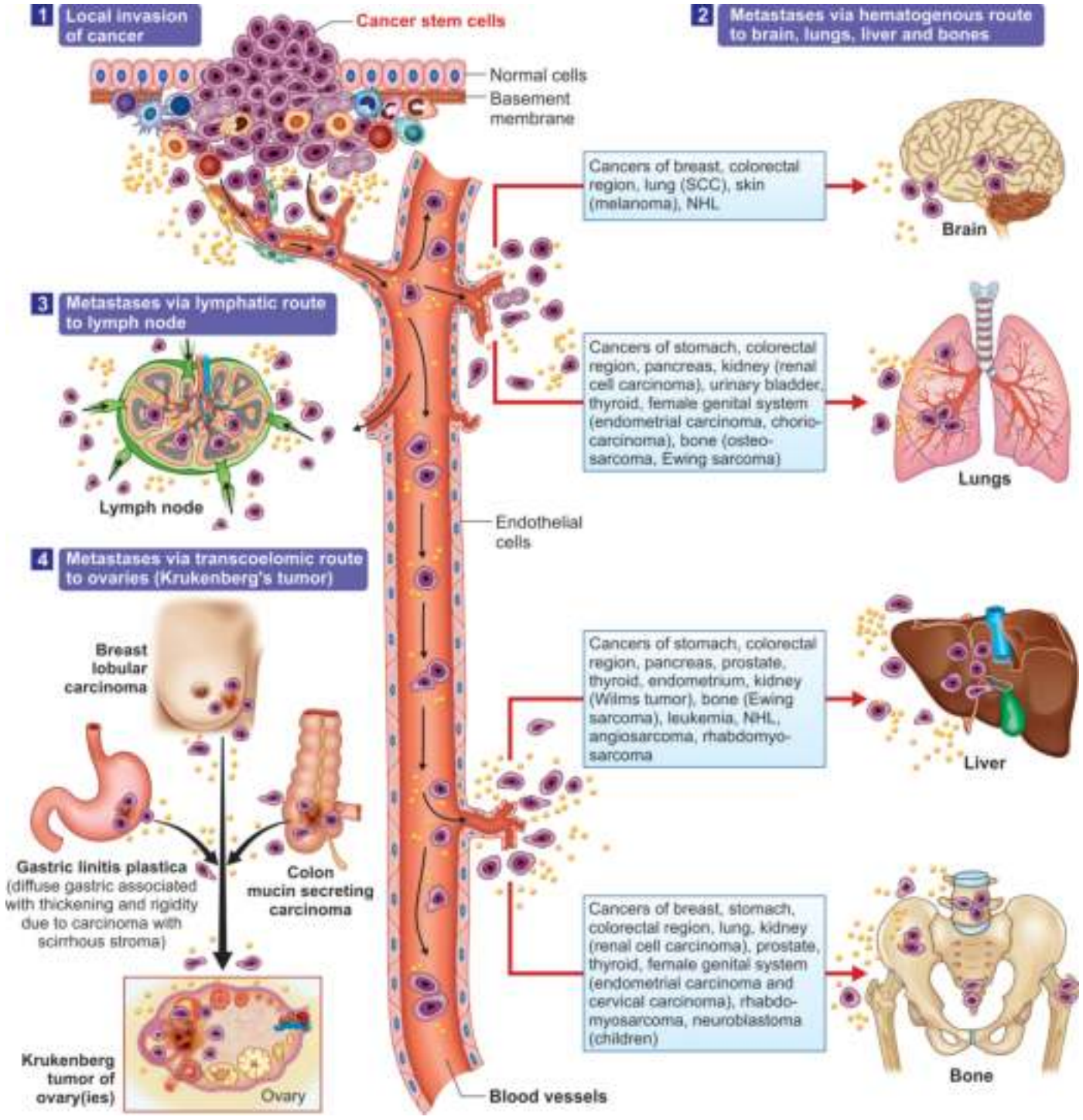


Fig. 6.61: Local invasion and metastasis of various cancers via various routes. Metastatic cascade represents a multistep process which includes CSC invasion, entry into the vasculature followed by the exit of cancer stem cells from the circulation and colonization in the distant organs.

thoracic duct. CSCs can enter the blood circulation from the lymphatic circulation through plentiful connections between the lymphatic and venous systems.

- Breast carcinoma in the outer quadrant involves axillary lymph nodes, whereas breast carcinoma present in the inner quadrant metastasizes to internal mammary lymph nodes. The first lymph node involved in a regional lymphatic drainage is called 'sentinel lymph node'. Histologic examination of sentinel lymph node is performed to select the patients, who require 'extensive lymph node dissection' particularly in breast carcinoma, colon carcinoma and melanoma. Breast carcinoma metastasis in

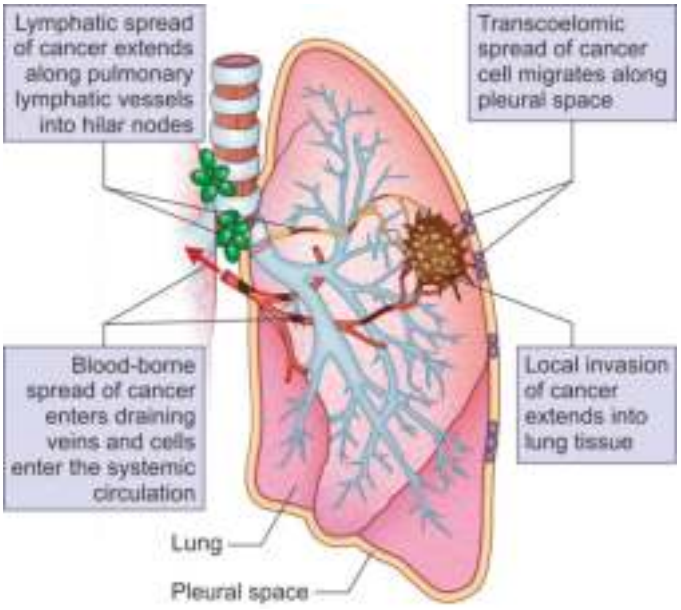


Fig. 6.62: Routes of metastasis of primary lung carcinoma. Routes of metastasis of primary lung carcinoma include local invasion, lymphatic, hematogenous and transcoelomic routes.

lymph nodes is shown in Fig. 6.63. Melanoma of skin metastasis in lymph nodes is shown in Fig. 6.64.

- Lung carcinoma metastasizes to perihilar tracheobronchial, and mediastinal lymph nodes. Colorectal carcinomas metastasize first to the mesenteric lymph nodes.
- Local chemotherapy and immunotherapy can sterilize a lymph node laden with CSCs. Histologic examination of sentinel lymph node is increasingly carried out to select the patients who require extensive lymph node dissection.

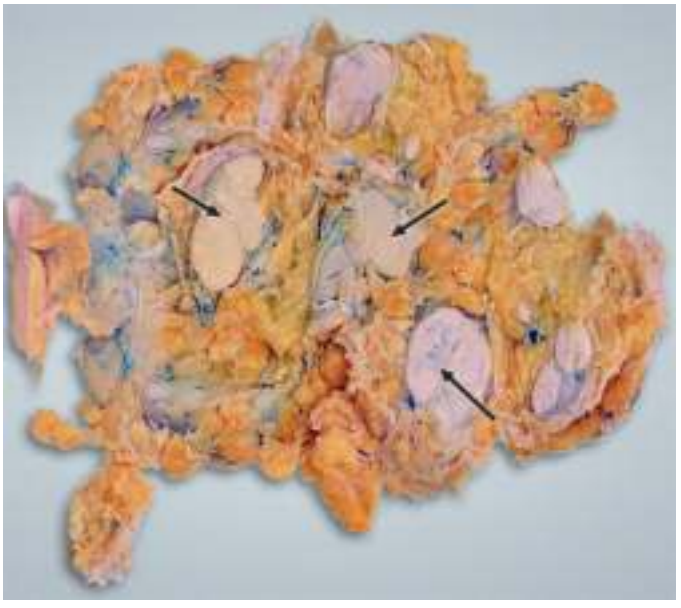


Fig. 6.63: Breast carcinoma metastasis in lymph nodes. Regional metastatic breast carcinoma refers to original tumor has spread to nearby lymph nodes most often axillary lymph nodes and sometimes internal mammary lymph nodes and supraclavicular lymph nodes (arrows).

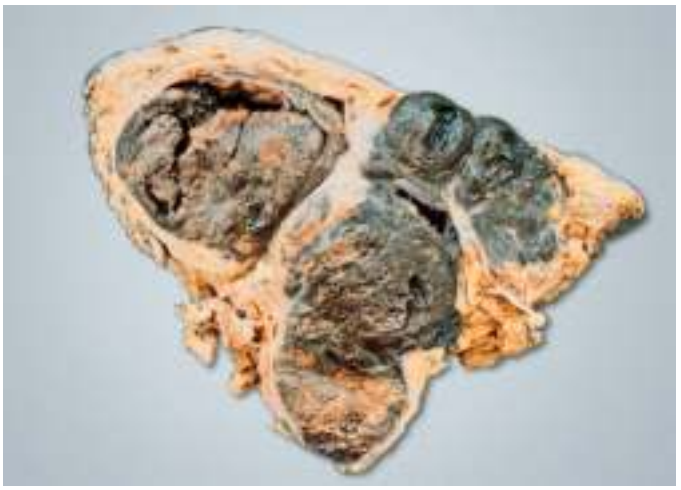


Fig. 6.64: Melanoma of skin metastasis in lymph nodes. Melanoma can grow extremely quickly and can become life-threatening within six weeks and associated with poor survival. (*Courtesy:* Dr. Sujata Kanetkar, Senior Professor and Head, Department of Pathology, Krishna Institute of Medical Sciences, Karad Maharashtra.)

Pathology Pearls: Lymph Node Metastasis

Tumor deposits measuring 0.2–2 mm are classified as micrometastases in lymph nodes, whereas tumor deposits measuring ≥2 mm is classified as macrometastases in lymph nodes.

Tumors Spreading Lymph Node Metastasis Against Lymphatic Flow

Tumors spreading against the lymph flow in lymphatic channels may cause metastases at unusual sites. For example, prostatic carcinoma metastasizes to the supraclavicular lymph nodes.

Sentinel Lymph Node

- The first lymph node involved in a regional lymphatic drainage is called sentinel lymph node, which can be identified by injecting blue dye. Sentinel lymph node mapping is done in breast carcinoma and colon carcinoma. If the sentinel lymph node is involved, the patient needs 'extensive lymph node dissection' surgery.
- In breast carcinoma, the sentinel lymph node is usually located in the axillary region under the armpit. In most cases, there is involvement of 1–5 sentinel nodes, and all are surgically excised and sent for histologic examination for signs of metastases.

Sinus Histiocytosis

- Necrotic products of the malignant tumor often evoke reactive changes in the lymph nodes such as enlargement and hyperplasia of the lymphoid follicles, proliferation of macrophages in the subcapsular sinuses, known as sinus histiocytosis.
- Sinus histiocytosis can also occur due to infections, immunodeficiency state, autoimmunity and Rosai-Dorfman disease.

Skipped Metastases

- Metastases to the regional draining lymph nodes may be bypassed (skipped metastases) due to involvement of lymphatic-venous anastomoses by inflammation or irradiation resulting in obliteration of these channels.
- For examples, carcinomas in the abdominal cavity may be first detected as enlarged supraclavicular lymph node.

HEMATOGENOUS ROUTE OF METASTASIS

Both malignant epithelial tumors (carcinomas) and malignant mesenchymal tumors (sarcomas) spread via hematogenous route to distant organ(s). Direct invasion of CSCs in arteries and arteriolar lumens is very rare due to the physical barrier provided by their thick muscular and elastic walls.

- Cancer stem cells (CSCs) invade the thin walls of venules, circulate as tumor emboli in lymphatic channels through thoracic duct enter blood circulation and via hematogenous route, metastasize to distant sites (e.g. liver, lung, bones, brain, adrenal glands). Conversely, rhabdomyosarcomas, although rich in capillaries, are rare site of secondary deposits via hematogenous route.
- Malignant mesenchymal tumors (sarcomas) usually metastasize via hematogenous route to distant organs(s). However, some carcinomas such as renal cell carcinoma, hepatocellular carcinoma, follicular thyroid carcinoma, prostatic carcinoma may also metastasize via hematogenous route. Renal cell carcinoma often invades the renal vein to grow in a snake-like fashion in the inferior vena cava sometimes reaches the right side of the heart.
- Cancer stem cells (CSCs) synthesize thromboplastin that may induce thrombosis of distant veins (thrombophlebitis migrans).
- Metastases irrespective of anatomic localization of the malignant tumors and natural pathways of venous drainage occurs by the following ways. Prostatic carcinoma preferentially spreads to bone. Bronchogenic carcinoma involves the adrenal glands and the brain. Neuroblastoma most often metastasizes to the liver and bones.
- Destination of tumor emboli depends on the anatomical drainage of the invaded blood vessel. CSCs reaching venous tributaries of the inferior vena cava flow to the right side of the heart and reach the lungs, whereas CSCs of colon carcinoma via portal vein drainage metastasize to the liver. Tumor emboli in lungs occur when solid malignant tumors seed the systemic circulation with individual CSCs, cluster of CSCs, or large tumor fragments. Tumor emboli pass to left-sided heart and systemic circulation.
- As in lymphatic channels, growth of tumor emboli within a vein causing 'reversal of blood flow' is called retrograde venous spread of tumor emboli. In addition, reversal of blood flow occurs in regions of the body where presence of rich plexus of veins are deficient in valves, e.g. in the pelvis and around vertebrae. Changes in intra-abdominal and intrathoracic pressures easily induce alterations in blood flow in these veins due to this reason, secondary tumors are relatively common in **vertebral bodies**.
- The distribution of tumor emboli is determined by anatomy and surface properties of the CSCs, e.g. increased expression of integrins and their receptors present on the endothelial cells of blood vessels at the metastatic site. Variations in immune response play important role in seeding of malignant tumor emboli.

LIVER METASTASIS

The liver is one of the most common site for malignant tumor metastasis accounting for nearly 25% of cases. A variety of malignant tumors may be the source for hepatic metastasis in adults and children, which include: (a) carcinomas of breast, lung, stomach, colorectal region, pancreas, kidney, prostate, endometrium, thyroid, (b) sarcomas of blood vessel, skeletal muscle, (c) melanoma (melanocyte), (d) round cell tumors (e.g. Wilms' tumor, neuroblastoma, Ewing sarcoma, bronchial carcinoid), and (e) hematolymphoid malignancies (e.g. leukemias, non-Hodgkin's lymphoma). Colorectal carcinoma metastasizes to liver via portal vein in 50% of diagnosed patients. Metastases in liver are shown in Fig. 6.65. Metastasis from invasive ductal adenocarcinoma of breast in liver is shown in Fig. 6.66.

PULMONARY METASTASIS

Pulmonary metastases are more common than primary lung carcinomas. In one-third cases, lung metastases are evident at autopsy. Blood-borne metastases from breast carcinoma (most common), renal cell carcinoma, colon carcinoma, thyroid carcinoma, osteosarcoma and testicular malignancies reach the lungs via systemic venous circulation to the right side of heart. Other malignant tumors from larynx, salivary glands, thyroid gland, uterus, ovaries, urinary bladder, prostate and skin (melanoma) may metastasize to the lungs. Metastasis from osteosarcoma in lung is shown in Fig. 6.67.

- On imaging study (X-ray chest, CT scan, positron emission tomography—PET scan), pulmonary

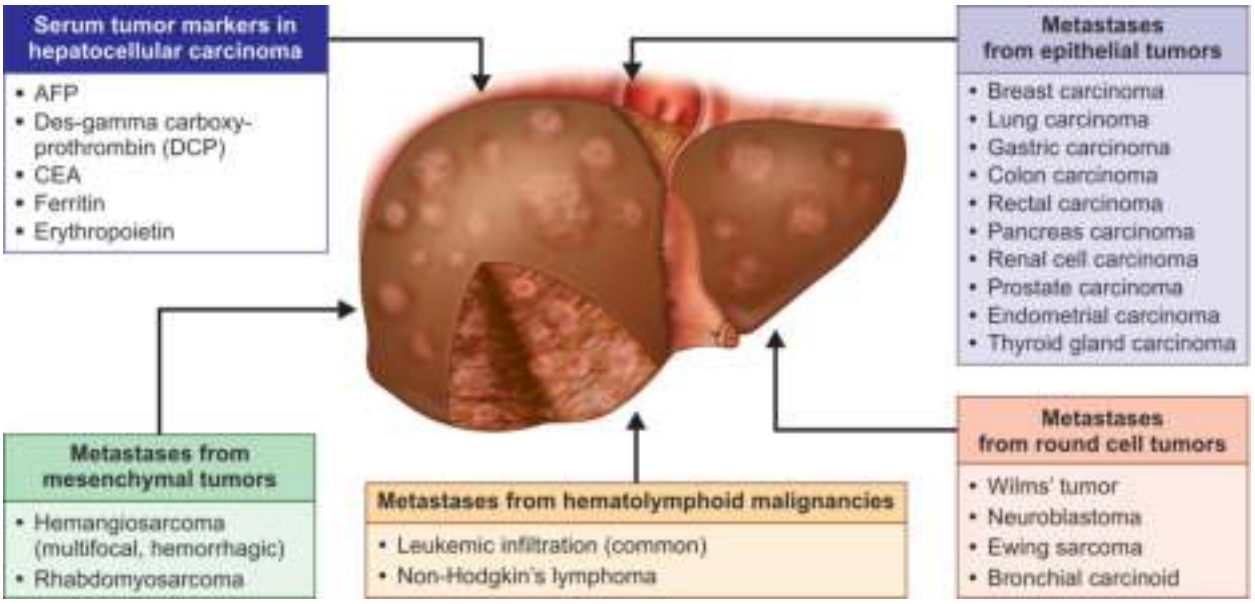


Fig. 6.65: Metastases in liver. Malignant tumors derived from epithelial, mesenchymal and hematolymphoid tissues can metastasize to liver.

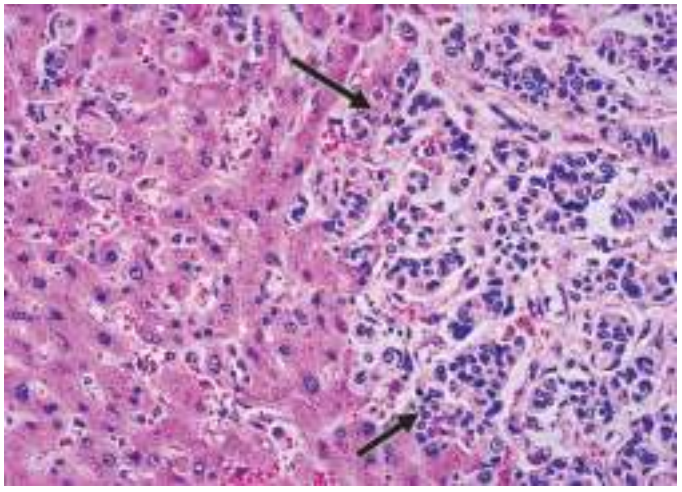


Fig. 6.66: Metastasis from invasive ductal adenocarcinoma of breast in liver. Histologic examination reveals metastatic infiltrating ductal carcinoma from breast is seen on the right side and normal liver parenchyma on the left side (arrows) (400X).

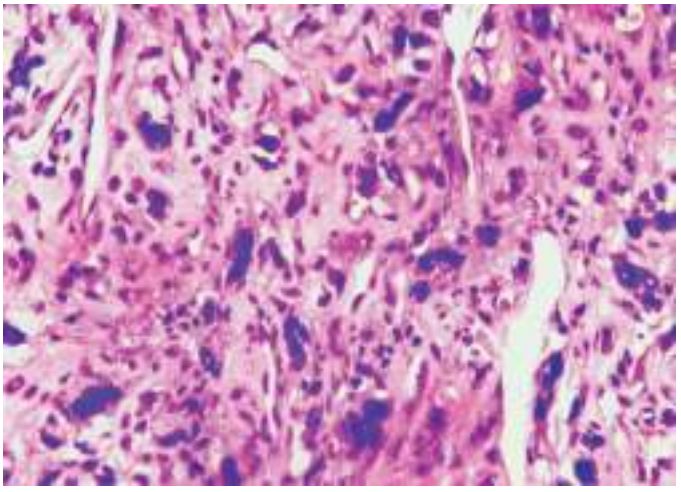


Fig. 6.67: Metastasis from osteosarcoma in lung. Osteosarcoma in lung is the most common mesenchymal bone tumor. Osteosarcoma metastasis in lung at diagnosis has a significantly poor prognosis, even when patient is treated with surgery and chemotherapy. Histologic examination shows pleomorphic atypical cells with hyperchromatic nuclei, numerous atypical mitotic figures and lace-like osteoid and calcified spicules produced by atypical cells in the pulmonary parenchyma (400X). (*Courtesy*: Dr. Tushar Kalonia)

metastases are most often multiple, and sharply circumscribed lacking cavitation in the lung. Calcification is unusual in the lungs except metastases from osteosarcoma and chondrosarcoma.

- Biopsy is obtained by bronchoscopy, transthoracic needle aspiration, and surgery and studied by histologic examination and immunohistochemistry.
- Chemotherapy is most common treatment for pulmonary metastases, which is administered to shrinkage of malignant tumors. Chemotherapy is sometimes induce along with surgical excision and targeted therapy to treat pulmonary metastases.

BONE METASTASIS

Batsons' paravertebral venous plexus has connection with the vena cava and vertebral venous system. Vertebral venous system is extensive and valveless, and has constant connection to breast, thyroid, lung, and prostate. In addition, the venous plexuses are connected to dural sinuses and vasa vasorum of humerus and femur. There are numerous interconnections

between vascular and lymphatic system. CSCs in blood circulation usually arrive in the bone via hematogenous route. Metastasis to bone occurs irrespective of anatomic localization of the tumor and natural pathways of venous drainage. It is important to know the major malignant tumors that metastasize to bone via hematogenous route, which include malignant epithelial tumors of the bronchus, breast, thyroid gland, kidney and prostate gland.

Age Group Distribution of Bone Metastasis

Metastatic carcinoma is the most common tumor of bone after 40 years of age. Most common malignant tumors metastasizing to the bones in male adults include prostatic carcinoma, small cell lung carcinoma, lung adenocarcinoma, gastric carcinoma, colorectal carcinoma, pancreatic carcinoma, renal cell carcinoma and transitional cell carcinoma of urinary bladder.

- Most common malignant tumors metastasizing to bones in female adults include breast carcinoma, thyroid carcinoma, renal cell carcinoma, endometrial carcinoma, uterine cervical carcinoma and uterine leiomyosarcoma.
- In children, metastatic tumors of bone include neuroblastoma, embryonal rhabdomyosarcoma, and retinoblastoma.
- Patient with metastatic bone disease presents with pain, swelling, deformity, encroachment of hemopoietic tissue in the bone marrow, compression of spinal cord or nerve roots and pathologic fractures. Bone metastases in adults and children are given in Table 6.36.

Sites, Solitary and Multifocal Metastases in Bones

Vertebral column is the most common site of metastases in hematopoietic bone marrow; followed by pelvis, ribs, skull and proximal long bones (femur humerus).

Table 6.36 Bone metastases in adults and children

Age Group	Tumors Metastasizing to Bones	
Male adults	▪ Prostatic carcinoma ▪ Small cell lung carcinoma ▪ Lung adenocarcinoma ▪ Gastric carcinoma ▪ Colorectal carcinoma	▪ Pancreatic carcinoma ▪ Renal cell carcinoma ▪ Transitional cell carcinoma of urinary bladder
Female adults	▪ Breast carcinoma ▪ Thyroid carcinoma ▪ Renal cell carcinoma	▪ Endometrial carcinoma ▪ Cervical carcinoma ▪ Leiomyosarcoma
Children	▪ Retinoblastoma ▪ Clear cell sarcoma of kidney	▪ Neuroblastoma ▪ Embryonal rhabdomyosarcoma

Skeletal metastases are uncommon below knee or elbow joints. Basal cell carcinoma of skin, gliomas, and soft tissue sarcomas (except embryonal rhabdomyosarcoma) rarely metastasize to bone.

- Patient may present with bone pain, swelling, deformity, pathological fracture, encroachment of hemopoietic tissue in the bone marrow, compression of spinal cord or nerve roots, urinary incontinence, bowel incontinence, weakness in arms and legs, and hypercalcemia-induced nausea.
- Metastatic deposits in hematopoietic bone marrow in descending order are given in Table 6.37. Solitary and multifocal bone metastatic disease is given in Table 6.38.

Type of Bone Lesions seen on Imaging Techniques

Bone metastases can be evaluated using radionuclide bone scanning, except in multiple myeloma where a skeletal survey is required. Osteolytic lesions are most often detected through radiographic examination, and are also known as X-rays.

- The use of modern imaging techniques such as bone scintigraphy, positron emission tomography (PET) and whole-body magnetic resonance imaging (MRI) to determine the extent of bone metastasis and its character, i.e. osteolytic or osteoblastic lesions.

Table 6.37 Metastatic deposits in hematopoietic bone marrow in descending order

Vertebral column (most common site)
Pelvis
Ribs
Skill
Humerus—proximal end
Femur—proximal end

Skeletal metastases are uncommon below knee or elbow joints. Tumors rarely metastasizing to bones include basal cell carcinoma, gliomas and soft tissue sarcomas except embryonal rhabdomyosarcoma.

Table 6.38 Solitary and multifocal bone metastatic disease

Solitary Lesion in Metastatic Bone Disease
▪ Lung carcinoma
▪ Renal cell carcinoma
▪ Well-differentiated thyroid carcinoma
Multifocal Lesion in Metastatic Bone Disease
▪ Breast adenocarcinoma
▪ Gastric adenocarcinoma
▪ Colorectal adenocarcinoma
▪ Neuroblastoma

Table 6.39 Osteolytic or osteoblastic lesions on radiograph in metastatic bone disease

Radiograph Findings	Tumors Metastasizing to Bone	Comments
Osteolytic lesions	▪ Renal cell carcinoma ▪ Well-differentiated follicular thyroid carcinoma ▪ Gastric adenocarcinoma ▪ Colorectal adenocarcinoma ▪ Malignant melanoma ▪ Non-Hodgkin's lymphoma ▪ Neuroblastoma	▪ Increased serum calcium (hypercalcemia) ▪ Increased excretion of calcium in urine (calciuria) ▪ Increased excretion of hydroxyproline containing peptide in urine indicates bone matrix destruction
Osteoblastic lesions	▪ Prostatic adenocarcinoma ▪ Ductal adenocarcinoma of female breast ▪ Hodgkin's disease	▪ Increased serum alkaline phosphatase ▪ Decreased serum calcium level
Osteolytic/osteoblastic lesions	▪ Small cell lung carcinoma ▪ Ductal carcinoma in female breast	Serum and urinary findings depend on type of lesion

- Breast carcinoma and small cell lung carcinoma may produce either osteolytic or osteoblastic lesion. Osteolytic or osteoblastic lesion on radiograph in metastatic bone disease is given in Table 6.39.

Laboratory Diagnosis of Bone Metastasis

A high level of blood alkaline phosphatase could suggest that patient has bone metastasis. An elevation of blood calcium can alert clinician to possible bone metastasis. Radiologic examination (radiograph, CT scan, MRI) and histologic examination of bone marrow aspiration and biopsies establish the diagnosis of bone metastasis.

- **CT/MRI-guided bone biopsies:** A biopsy needle retrieves a sample of bone and sent for histologic examination to determine the cause of bone pain and tenderness, and to distinguish between benign and malignant primary/secondary bone tumors including other bone abnormalities.
- **Liquid biopsies in bone tumors:** Liquid biopsies are done to analyze ctDNA, CTCs and EVs and detect tumor-specific DNA mutations in primary (osteosarcoma, Ewing sarcoma) and bone metastases (breast lung, prostate), which provide new avenues in early detection and monitoring treatment of disease.

BRAIN METASTASIS

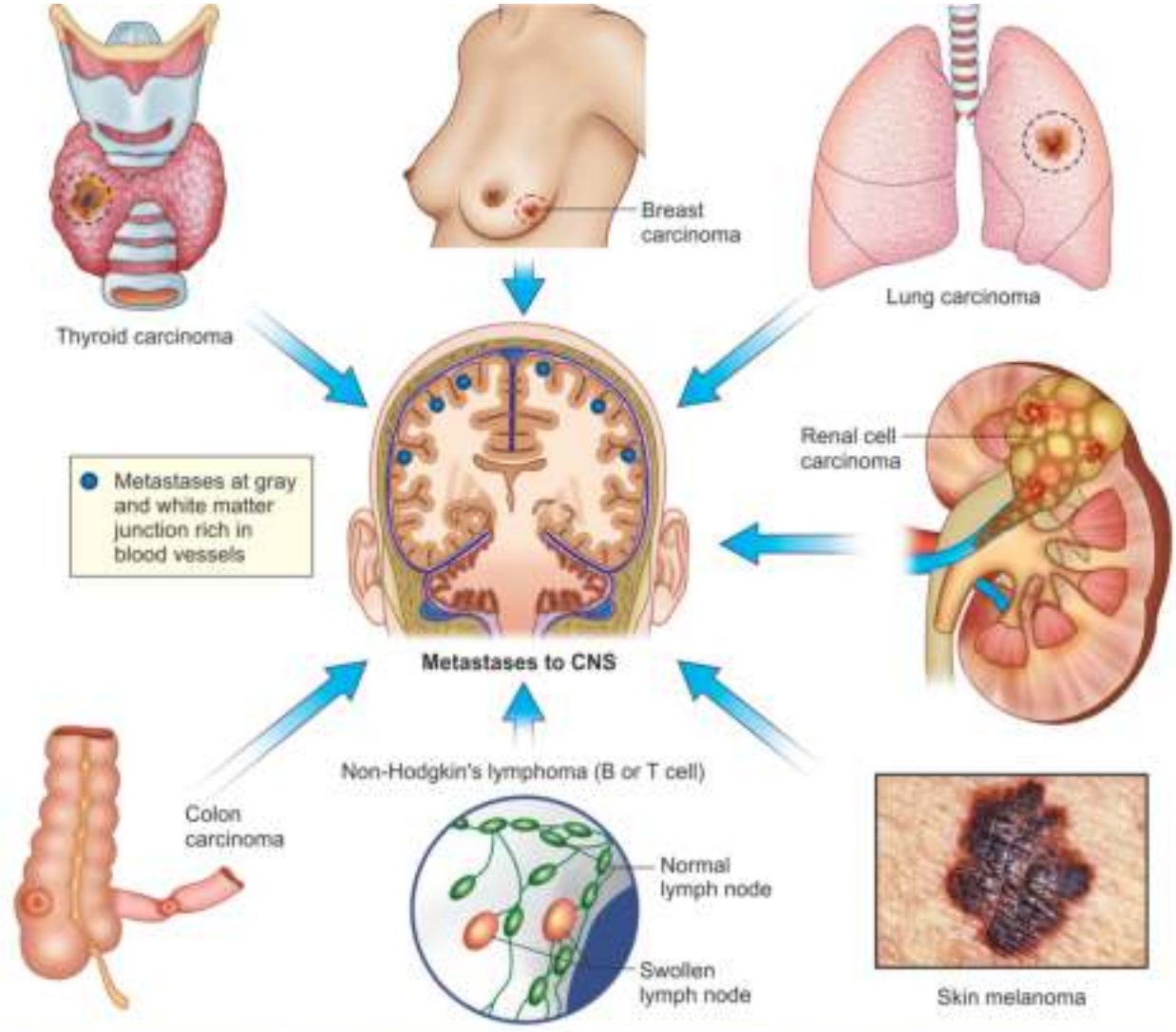
Metastatic tumors are more common than primary intracranial tumors of central nervous system. Certain malignant tumors metastasizing to the brain in the region at the junction of gray and white matter rich in blood vessels include thyroid carcinoma, breast carcinoma, lung carcinoma, renal cell carcinoma, colon carcinoma, non-Hodgkin's lymphoma, and melanoma in 30% of adults. Common brain metastases in adults are shown in Fig. 6.68.

- Brain metastasis occurs in 6–10% children from osteosarcoma, rhabdomyosarcoma, acute lymphoblastic leukemia (ALL) and testicular germ cell tumors. As the metastatic brain tumor grows, it creates pressure on surrounding brain tissue and disrupt functions. Brain metastases can cause many signs and symptoms.
- Pathologic patterns of brain metastases include dural metastasis, leptomeningeal carcinomatosis, miliary metastasis, intravascular metastases, and tumor-to-tumor metastasis in which CSCs from primary malignant tumor metastasize via hematogenous route to another malignant tumor. For example, primary renal cell carcinoma metastasis into a pancreatic neuroendocrine tumor exhibiting a tumor-to-tumor metastasis has been reported. Immunohistochemical markers can be useful to separate metastatic tumors from primary brain tumors.

ADRENAL GLAND METASTASIS

Metastatic spread to bilateral adrenal glands occurs mainly by hematogenous route, involving bilateral adrenal glands. Primary malignant tumors metastasizing to the adrenal glands include renal cell carcinoma, lung carcinoma, colon carcinoma, melanoma and non-Hodgkin's lymphoma. Adrenal gland metastases from ovarian carcinomas are extremely rare.

- Most patients with adrenal gland metastases are asymptomatic. Adrenal gland metastases have been primarily detected on autopsy.
- Computed tomography (CT) scan, magnetic resonance imaging (MRI), and positron emission tomography (PET) scan are done in diagnosing, staging and follow up. Adrenal gland metastases are increasingly detected as incidental finding. Occasionally, patients may present with back pain or abdominal pain due



Primary cancer metastases to central nervous system	Immunohistochemistry
• Thyroid carcinoma	Thyroglobulin, CA 125 cell surface glycoprotein demonstrated by monoclonal antibody OC-125 and TTF1
• Breast carcinoma	ER, PR, HER2/neu, CK7, GCDFP-15 and mammoglobulin
• Lung carcinoma (squamous cell carcinoma)	EMA, CK7, TTF1, p63
• Renal cell carcinoma	Cytokeratin, vimentin
• Skin melanoma	HMB-45, melan-A (MART-1), CAM 5.2, S-100
• NHL (B cell type)	CD10, CD19, CD20
• NHL (T cell type)	CD3
• Colon carcinoma	CK20, CDX2 and β-catenin

Fig. 6.68: Common brain metastases in adults from thyroid, breast, lung, kidney, colon, skin melanoma and NHL.

to rapidly growing metastatic tumor in adrenal gland and causing adrenal insufficiency.

- The best treatment for metastatic adrenal gland tumor is chemotherapy. Adrenalectomy may be recommended, when the primary disease is well controlled and adrenal gland metastatic disease. On gross pathology, arenal gland metastases are gray-white or black colored, solitary or multiple, firm heterogenous masses with occasional area of hemorrhage and necrosis.
- Cytologic and histologic examination of involved adrenal gland demonstrates features of metastatic disease.
- Immunohistochemical markers could be useful to separate metastatic tumors from primary adrenocortical tumors.

SEEDING OF CANCER STEM CELLS AND TRANSCOELOMIC ROUTE OF METASTASIS

Transcoelomic (meaning 'across the peritoneal cavity) metastasis route refers to the dissemination of malignant tumors throughout the surfaces and organs of the abdominal cavity and pelvic cavity covered by peritoneum.

- Cancer stem cells (CSCs) originating in organs (i.e. stomach, colon, breast, endometrium, appendix, pancreas and ovary) can invade into the peritoneal cavity.
- The omentum is the most common site of ovarian carcinoma metastasis via the transcoelomic and hematogenous routes.
- Advanced breast carcinoma can invade peritoneum via the lymphatic and hematogenous routes.
- Surgical resection of colorectal carcinoma can cause intraperitoneal seeding.
- As the CSCs from various malignant tumors of breast, gastrointestinal tract and lung sink within the peritoneal cavity. They settle in various sites, and cause inflammatory reaction with fibrin formation, malignant effusion and adhesion of organs. There can be seeding of CSCs in the pleural and pericardial sacs.

PERITONEAL CAVITY SEEDING

Primary carcinomas of stomach, colon, breast, endometrium, appendix and pancreas may involve the peritoneum and ovaries. Krukenberg tumor, which refers to bilateral ovarian neoplasms and is composed of atypical, mucin containing signet-ring cells. Malignant tumors can spread to the ovary by several mechanisms: direct, transcoelomic spread, and lymphovascular routes.

- **Clinical features:** Peritoneal seeding is common in patients with ovarian serous or mucinous cystadenocarcinomas. Mucinous cystadenocarcinoma secretes large amount of mucinous material and filling the abdominal cavity is known as 'pseudomyxoma peritonei'.
  - Woman with Krukenberg tumor presents with bilateral ovarian enlargement.
  - Final diagnosis of Krukenberg tumor is established by histologic examination and immunohistochemistry. Patients are treated by debulking surgery with or without chemotherapy.
- **Gross morphology:** Gross morphologic examination of 'Krukenberg tumor' reveals most often bilateral ovarian involvement <10 cm in size with multiple small nodules on ovarian surface and extensive intra-abdominal spread. Hematogenous spread of primary malignant tumor to ovary most often involves hilar region of the ovary. Gross morphology of Krukenberg tumor is shown in Fig. 6.69.
- **Histologic examination:** Histologic examination of 'Krukenberg tumor' shows multinodular growth pattern with intervening normal ovarian parenchyma, multiple vascular emboli, specific pattern favoring metastasis such as signet cell carcinoma, pseudomyxoma peritonei, colloid carcinoma, infiltrative pattern of small glands with desmoplastic reaction and single cell infiltration. Histology of Krukenberg tumor is shown in Fig. 6.70.
- **Immunohistochemistry:** Gastric and colon carcinoma metastasis to ovaries are positive for immunohistochemical markers (CK7, CK20, CDX2, SATB to MUC), and breast carcinoma is positive for GCDFP-15.

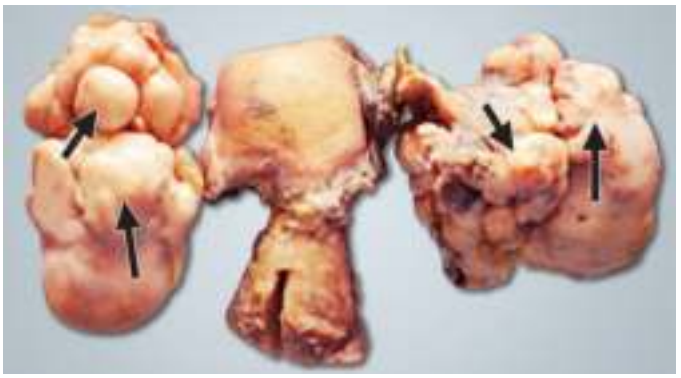


Fig. 6.69: Krukenberg's tumor gross morphology: Both ovaries show involvement as a result of metastases from primary cancers of stomach or colon or breast (arrows).

PLEURAL CAVITY SEEDING

Malignant pleural effusion refers to the presence of CSCs in the pleural cavity associated with significant morbidity and an overall poor prognosis. The most

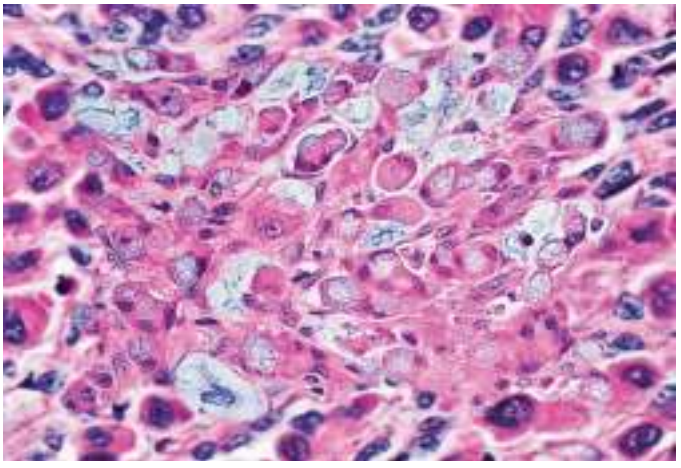


Fig. 6.70: Krukenberg's tumor histology. Tumor is composed of nests of mucin-filled "signet ring" epithelial cells with eccentric nuclei within a cellular stroma derived from the ovary (400X).

common etiologies of malignant pleural effusion are lung carcinoma, breast carcinoma, non-Hodgkin's lymphoma, ovarian carcinoma, gastric carcinoma and mesothelioma in decreasing frequency.

- **Pathogenesis:** Malignant pleural effusion occurs as a result of loss of cell adhesion molecules, detachment of CSCs from the primary tumor invades surrounding tissue. CSCs penetration in venules, migration through the pleura, production of autocrine growth factors induce angiogenesis.
- **Clinical features:** Patient presents with progressive dyspnea, chest pain, cough, constitutional symptoms including weight loss, malaise and anorexia. Severity of symptoms depends on the rate of fluid accumulation in the pleural cavity rather than on the total quality of fluid that might have accumulated over a prolonged time period. Clinical history and physical examination assist in further investigations.
- **Physical examination:** Physical examination may demonstrate stony dullness on percussion, decreased vocal resonance, and tactile fremitus and decreased intensity of breath sounds over the affected region.
- **Diagnostic modalities:** Pleural effusion can be detected by radiograph, ultrasonography, computed tomography (CT) scan and magnetic resonance imaging (MRI). Therapeutic thoracentesis should be completed prior to any definitive pleural procedure to ensure the patient benefits from aspiration of pleural fluid. Pleural fluid aspiration is performed for diagnostic and combined with a therapeutic procedure to relieve symptoms.
  - Cancer stem cells (CSCs) may be demonstrated in pleural fluid on cytological examination.
  - Malignant pleural effusions are generally exudates and lymphocytic predominant. Pleural biopsy is obtained and sent for histologic examination, which should be directed to look for primary tumor.
- **Treatment:** The primary malignant tumor cell type will predict responsiveness to chemotherapy or radiation in the setting of malignant pleural effusion. An indwelling catheter may be inserted into the pleural cavity, which allows intermittent drainage with a vacuum bottle.

PERINEURAL AND LEPTOMENINGEAL ROUTES OF METASTASIS

Cancers that spread by perineural and leptomeningeal routes rely on the nervous system.

- **Perineural spread of cancers:** CSCs may also grow along perineural spaces leading to compression of nerves causing pain and loss of function. Observations reveal that perineural spread is detected in the prostatic carcinoma, colorectal carcinoma and head and neck cancers, where these sites are heavily innervated.
- **Leptomeningeal route of metastasis:** In contrast to perineural spread of cancers, leptomeningeal spread refers to the spread of malignant tumors by the cerebrospinal fluid involving leptomeninges overlying brain parenchyma, and spinal cord; and subarachnoid space. Glioblastoma multiforme, medulloblastoma and ependymoma follow leptomeningeal route originate in the brain, but not all. Non-Hodgkin's lymphoma, breast carcinoma and lung carcinoma especially small cell lung carcinoma are associated with the greatest predilection to metastasize to the subarachnoid space via leptomeningeal route.
  - Leptomeningeal route of metastasis consists of metastatic CSCs growing either attached to the pia mater of brain, and spinal cord or floating in the cerebrospinal fluid (CSF) in the subarachnoid space, that provides favorable environment for the growth of metastatic tumor. Rich vascular supply of meninges provides hematogenous metastatic access to the subarachnoid space.
  - Cerebrospinal fluid has high content of glucose and oxygen to support metastatic CSCs with high metabolic activity that is the reason that metastatic CSCs escape angiogenesis.
  - Patient with leptomeningeal metastasis has overall median survival of 2–4 months. There are many new approaches to the diagnosis and treatment of leptomeningeal meningitis, that promise to extend life and prevent disability.
  - Leptomeningeal meningitis is diagnosed by molecular techniques. Patients are treated by drugs available for intrathecal administration, using systemic chemotherapy; and applying strategies such as gene therapy and immunotoxins to the management of leptomeningeal metastases.

INTRAEPITHELIAL ROUTE OF METASTASIS

Intraepithelial route of metastasis can occur, where malignant epithelial tumor originates from duct in the breast. Patient presents with eczematous, scaling, pruritic eruption of the skin that clinically mimicks inflammatory dermatoses. Paget's disease of the female breast is shown in Fig. 6.71.

- Paget's disease of the female breast involving nipple and areola originates by intraepidermal migration of CSCs from *in situ* carcinoma or invasive ductal carcinoma of breast through the lactiferous ducts to the surface epithelium of the nipple and areola.

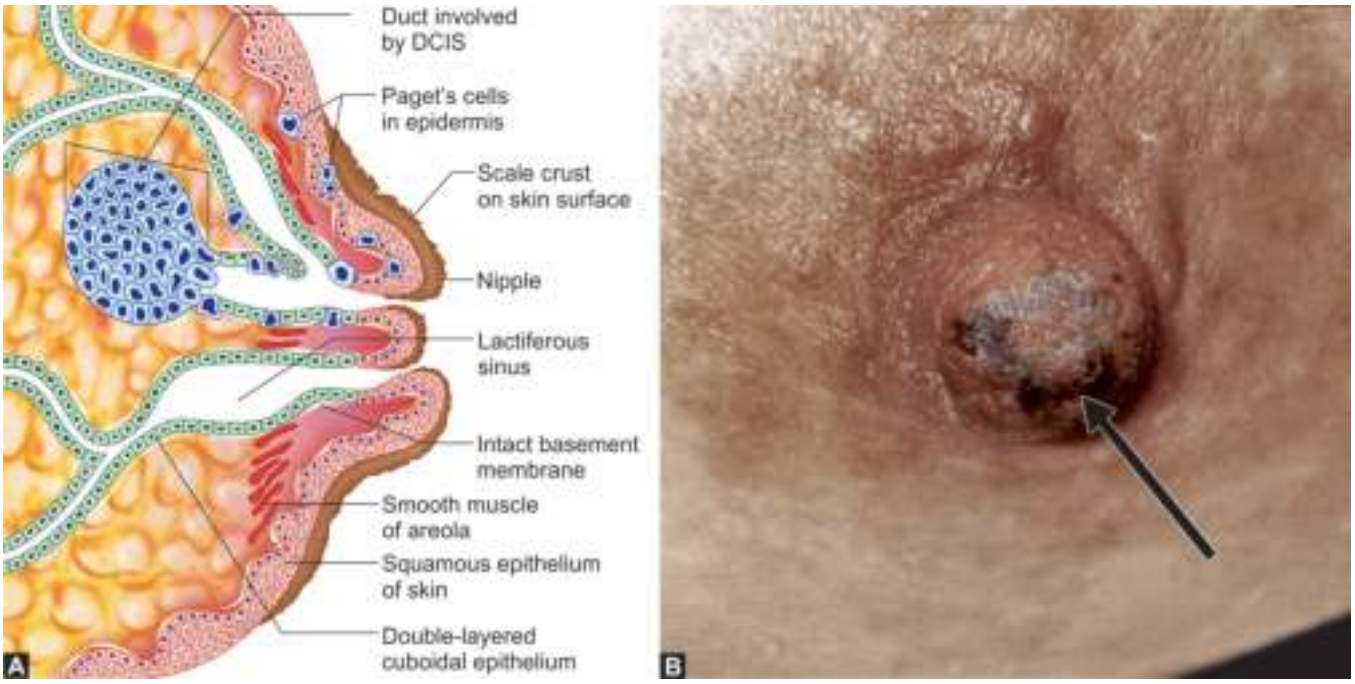


Fig. 6.71: Paget's disease of the female breast. It is ductal carcinoma that affects skin of the nipple, which sometimes is accompanied by more invasive breast carcinoma. Patient presents with crusting of the skin and areola and discharge mimicking eczematous lesion (arrow).

- Paget cells infiltrate the entire thickness of epidermis singly or in nests and laterally in the surface epithelium with clear or pale eosinophilic cytoplasm, vesicular nuclei, prominent nucleoli, and increased mitotic activity. Glandular and acinar structures and signet ring cells with intracytoplasmic mucin may be present.
- Paget cells are positive for immunohistochemical markers such as LMWCK (CAM 5.2, CK7), AE1/AE3, epithelial membrane antigen (EMA), HER2/neu, and GATA3.

IATROGENIC MALIGNANT TUMOR IMPLANTATION DURING SURGERY

Iatrogenic malignant tumor implantation results from various medical or surgical procedures used for diagnosis and treatment of a malignancy. It involves accidental spillage of CSCs by direct contact during surgical procedures. It is significant potential hazard during malignant tumor surgery. Dissemination of CSCs can result in local recurrence or distant metastasis from the tumor under treatment.

MOLECULAR BASIS OF CANCER

Cell division occurs by two processes: (a) mitosis in somatic cells and (b) meiosis in gametes (ova and sperms). Mitotic activity is restricted to somatic cells that eventually repair injuries, and the committed stem cells have the ability continuously to regenerate new cells and differentiate for maintenance of hemostasis. **Cell division** is the process by which a parent somatic cell divides and duplicates accurately the vast amount of deoxyribonucleic acid (DNA) into the chromosomes and then segregate the copies precisely into two genetically identical daughter cells.

- **Cell cycle–cell division major activities:** Normal cell cycle consists of two major activities: interphase (G1, S, and G2 phases) and mitotic phase (M phase). During interphase, cells are metabolically active, which accumulate nutrients needed for mitosis and duplicating its DNA in somatic cells. Mitotic (**M**) phase consists of prophase, metaphase, anaphase, and telophase.
  - Cell division is tightly regulated by two sets of opposite functioning genes, i.e. proto-oncogenes (growth-promoting), and tumor suppressor (growth-inhibiting) genes.
  - Apoptosis regulatory genes, and DNA repair genes also play important role in cell division.
  - Activation of proto-oncogenes (oncogenes), and inactivation/biallelic loss of tumor suppressor

genes leads to unrestricted cell proliferation and carcinogenesis to produce malignant tumors.

- **Cell cycle regulation:** Cell cycle is tightly regulated by positive regulators (i.e. cyclins and cyclin-dependent kinases), and negative regulators, i.e. RB, TP53, INK family of proteins ($p16^{INK}$, $p15^{INK}$, $p18^{INK}$, $p19^{INK}$), CIP/KIP family of proteins ($p27^{KIP}$, $p\ 21^{KIP}$, $p57^{KIP}$), and BRCA1 and BRCA2 acting at various phases of the cell cycle.
  - Extracellular regulation of cell cycle is controlled by mitogens (growth factors), growth factor receptors, and survival factors. A lone cyclin or cyclin-dependent kinase (CDK) exists in inactive state until cyclin binds to CDK.
  - Binding of cyclin to CDK results in formation of cyclin–CDK complex, that gets phosphorylated by receiving phosphate protein by kinase. Activated cyclin–CDK complex phosphorylates target functional proteins in the genome and activate the cell cycle.
- **Cell cycle–cell division dysregulation and cancer:** Cell cycle dysregulation is a hallmark of cancer. Following DNA damage, the ability of normal cells to undergo cell cycle arrest is crucial for the maintenance of genome integrity.
  - The biochemical pathways that inhibit the cell cycle response to cellular stressors are called checkpoints. Defective checkpoint function leads to acquisition of genetic alterations (chromosome mutations), genomic instability that contribute to carcinogenesis, development of malignant tumor and chemoresistance.
  - Transformation of normal cell to CSC involves disruption of key processes such as cell cycle regulation, growth factor-induced cell cycle progression, apoptosis and senescence.
  - Loss of function of tumor suppressor genes (TP53, SMAD4, p14, p16, CDKN2A, and p27), and gain of function of oncogenes (i.e. K-RAS, HER2/neu, MDM2, cyclin D-CDK4 complex) are linked to human cancers.
  - Cancer stem cells (CSCs) can alter growth factor signaling to unrestricted cell proliferation by following mechanisms, which include: (a) CSCs may synthesize growth factors to stimulate self-growth in an autocrine fashion, (b) CSCs may also harbor growth factor receptor mutations that make the receptor constitutive active, i.e. active without having a growth factor bound to it, which permits continuous stimulatory signal to proliferate malignant neoplastic cells, i.e. CSCs, and (c) CSCs may overexpress growth factor receptors resulting in increased signaling. For example, a type of epidermal growth factor (EGF) receptor, known as HER2/neu, is overexpressed in breast carcinoma (HER2/neu-positive). Trastuzumab (**Herceptin**) is a monoclonal antibody directed against HER2 receptor that is used to treat HER2/neu-positive breast cancers. Oncology is a branch of medicine that specializes in the diagnosis and treatment of cancer.
- **Genes most commonly involved in the process of carcinogenesis:** Several regulatory genes regulate the normal cell growth in human body, which include: proto-oncogenes, tumor suppressor genes, apoptosis regulatory genes, and DNA repair genes. Dysregulation of regulatory genes is linked to carcinogenesis and development of malignant tumor.
  - Proto-oncogenes are growth-promoting genes (HER2/neu, RAS, Myc, SRC, hTERT, BCL-2, and EGFR), which code for proteins that promote cell division, growth and development. The viruses have appropriated their oncogenes from normal cellular DNA by genetic recombination. Retrovirus associated oncogenes that have been discovered in altered form in human cancers are given in Table 6.40.
  - Tumor suppressor genes (**growth-inhibitory genes**) code for tumor suppressor proteins that inhibit cell division. Caretaker tumor suppressor genes code for proteins that engage in maintaining the genome stability and DNA repair thus prevent carcinogenesis. Examples of caretaker tumor suppressor genes are BRCA1, BRCA2, DNA mismatch repair (MLH1, MSH2, MSH3, MSH6, PMS1, PMS2), Fanconi anemia, DNA repair and DNA nucleotide base pairs excision repair genes. In contrast, gatekeeper tumor suppressor genes regulate cell cycle. Gatekeeper tumor suppressor genes (TP53 guardian of the genome, RB1, APC, BRCA, APC/β-catenin genes) code for proteins, which regulate cell cycle. Dysregulation of tumor suppressor genes is linked to carcinogenesis.
  - Apoptosis regulatory genes include pro-apoptotic genes (TP53, BAX, c-Myc) and anti-apoptotic B cell lymphoma (BCL-2) family of genes. Apoptosis is a programmed cell death. There are two primary pathways that lead to apoptosis: extrinsic (death receptor-mediated) pathway and intrinsic (mitochondrial-mediated) pathway. Both apoptosis pathways result in the activation of caspases, which are final effectors of apoptosis and cleave several proteins leading to programmed cell death.
  - DNA mismatch repair genes include BRCA1, BRCA2, ATM, CHEK2, MLH1, MSH1, MSH3, MSH6, PMS1, PMS2, XP, MLM, and TERT, Fanconi anemia genes and DNA nucleotide excision repair genes, which encode DNA repair proteins.

Table 6.40 Retrovirus associated oncogenes that have been discovered in altered form in human cancers

Name of the Virus	Virus Present in Species	Viral Oncogene	Oncoprotein	Homologous Oncogene Observed in Human Cancers
Rouse sarcoma	Chicken	v-Src	Nonreceptor tyrosine kinase	Colon carcinoma, breast carcinoma
Abelson murine leukemia virus	Mouse	v-Abl	Nonreceptor tyrosine kinase	Precursor B cell lymphoid blast crisis of chronic myelogenous leukemia (CML)
Avian erythroblastosis	Mouse	v-Erb2	Receptor tyrosine kinase (RTK), also known as epidermal growth factor receptor (EGFR)	Gastric carcinoma, lung carcinoma, breast carcinoma (receptor for EGF, related erbB2/HER2/neu protein overexpressed in breast carcinoma)
McDonough feline sarcoma	Cat	v-Fms	Receptor tyrosine kinase (RTK)	Acute myelogenous leukemia (Fms receptor for colony stimulating factor 1 found in few cases of AMLs)
H-Z feline	Cat	v-Kit	Receptor tyrosine kinase (RTK) for stem cell factor	Gastrointestinal tract stromal tumors
Murine sarcoma 3611	Mouse	v-Raf	Serine threonine kinase (B-Raf mutation in melanoma)	Urinary bladder carcinoma
Simian sarcoma	Monkey	v-Sis	Platelet-derived growth factor (PDGF)	Many human cancers
Harvey sarcoma	Mouse/rat	H-RAS (mutant H-RAS in human cancers)	Small GTP proteins	Urinary bladder carcinoma
Kirsten sarcoma	Mouse/rat	K-RAS (mutant H-RAS in human cancers)	Small GTP proteins	Many human cancers
Avian erythroblastosis	Chicken	v-erbA	Nuclear receptor (receptor for thyroid hormones)	Hepatocellular carcinoma, renal cell carcinoma, pituitary tumors
Acute myeloblastosis E26	Chicken	v-ets	Transcription factor	Leukemias
Avian myelocytoma	Chicken	v-Fos, v-Jun, v-Myc (related N-Myc gene in pediatric neuroblastoma)	Transcription factor	Many human cancers, e.g. osteosarcoma and fibrosarcoma
Reticuloendotheliosis	Turkey	rel (constitutes NF-κB transcription factor)	Transcription factor	Lymphoma

- **Carcinogenesis:** Malignant tumors most often have both activation of oncogenes and inactivation/biallelic loss of tumor suppressor genes. Transformation of normal cell to CSC results from accumulation of successive somatic nonlethal gene mutations, so that cell can survive and undergo unrestricted cell division and thus propagate multiple gene mutations and expression of mutant forms of growth factors, growth factor receptors, signal transducing proteins, transcription factors, DNA repair proteins, chromatin remodelers, regulatory proteins of apoptosis, positive and negative regulatory proteins of the cell cycle, generally give rise to dominantly active oncogenes resulting in carcinogenesis and development of malignant tumor. Conversion of one of the two alleles of proto-oncogene to an oncogene, is sufficient for initiation, promotion and progression of malignant tumor. However, it requires inactivation/biallelic loss of tumor suppressor genes to promote malignant tumor growth. Transformed CSC acquires self-sufficiency in sustained growth signals, inactivation/biallelic loss of tumor suppressor signaling, evasion of apoptosis, defective DNA repair mechanisms, uncontrolled cell division, angiogenesis, escape immune destruction of CSCs, invasion into surrounding tissues, and metastasis to distant organ(s) through stepwise acquisition of complementary gene mutations.

- **Initiation, promotion and progression to malignant phenotype:** Genetic alterations of cellular proto-oncogenes (i.e. oncogenes) lead to initiation of transformation of normal cell to CSC. Activation of a proto-oncogene into an oncogene occurs due to several different mechanisms: numerical and/or structural alterations in chromosomes, chromothripsis, gene amplification, gene overexpression, gene deletion, and point mutation. Promotion occurs due to mutations in tumor suppressor genes, which induce cell growth through activation of cell cycle and propagation of gene mutations induced by initiator oncoproteins. Both initiator and promoter oncoproteins must act together to transform normal cell to CSC. Activation of oncogenes promote progression of malignant tumor, i.e. angiogenesis, invasion, metastasis to distant organs(s) and immunologic tolerance. Tumor angiogenesis is the process of new blood vessels formation from pre-existing blood vessels. Tumor invasion is the mechanism by which CSCs invade and expand into nearby environments. Tumor invasion is the process by which malignant neoplastic cells, i.e. CSCs in malignant epithelial tumor (carcinoma invade basement membrane and then surrounding tissues. Tumor metastasis is the process by which malignant tumor spreads from its origin to another site of the body via lymphatic, hematogenous and transcoelomic routes.
- **Gain-of-function (dominant) versus loss-of-function (recessive) mutations:** Gain-of-function ('dominant') mutation in proto-oncogene encodes oncoprotein. Inactivation/biallelic loss of tumor suppressor genes (recessive mutations) results in unrestricted cell cycle progression and malignant phenotype.
- **Oncogenic driver versus passenger mutations in cancer evolution:** Multistep carcinogenesis results from accumulation of successive driver and passenger mutations in genes. Driver gene mutations perturb regulatory or metabolic signaling pathways that promote unrestricted CSC proliferation, and progression to form malignant epithelial tumor. Numerous additional gene mutations taking place in malignant tumor are called 'passenger gene mutations', which confer malignant phenotype.
- **Genetic and epigenetic alterations and cancer:** Genetic and epigenetic alterations in DNA contribute to abnormal gene expression and genomic instability in cells and development of malignant tumors. The link between the genetic and epigenetic alterations in cancer is demonstrated by the existence of aberrant cellular metabolism and biochemical pathways in CSCs. Genetic alterations in proto-oncogenes, tumor suppressor genes, apoptosis regulatory genes and DNA repair genes are induced by aging process, mutagenic chemical agents, physical carcinogens (ultraviolet rays, ionizing radiation, i.e. X-rays, and γ-radiation), oxygen-derived species (ROS), ethanol metabolic product acetaldehyde, tobacco smoke and incomplete combustion of organic substances. Epigenetic alterations occur due to advancing age and chronic inflammation in the settings of biological carcinogens (HBV, HCV, EBV, HPV, *Helicobacter pylori, Schistosoma haematobium, Opisthorchis viverrini, Clonorchis sinensis, Aspergillus flavus*). Epigenetic alterations such as DNA methylation, histone modifications, nucleosome remodeling and microRNAs dysregulate gene expression leading to development of malignant tumors.
- **Targeting therapy in cancers:** Debulking of malignant tumor performed by surgery is one of the strategies in the management of human cancers. Targeted therapy is the foundation of precision medicine to treat human cancers with medications that disrupt cellular mechanisms, which induce unrestricted proliferation of CSCs and promotion of malignant tumor growth. Targeted therapy does not directly kill CSCs, but alter their biological composition and thus inhibit their growth.
  - Basic aim of targeted therapy is to antagonize specific hallmark of cancer. As researchers learn more about changes in DNA and aberrant proteins involved in driving malignant phenotype, they are in a position to design promising treatments that target aberrant proteins.
  - Most targeted therapies are either small molecule inhibitors or monoclonal antibodies. Small molecule inhibitors are minute organic compounds that attach to the surface of CSC, then enter the CSCs and interfere with their activity. Monoclonal antibodies are laboratory-manufactured cancer-specific antibodies which attach to the surface of CSCs and activate the immune system to induce destruction of CSCs.

CHROMOSOMAL ABNORMALITIES AND DYSREGULATION OF GENES: MECHANISMS

Normal gene expression is the process by which a gene gets '**turned on**' in a cell to make RNA and proteins essential for cellular processes. Four classes of normal

cellular regulatory genes include proto-oncogenes (growth-promoting), tumor suppressor (growth-inhibiting) genes, apoptosis regulatory genes and DNA repair genes, which are the principal targets of mutations causing malignant phenotype. Deregulated function of proto-oncogenes and tumor suppressor genes leads to transformation of normal cell to CSC and tumorigenesis.

- **Proto-oncogenes:** Proto-oncogenes are normal growth-promoting genes, which include HER2/neu (ERBB2), RAS family of genes, Myc, SRC family of tyrosine kinase, hTERT gene (codes for telomerase reverse transcriptase), BCL-2 (B cell lymphoma), EGFR and CCND1 proto-oncogene (codes for cyclin D protein).
  - Each proto-oncogene codes for specific growth factor, growth factor receptor and intracellular regulatory protein responsible for providing positive signals that lead to cell growth, cell division, apoptosis and maintenance of hemostasis.
  - In general, extracellular growth factor binds with growth factor receptor, which activate intracellular downstream signal transducers. The signaling pathway initiates DNA transcription of genes involved in cell growth, which involves binding of transcription factors to DNA regulatory proteins and recruiting chromatin remodelers to carry out gene transcription.
- **Oncogenes:** Oncogenes are mutated forms of normal cellular proto-oncogenes generally involved in promoting cell growth and unrestricted CSC proliferation.
  - Oncoproteins are the products of oncogenes formed when a gene is transcribed and translated to RNA and synthesis of aberrant proteins.
  - Oncogene gets permanently 'turned on', which is known as 'gain-of-function', i.e. dominant gene mutation. This means that only one copy of gene needs to be mutated to induce carcinogenesis.
  - Malignant tumors most often have both activation of oncogenes and inactivation/biallelic loss of tumor suppressor genes, which can invade basement membrane, surrounding tissues and metastasize to distant organ(s).
  - Most malignant tumors occur due to somatic (acquired) rather than germline gene mutations.
- **Viral oncogenes:** Viral oncogenes are responsible for oncogenesis resulting from persistent viral infection when integrated into host genome. Virus-encoded oncoproteins that activate growth factor receptors can induce human cancers. Although different human viruses express different viral oncogenes that induce different tumors, their oncoproteins most often target similar sets of cellular tumor suppressors or signal pathways to immortalize and/or transform infected cells to become CSCs.
- **Proto-oncogene conversion into oncogene—mechanisms:** Activation of a proto-oncogene into an oncogene occurs due to several different mechanisms: numerical alterations in chromosomes, structural alterations in chromosomes, chromothripsis, gene amplification, gene overexpression, gene deletion, and point mutation. Chromosomal rearrangements can be either balanced or imbalanced. Balanced chromosomal rearrangements occur in an even exchange of chromosome material (segment) between two chromosomes without genetic information extra or missing genes, and ideally fully functional. In imbalanced chromosomal rearrangements occur where the exchange of chromosome material is unequal resulting in extra or missing genes.
  - **Numerical alterations in chromosomes:** Numerical alterations in chromosomes (aneuploidy and polyploidy) occur due to segregation errors during mitosis. There may be gain or loss of chromosome. Notability, approximately 90% of human cancers have lost or gained at least one chromosome, e.g. trisomy 12 in chronic lymphocytic leukemia (CLL), trisomy 8 in AML, monosomy 7 in MDS/AML, and monosomy 17 in breast carcinoma.
  - **Structural alterations in chromosomes:** A chromosome's structure can be altered in several ways: translocations (e.g. reciprocal and Robertsonian), deletion, duplications, inversion and ring chromosome. Chromosomal rearrangements can lead to cancer by forming a hybrid gene or by causing dysregulation of a gene. Philadelphia chromosome, which is formed due to rearrangement (reciprocal chromosomal translocation to a transcriptionally active site) that creates the hybrid BCR-ABL fusion gene. The aberrant protein encoded by the hybrid gene accelerates cell division and is associated with chronic myelogenous leukemia (**CML**). Both reciprocal and Robertsonian translocations are one of the most common balanced structural chromosomal abnormalities in the general population accounting for 0.2–4%.

Pathology Pearls: Proto-oncogene Conversion to Oncogene by Imbalanced Chromosomal Rearrangements

- **Chromosomal deletion:** Chromosomal deletion can involve the loss of any number of nucleotides from a single nucleotide to an entire segment of a chromosome.
- **Chromosomal duplication:** Chromosomal duplication simply means that a segment of a chromosome is duplicated, or present in two copies. This results in having extra genetic material, even though total number of chromosomes is usually normally.

- **Chromosomal inversion:** Chromosomal inversion refers to portion of chromosome is broken off, turned upside down, and reattached resulting in inversion of genetic material.
- **Chromosomal insertion:** Chromosomal insertion is a type of mutation that involves the addition of one or more nucleotides into segment of DNA.
- **Ring chromosome:** Ring chromosome can result when a chromosome undergoes two breaks and the broken ends fuse into a circular/ring fashion with or without loss of genetic material.
- **Isochromosome:** An isochromosome is formed when an arm of the chromosome is missing and the remaining arm undergoes duplication.
- **Chromothripsis:** Chromothripsis is a mutational phenomenon characterized by massive clustered genomic rearrangements that are often generated in a single catastrophic event and localized to isolated chromosomal regions leading to cancers and other diseases.
- **Point mutation in proto-oncogene:** Point mutation in proto-oncogene brings a growth-regulatory gene under the control of a different promoter and causes inappropriate expression of the gene. Point mutation in RAS proto-oncogene is linked to lung carcinoma, colon carcinoma and pancreatic carcinoma.
- **Gene amplification of proto-oncogene:** Gene amplification of proto-oncogene is a localized reduplication of a DNA segment, i.e. additional copies of a proto-oncogene.
- **Overexpression of proto-oncogene:** Proto-oncogene is not mutated but overexpressed. For example, HER2/neu proto-oncogene is overexpressed in breast carcinoma.

- **Oncogenes hyperactivate signaling pathways:** Oncogenes can cause hyperactivation of signaling pathways leading to unrestricted cell proliferation, whereas inactivation/biallelic loss of tumor suppressors (p53, pRB, cyclin-dependent kinase inhibitors such as $p16^{INK4A}$, $p15^{INK4B}$, $p21^{CIP1}$) eliminate critical negative regulators of cellular signaling pathways. During the course of tumor progression, CSCs acquire genetic and epigenetic alterations resulting in invasion, angiogenesis, metastasis, evasion of apoptosis. Many of the oncoproteins currently under investigation as possible targets for targeted therapy.

NUMERICAL ALTERATIONS IN CHROMOSOMES

Haploid cells contain only one set of chromosomes. Gametes (ovum and sperm) are most common type of haploid cells produced through meiosis. Haploid cells are vital and important for genetic diversity and sexual reproduction. When the haploid cells from both male and female gametes fuse together during fertilization, it results in formation of diploid cells, which are vital for growth and development.

- Chromosomes are thread-like structures in which DNA is tightly packaged many times around associated histone octamer proteins within the nucleus, that carries genetic information.
- Human beings have 23 pairs of chromosomes for a total of 46 chromosomes in diploid cell.
  - Each cell contains 22 pairs of chromosomes called autosomes and one pair of sex chromosomes (gametes): female has XX pattern and male has XY pattern.
  - In diploid cells, one set of chromosomes is inherited from the individual's father, while the second set of chromosomes is inherited from mother.
  - The total number of chromosomes in diploid cells is described (2n), which is twice the number of chromosomes in a haploid cell (n).
- Numerical alterations in chromosomes (aneuploidy or polyploidy) occur due to errors in segregation of chromosomes during mitosis resulting in **genomic instability** due to gain or loss of chromosome. Notability, approximately 90% of human cancers have lost or gained at least one chromosome.
- The chances of spontaneous gene mutations are higher in somatic diploid cells (2n) than haploid cells (n).
  - Numerical alteration in chromosomes means an individual is either missing one of the chromosomes from a pair or has more than two chromosomes instead of a pair.
  - Numerical alterations in chromosomes result from errors in segregation of chromosomes.
  - Trisomy, monosomy and polyploidy are among the major causes of spontaneous abortions. Trisomies are compatible with survival that often result in multiple defects. Trisomies, monosomies and segmental aneuploidies have impact on chromosomal stability.
  - Aneuploidy and chromosomal instability are commonly found in human cancers.
  - Chromosomal instability leads to karyotype heterogeneity in malignant tumors and is associated with chemoresistance, metastasis and poor prognosis.

Aneuploidy

Alterations in chromosomes involving addition or loss of chromosome in a cell leading to unbalanced chromosome component is termed aneuploidy. Human cell has 47 or 45 chromosomes instead of 46. Trisomy and monosomy are examples of aneuploidy.

- Specific aneuploidy has been observed in various non-cancer genetic orders such as **Down syndrome** (trisomy 21), **Patau syndrome** (trisomy 13) associated with advanced maternal age, **Edwards syndrome**

(trisomy 18, **Klinefelter syndrome** (trisomy XXY), Jacob syndrome (trisomy XYY), and XXY syndrome; and Turner syndrome (monosomy 45X, i.e. absence of one X chromosome). While, clonal aneuploidy is detected in some human cancers, i.e. chronic lymphocytic leukemia (trisomy 12 in CLL) and acute myelogenous leukemia (trisomy 8 in AML).

- Complete loss of chromosome 17 (monosomy 17) has been detected in breast carcinoma. Complete or partial loss of chromosome 7 (monosomy 7) is linked to pediatric acute myelogenous leukemia (AML).
- Aneuploidy can later induce carcinogenesis, which is considered a hallmark of cancer. If extra copies of chromosome containing an oncogene are present, more of the protein may be produced. Alternatively, loss of chromosomes that code for tumor suppressor proteins will lead to less expression of tumor suppressor proteins resulting in carcinogenesis.
- Aneuploidy is demonstrated by fluorescence *in situ* hybridization (**FISH**) in many human cancers, i.e. trisomy 21 in chronic lymphocytic leukemia (**CLL**) and trisomy 8 in acute myelogenous leukemia (**AML**) in 8.5% of cases.

Polyploidy

Polyploidy refers to increase in number of chromosomes (a whole set of chromosomes) more than as in diploid cell (2n), which occurs due to failure of cytokinesis after telophase stage of cell division. Polyploidy is rarely compatible with life, and usually results in spontaneous abortion. Triploidy (3X) is three times the haploid number; tetraploidy (4X) is four times the haploid number and so on.

STRUCTURAL ALTERATIONS IN CHROMOSOMES

Structural alterations in chromosomes occur by relocation of a gene into incorrect promoter region of the gene to a new chromosomal site that results in the higher expression of abnormal proteins/chimeric proteins leading to increased oncogenic potential and thus inducing disruption in normal cell regulation.

- Structural alterations in chromosomes arise during DNA replication of the chromosomes just prior to cell division, which can occur in several ways: (a) deletion of a section of chromosome, (b) duplication of chromosome, (c) chromosomal rearrangement and creation of chimeric (fusion gene) and chromosomal rearrangement to a transcriptionally active site, (d)Robertsonian translocation, (e) chromosome inversion, and (f) formation of ring chromosome.
- Philadelphia chromosome in chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL) is an example of reciprocal chromosomal translocation t(9;22). Philadelphia chromosome is a t(9;22) that juxtaposes the BCR gene on chromosome 22 with the c-ABL proto-oncogene on chromosome 9. The **BCR-ABL fusion** creates a constitutively active tyrosine kinase product that promotes unrestricted cell proliferation independent of extrinsic regulation.
- Chromosomal translocation to a transcriptionally active site involves c-Myc gene mapped on 8q24 and IgH locus mapped on 14q32 that results in fusion of BCL-2 gene located near to IgH locus leading to over expression of BCL-2 anti-apoptotic protein in the settings of B cell lymphoid malignancies, e.g. Burkitt's lymphoma (90%), diffuse large B cell lymphoma (DLBCL) and acute lymphoblastic leukemia (ALL).
- Structural alterations in chromosomes and exchange of sister chromatids give rise to structural abnormalities that can be easily marked by employing various chromosomal banding techniques used as biomarkers for human cancers. Further, homogenously stained regions (HSR) and double minute chromosomes (small fragments of extrachromosomal DNA) are frequently associated with gene amplification in breast carcinoma, lung carcinoma, ovarian carcinoma, colon carcinoma and neuroblastoma.

Balanced Chromosomal Rearrangement

In genetics, balanced chromosomal rearrangement is a phenomenon that results in unusual rearrangement of chromosomes that can lead to solid and lymphoid malignancies either by creating hybrid gene or dysregulation of gene (i.e. activation of proto-oncogene or inactivation/biallelic loss of tumor suppressor gene). Common balanced chromosomal rearrangements in human malignancies are given in Table 6.41.

- Balanced chromosomal rearrangement is defined as the genomic abnormality resulting from either breaking of the whole chromosome or breaking of a portion of chromosome that reattaches to a different chromosome. Depending on the location of chromosome breaks, balanced chromosomal rearrangement may lead to formation of fusion gene, or may disrupt gene or its regulatory sequences, and in this way may cause dysregulation of gene. Balanced chromosomal rearrangement contributes to molecular **carcinogenesis** by overexpression of oncogenes or generation of novel fusion proteins with altered functions.
- Balanced chromosomal rearrangement can be detected by karyotyping of the CSCs. Cancer therapeutics may cause chromosome translocations that generate secondary therapy-induced cancers.

Table 6.41 Common balanced chromosomal rearrangement in human malignancies

Cancer	Molecular Mutations and Fusion Gene Product	Chromosomal Rearrangements
Hematolymphoid malignant tumors		
CML (chronic myelogenous leukemia)	BCR-ABL	t(9;22)
ALL (acute lymphoblastic leukemia)	▪ BCR-ABL ▪ MLL-AF4 ▪ ELA-PBX ▪ TEL (ETV6)-AML1 (CBFA2) ▪ Myc-IgH	▪ t(9;22) ▪ t(4;11) ▪ t(1;19) ▪ t(12;21) ▪ t(12;14)
AML-M2 (FAB)	ETO-AML1	t(8;21)
AML-M3 (FAB), i.e. acute prolymphocytic leukemia	PML-RARA	t(15;17)
AML-M4	CBFB-MYH11	t(16;16) inversion
Plasma cell myeloma	▪ FGFR3-IGH ▪ c-MAF-IGH ▪ c-MAF-IGL	▪ t(4;14) ▪ t(4;16) ▪ t(4;22)
Burkitt's lymphoma	IgH-Myc	t(8;14)
Follicular lymphoma	BCL-2-IgH	t(14;18)
Mantle cell lymphoma	BCL-1-IgH	t(11:14)
Diffuse large cell B cell lymphoma (DLBCL)	▪ BCL-2-IgH ▪ BCL-6-IgH ▪ ALK-clathrin	▪ t(14;18) ▪ t(3;14) ▪ t(2;17)
Marginal zone lymphoma	▪ ALP-12-MLT ▪ BCR-10-IGH	▪ t(11;18) ▪ t(11;14)
Small cell lymphocytic lymphoma/CLL	BCL-3-IGH	t(14;19)
Mesenchymal tissue-derived malignant tumors		
Fibrosarcoma	ETV6-NTRK3	t(12;15)
Alveolar rhabdomyosarcomas	▪ PAX3-FKHR ▪ PAX27-FKHR	t(2;13) t(1;13)
Clear cell sarcoma	EWS-ATF1	t(12;22)
Dermatofibrosarcoma protuberans	COL1A1-PDGF	t(17;22)
Desmoplastic small round cell tumor	EWS-WT1	t(11;22)
Extraskeletal myxoid chondrosarcoma	EWSR1-CHN	t(9;22)
Synovial sarcoma	SYT-SSX1 or SYT-SSX2	t(X;18)
Epithelial tissue-derived malignant tumors		
Follicular thyroid carcinoma	PPARG-PAX8	t (2;3)
Papillary thyroid carcinoma	RET-PTC	Rearrangement on 10q11.2
Primitive neuroendocrine tumor (PNET)		
Ewing sarcoma	▪ EWSR1-FLT1 ▪ EWSR1-ERG ▪ EWSR1-ETV1 ▪ EWSR1-ETV4 ▪ EWSR1-FEV ▪ FUS-ERG	▪ t(11;22) ▪ t(21;22) ▪ t(7;22) ▪ t(17;22) ▪ t(2;22) ▪ t(16;21)

BCR-ABL Fusion Gene Positive Chronic Myelogenous Leukemia

The best-known example of an acquired chromosomal translocation in a human cancer is the 'Philadelphia chromosome', which is found in 95% of patients with chronic myelogenous leukemia (**CML**) and some patients with acute lymphoblastic leukemia (ALL). Schematic representation of Philadelphia chromosome in chronic myelogenous leukemia is shown in Fig. 6.72.

- Reciprocal translocation between human chromosomes 9 and 22, which carries ABL (Abelson mouse leukemia) proto-oncogene on chromosome 9 and BCR gene on chromosome 22, result in the fusion and creation of BCR-ABL hybrid genes, that code for hybrid BCR-ABL proteins commonly observed in 95% cases of CML. BCR-ABL fusion protein product provides a growth factor by virtue of increased nonreceptor protein kinase, that cannot '**turn off**' and codes for abnormal fusion protein, that results in cell growth, unrestricted leukemic cell proliferation and progression in unregulated manner independent of extrinsic regulation.
- 'Philadelphia chromosome' is the hallmark of CML and also occurs in about 5% of children and 20% of adults with ALL. Philadelphia-positivity in ALL is associated with aggressive disease and has been associated with poor prognosis, especially in children.
- Philadelphia chromosome can be detected by cytogenetic analysis and polymerase chain reaction (PCR) to analyze DNA sequence and detect BCR-ABL fusion gene. PCR testing may be used to monitor a patient's clinical course.
- Patients with CML are treated by chemotherapeutic agent 'imatinib', that inhibits the activity of BCR-ABL fusion protein, thus prevents cell growth, progression and metastasis.

Myc-IgH Fusion Gene Positive Burkitt's Lymphoma

In Burkitt's lymphoma, reciprocal translocation t(8;14) (q24;q32), which involves the c-Myc gene (8q24) and immunoglobulin heavy-chain (IgH) gene locus (14q32). Burkitt's lymphoma is characterized by a high-turnover rate of malignant B cells and the c-Myc gene deregulation. Schematic representation of chromosomal translocation (14;18) (q32.q21) in Burkitt's lymphoma is shown in Fig. 6.73.

- The immunoglobulin heavy-chain (IgH) gene acts as a promoter for the c-Myc gene. Myc is a transcription factor that is aberrantly activated in Burkitt's lymphoma. Balanced chromosomal rearrangement is the basis of malignant transformation of lymphocyte in Burkitt's lymphoma. Patients can develop Burkitt's lymphoma in the jaw region or in the mouth region that interfere with the ability to eat.
- Cytological analysis is performed on bone marrow and blood samples in these patients. Most Burkitt's lymphoma cases demonstrate t(8;14) (q24;q32) with Myc-IGH fusion gene product, and less commonly t(8;22) (q24;q11) or t(2;8)(p12;24). The Myc breakpoints are diverse and distributed over 2Mb region. High quality metaphases are required to detect t(8;14) and t(8;22). Fluorescence *in situ* hybridization (FISH) technique cannot detect all Myc rearrangements.

BCL-2-IgH Fusion Gene Positive Follicular Lymphoma

Chromosome translocation (14;18) (q32.q21) is the hallmark of follicular lymphoma. Translocation of chromosomes 14 and 18 places potent antiapoptotic

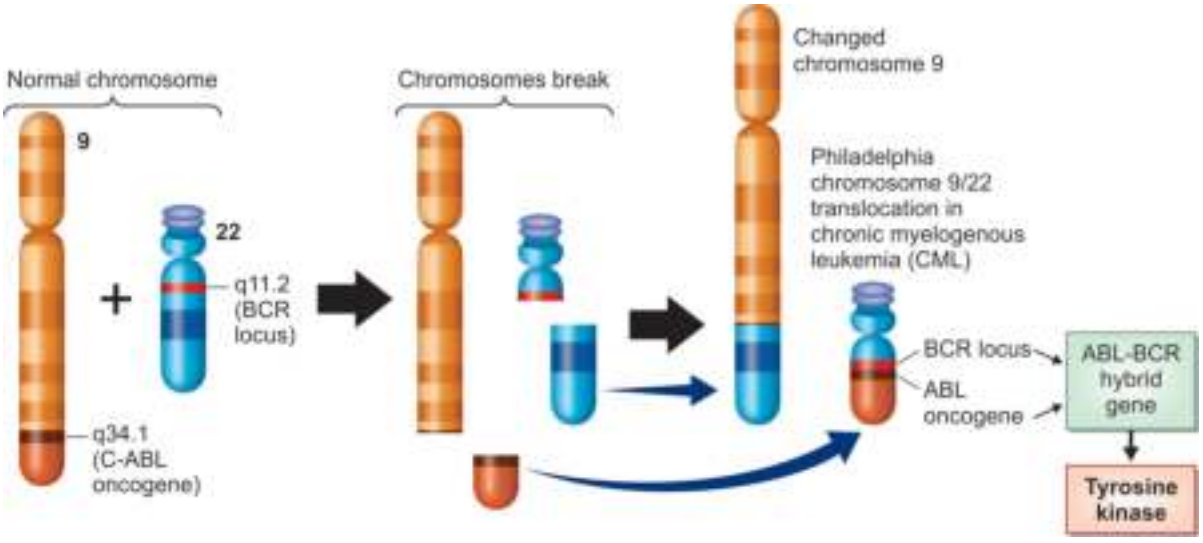


Fig. 6.72: Schematic representation of Philadelphia chromosome in chronic myelogenous leukemia. Reciprocal chromosomal translocation t(9;22) of ABL (Abelson mouse leukemia on chromosome 9) into the BCR site on chromosome 22 creates the 'BCR-ABL fusion protein product' in 90% cases of chronic myelogenous leukemia.

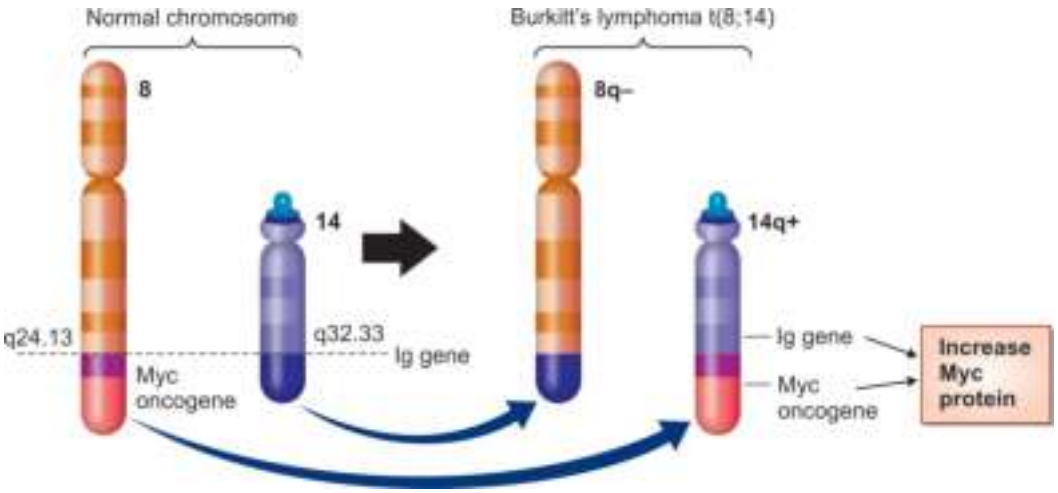


Fig. 6.73: Schematic representation of chromosomal translocation (14;18) (q32.q21) in Burkitt's lymphoma. Translocation of c-Myc oncogene from chromosome 8 to an immunoglobulin heavy gene locus on chromosome 14 results in expression of c-Myc gene.

BCL-2 gene, next to the promoter region of immunoglobulin heavy-chain (IgH) gene leading to development of B cell-derived **follicular lymphoma**. B cell-derived follicular lymphoma evades apoptosis by overexpression of antiapoptotic BCL-2 protein.

EWSR1/FLT1 Fusion Gene Positive Ewing Sarcoma/PNET

Approximately 20% cases of Ewing sarcoma show features of neural differentiation. Diagnostic criteria of Ewing sarcoma/peripheral primitive neuroectodermal tumor (PNET) includes (≥2 major features of neural differentiation): (a) Homer Wright rosettes, (b) MIC2 expression, (c) vimentin expression, (d) expression of neuron-specific enolase (NSE), synaptophysin and chromogranin.

- In Ewing sarcoma/PNET, reciprocal recurrent chromosomal between chromosome 11 and 22 results in fusion of the amino terminus of EWSR1 gene to the FLT1 gene, which codes for a nuclear transcription factor leading to unrestricted CSC proliferation in 100% of cases.
- Approximately 5–15% of these tumors have chromosomal translocation q22; q12. Occasionally chromosomal translocation (21; 22) may also be observed in these patients.
- Ancillary laboratory tests, i.e. conventional cytogenetic analysis, fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR) are performed to analyze EWSR1/FLT1 and EWSR1/ERG fusion gene.

AML1/ETO Fusion Gene Positive Acute Myelogenous Leukemia (AML2-FAB)

Acute myelogenous leukemia (AML2-FAB) is most common childhood hematologic malignancy, and the cases with the chromosome t(8;21) (q22,q22) aberration stand for a subset with the biological and clinical features. AML1/ETO fusion represents one of the first fusion genes employed in monitoring of minimal residual disease (MRD).

- AML1 gene mapped on chromosome 21 is involved in several leukemias. In acute myelogenous leukemia (AML2) with chromosomal translocation t(8;21) (q22,q22), AML1 gene is juxtaposed to the ETO gene mapped on chromosome 8 generating AML1/ETO fusion protein leading to cell proliferation, differentiation and the viability of leukemia cells. Both AML1/ETO and AML1 proteins recognize the same consensus DNA-binding motif (TGT/CGCT), which is found in the promoter regions of several genes involved in hematopoiesis.
- Acute myelogenous leukemia (AML2-FAB) can be diagnosed by cytogenetic analysis by G-binding technique and fluorescence *in situ* hybridization (**FISH**) technique. Detection of AML1/ETO fusion gene can be detected by reverse transcriptase polymerase chain reaction (**RT-PCR**).

RARA-PML Fusion Gene Positive Acute Promyelocytic Leukemia (AML3-FAB)

Acute promyelocytic leukemia (APL) is a unique subtype of acute myelogenous leukemia (AML3-FAB) characterized by abnormal proliferation of promyelocytes, life-threatening coagulopathy due to chromosomal translocation t(15;17) (q24:q21) resulting in the RARA-PML fusion gene, that codes for an oncoprotein responsible for disruption of myeloid distinction.

- Rapid molecular testing is performed for fluorescence *in situ* hybridization (FISH) to detect RARA-PML fusion gene in acute promyelocytic leukemia (AML3).

- Acute promyelocytic leukemia patients are treated by all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO) therapy.

IgH-CCND1 Fusion Gene Positive Hematolymphoid Malignancies

Chromosomal translocation juxtaposes the CCND1 gene mapped on chromosome 11q13 with the IgH gene mapped on chromosome 14q32 results in creation of IgH-CCND1 fusion gene chiefly found in B cell-derived mantle cell lymphoma, and less commonly in multiple myeloma, plasma cell leukemia, splenic lymphoma with villous morphology, B-prolymphocytic leukemia and chronic lymphocytic leukemia (CLL). Molecular testing strategy for mantle cell lymphoma includes immunohistochemistry (strong cyclin D1 positivity on formalin-fixed paraffin-embedded tissue), cytogenetic analysis/fluorescence *in situ* hybridization technique (IgH-CCND1 fusion gene, t(11;14) on fresh tissue), flow cytometry for leukemia/lymphoma phenotyping (bright CD20, monoclonal light chains, CD5+, CD10–, CD23–), and bone marrow analysis for staging and monitoring disease.

ETV6/RUNX1 Fusion Gene Positive Pediatric Acute Lymphoblastic Leukemia

In pediatric B cell acute lymphoblastic leukemia (ALL), the chromosome translocation t(12;21) (p13;q22) results in ETV6/RUNX1 (also called TEL/AML1) chimeric fusion gene that codes for creation of ETV6/RUNX1 chimeric fusion transcript. This structural alteration in chromosome occurs in 25% of pediatric acute lymphoblastic leukemia cases between 2 and 10 years of age group, with a median of 4 years.

- ETV6/RUNX1-positive pediatric ALL is a 'two-hit' disease for molecular carcinogenesis, i.e. first hit (ETV6/RUNX1) and second hit (ETV6 deletion, RUNX1 gain, CDK2NA, PAX5).
- **Conventional molecular cytogenetic analysis** is performed to detect ETV6 and RUNX1 related chromosomal abnormalities. Recently, t(12;21) and other related abnormalities are analyzed by using fluorescence *in situ* hybridization (**FISH**) technique and real-time polymerase chain reaction.

SYT-SSX1/SSX2 Fusion Gene Positive Synovial Sarcoma

Synovial sarcoma is a highly malignant mesenchymal tumor. Tumor originates in the region of a joint rather within joint cavity. Demonstration of chromosomal translocation t(X;18) (p11, q11) is characteristic finding in synovial sarcoma in 95% of cases. SYT gene from chromosome 18 is fused to SSX1 and SSX2 gene mapped on X chromosome. There is possible ectopic expression of SSX1 and SSX2 gene products in the patients.

PRCC-TFE3 Fusion Gene Positive Pediatric Renal Cell Carcinoma

Pediatric renal cell carcinoma is characterized by various chromosomal translocations involving Xp11.2, all resulting in creation of gene fusion involving the transcription factor E3 (TFE3) gene recognized by World Health Organization. While TFE3 translocations involving a multitude of various partners have been reported, i.e. chromosome 17 [t(X;17) (p11.2; q25), ASPL-TFE3], and chromosome 1 [t(X;1) (p11.2; q21), PRCC-TFE3 fusion] in the patients.

ASPSCR1-TFE3 Fusion Gene Positive Alveolar Soft Part Sarcoma

Alveolar soft part sarcoma is a rare slow-growing malignant tumor of young adults. Tumor is defined by a specific chromosomal alteration, der(17) t(X;17) (p11;q25) resulting in fusion of transcription factor E3 (TFE3) with alveolar soft part sarcoma critical region 1 (ASPSCR1) at 17q25 leading to creation of ASPSCR1 TFE3 fusion protein and unrestricted proliferation of CSCs.

Robertsonian Translocation

Robertsonian translocation is centric fusion of two acrocentric chromosomes resulting in the formation of one large metacentric chromosome and one small fragment. Robertsonian translocation is linked to Down syndrome (trisomy 21) and Patau's syndrome (trisomy 13). Robertsonian translocation is shown in Fig. 6.74.

- Acrosomal chromosomes with very short p arms break very close to centromere leading to subsequent fusion of long q arms. The human genome includes five acrocentric chromosomes: 13, 14, 15, 21, 22.
- The Y chromosome is also acrocentric chromosome. Robertsonian translocation predisposes to Down syndrome (14q;21q).
  - Chromosome 21 is joined to a second acrocentric chromosome, commonly chromosome 14 or 22.

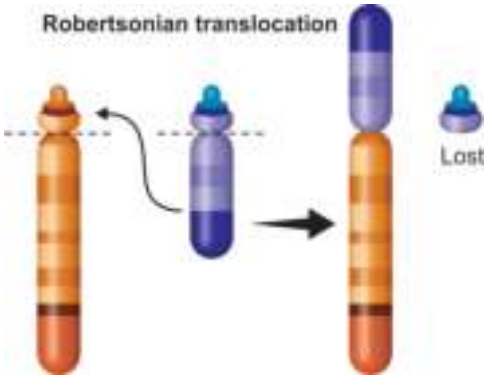


Fig. 6.74: Robertsonian translocation. In Robertsonian translocation, two nonhomologous acrocentric chromosomes break near centromeres, after which the long arms fuse to form one large metacentric chromosome. It is the most common type of chromosomal translocation in human beings.

- The union of a gamete with this translocation with a gamete from an unaffected person can result in trisomy 21. There is high incidence of spontaneous abortion of fetuses.
- Robertsonian translocation confers a high-risk of childhood leukemia, non-Hodgkin's leukemia, and breast cancer with Robertsonian translocation (13;14).

Imbalanced Chromosomal Rearrangements

Imbalanced chromosomal rearrangements occur, where the exchange of chromosome material (segment) is unequal resulting in extra or missing genes by various mechanisms: deletion, duplication, insertion, ring chromosome and isochrome creation.

Chromosome Deletion

In genetics, loss of chromosomal fragment containing numerous genes ('p' short arm or 'q' long arm) or entire chromosome during DNA replication just prior to cell division is termed 'chromosome deletion'.

- Any number of nucleotide base pair(s) can be deleted ranging from single nucleotide base pair to an entire piece of chromosome involving tumor suppressor genes. Examples of chromosomal deletion syndromes include 5p– deletion (**cri-du-chat syndrome**), 4p– deletion (**Wolf-Hirschhorn syndrome)**.
- Chromosome deletion appears to be the earliest as well as the most frequent somatic (acquired) genetic alteration during carcinogenesis, which occurs by three mechanisms: (a) loss of heterozygosity (**LOH**) as proposed in the Knudson' two-hit hypothesis (inactivation of both alleles of RB tumor suppressor gene resulting in retinoblastoma), (b) haploinsufficiency through quantitative deletion and associated with loss of expression, and (c) truncating of the genome by homozygous deletions. Chromosomes 1p and 16q are commonly lost in solid malignant tumor.

Pathology Pearls: Chromosome Deletion Mechanisms

Loss of Heterozygosity

Loss of heterozygosity (LOH) is defined as the loss of one copy of a segment of DNA (second allele) assuming the knocking out of the first allele. Haploinsufficiency refers to poorly functional second allele assuming the knocking out of the first allele of tumor suppressor gene. Human cells have two copies of any genomic segment-one from each parent, so in the case of loss of heterozygosity, only one copy would still be present.

- Loss of heterozygosity is a common form of allelic imbalance of essential genes by which a heterozygous somatic cell becomes homozygous because one of the two alleles gets lost.
  - Loss of heterozygosity (LOH) causes chromosomal instability resulting to potential cancer vulnerabilities.
  - When tumor suppressor genes are absent or nonfunctional due to loss of heterozygosity, normal cell may begin to divide abnormally and becomes cancer stem cell (CSC).
  - The classical example of loss of heterozygosity is the loss or inactivation of both alleles of the retinoblastoma (RB) gene in retinoblastoma. The pRB protein is inactivated either by somatic or germline mutations leading to the pediatric tumor of the eye 'retinoblastoma'.
- Knudson initially proposed that retinoblastoma was initiated by inactivation of putative RB1 tumor suppressor gene. Knudson' two-hit hypothesis was subsequently confirmed by demonstration of loss of heterozygosity (LOH) at chromosome 13q14 in retinoblastoma gene and the cloning of the first RB1 tumor suppressor gene. Retinoblastoma is virtually always fatal due to metastasis.

Haploinsufficiency

Haploinsufficiency of tumor suppressor genes (TSGs) occurs when single copy of the **wild-type** allele in the tumor suppressor gene at a genomic locus is **inactivated** or **deleted** and the remaining functional copy of the gene is not adequate to produce the needed gene product to execute normal cellular functions, and thus contributing to development of malignant tumor.

Truncating the Genome by Homozygous Deletions

Homozygous deletion is defined as the loss of both alleles of the gene at given locus. A deletion changes the DNA sequence by removing at least one nucleotide base pair in a gene. Deletion of chromosomal material is the most common feature of cancer genomes. The landscape of somatic homozygous deletions in CSCs is shaped by positive and negative selection. Recurrent deletions of chromosomal material typically target tumor suppressor genes resulting in positive selection. Simultaneously, loss of a nearby essential gene can lead to negative selection, and introduce latent vulnerabilities specific to CSCs.

- **Chromosome 8p deletion:** The short arm of chromosome 8 (8p) is one of the most recurrently deleted genomic regions in the majority of advanced epithelial cancers, i.e. urinary bladder carcinoma, colorectal carcinoma, prostatic carcinoma, lung carcinoma, and hepatocellular carcinoma.
- **Chromosome 5p deletion:** Multiple early genetic events on chromosome 5p deletion have been identified in the progression of nonsmall cell lung carcinomas (e.g. squamous cell lung carcinoma and lung adenocarcinoma). Lung carcinoma is most widely diagnosed malignancy in the world. Using a high-resolution chromosome 5p– specific genome array, which contains a tilting patch of DNA segments for comparative genomic hybridization, nine novel minimal regions of loss and gain have been demonstrated in bronchial carcinoma *in situ* specimens.
- **Chromosome 1p32 deletion:** Chromosome 1p32 is the largest human chromosome, spanning about 249 million DNA building blocks (nucleotide base pairs) and representing approximately 8% of the total DNA in cells.

- Chromosome 1p32 contains 2,000 to 2,100 genes that provide instructions for making proteins, which perform a variety of different biological functions in the body.
- **TAL1 gene** is mapped on short arm of chromosome 1p32 that codes for a hematopoietic transcription factor essential for normal hematopoiesis. Disruption of TAL1 via chromosomal translocation of a site-specific deletion has been reported in 30–50% of T cell acute lymphoblastic leukemia (**T-ALL**).
- Chromosome 1p32 deletion can be detected by conventional cytogenetics, fluorescence *in situ* hybridization, southern blot analysis and real-time polymerase chain reaction (RT-PCR).

Chromosome Duplication

The term 'chromosome duplication' means duplication of chromosomal segment resulting in extra genetic material, even though the total number of chromosomes is usually normal. Chromosome duplication is the opposite of deletion.

- During a disease process, extra copies of the gene in the human genome can contribute to carcinogenesis. Genes can also duplicate in the evolution of human genome, where one copy of the original gene function and other copy of the gene produces new function. Duplication of whole chromosome induces disease.
- Chromosome duplication happens during the crossing-over (recombination) between misaligned homologous chromosomes stage of meiosis.
- Duplication of chromosome results in trisomy (e.g. Down syndrome, trisomy 21) and Pallister-Killian syndrome (#12). Duplication of chromosome is detected by high-resolution banding, fluorescence *in situ* hybridization (FISH) and array comparative genomic hybridization (array CGH).

Chromosome Inversion

An inversion is a rearrangement in chromosome, in which a segment of a chromosome breaks at two regions is reversed end to end position in original chromosome after rotating by 180°. Inversion occurs when a single chromosome undergoes breakage and rearrangement within itself.

- As many as 1% of the newborn babies may carry an inversion in chromosome that can be detected by G-banding chromosome karyotyping. Chromosome inversion produces unbalanced meiotic products; thereby resulting in sterility.
- Chromosome inversion leading to the formation of the **EML4-ALK fusion oncogene** is most commonly present in lung adenocarcinoma. EML4-ALK fusion oncogene is also demonstrated in **breast carcinoma** and **colorectal carcinoma**. There are two types of inversion: paracentric and pericentric.
  - **Differences between paracentric and pericentric inversion:** Paracentric inversion does not include the centromere and both breaks occur in one of the chromosomes. On the contrary, pericentric inversion includes the centromere and breaks occurs in both arms of chromosome.
  - **Similarities between paracentric and pericentric inversion:** Both paracentric and pericentric inversions are large-scale chromosomal mutations occurring within a single chromosome. Both types of inversion do not cause a loss of genetic information. They simply rearrange the linear gene sequence in the chromosome.
- Researcher reported a related chromosome 16 inversion in acute myelomonocytic leukemia (AMML), i.e. AML-M4 (FAB), that is defined with differentiation along with both myeloid and monocytic lines in bone marrow and high number of circulating monocytes.
  - Bone marrow contains myeloblasts and monoblasts constitute 20%.
  - Monoblasts must be <80% of total nucleated cells. Cytogenetic analysis detects chromosome 16 inversion. Flow cytometry is performed to confirm the diagnosis. In general, myeloblasts are moderate CD45 positive, low SSC and express CD34, CD13, CD33, CD117 and HLA-DR.
  - Monoblasts and promonocytes are CD45 positive bright and SSC slightly higher than myeloblasts; and these cells express CD11b, CD11c, CD13, CD14, CD64 and HLA-DR.

Chromothripsis (Shattering of Chromosomes)

Chromothripsis means shattering of chromosomes into small fragments that leads to remarkable gene rearrangements in localized regions. During mitosis, chromosomes undergo condensation, and focal DNA damage can be induced by exogenous (radiation) or endogenous factors such as microhomology-mediated break-induced replication (MMIR) or chromosome lagging, micronuclei formation, and pulverization as a result of inappropriate chromosome segregation.

- After chromosome shattering, DNA fragments are joined by nonhomologous end joining (NHEJ) within chromosomal regions. Repaired chromosomes contain chromosomal rearrangements such as deletions, head-to-head and tail-to-tail inversions, and tandem duplications.
- With exit of mitosis, shattering of chromosome (chromothripsis can be incorporated into the nucleus. Lack of DNA damage checkpoints, such as TP53 (p53 protein), facilitates the survival of cells during the chromothripsis process.
- As a result, chromothripsis can induce activation of oncogene by chromosome translocations or duplications as well as inactivation/biallelic loss of tumor

suppressor genes by deletions or locus disruptions, thus facilitating progression of human cancers (e.g. **osteosarcoma** and **glioma**).

GENES DYSREGULATION AND CARCINOGENESIS

Carcinogenesis is the multistep process involving genetic alterations (e.g. gene amplification, point mutation, frameshift mutations and insertion) in proto-oncogenes, and tumor suppressor genes, that regulate normal functions and leading to transformation of healthy cell into CSCs.

Gene Amplification

Gene amplification refers to an increase in additional copies of the same proto-oncogene without a proportional increase in other genes within the genome that leads to increase in production of the associated protein and then self-sufficiency in growth signals may be achieved. Gene amplification can result from duplication of a region of DNA, that contains a gene through errors in DNA replication and repair machinery as well as through insertion of oncogenic retroviruses. Gene overexpression occurs due to gene amplification, activating gene mutation or epigenetic activation.

- Oncogenes such as ERBB2, CCND1, c-Met, N-Myc, EGFR, and MDM2 are amplified in human cancers and can be associated with increased expression of their respective aberrant proteins. Amplification of N-Myc oncogene is linked to neuroblastoma. Amplification of HER2/neu is linked to breast carcinoma. Gene amplification and associated human cancers are given in Table 6.42.

Table 6.42 Gene amplification and associated human cancers

Name of Oncogene	Oncoprotein	Human Cancers
K-RAS gene	Small GTP proteins	Lung carcinoma, ovarian carcinoma, colorectal carcinoma, urinary bladder carcinoma (20%)
N-RAS gene	Small GTP proteins	Head and neck cancers
H-RAS gene	Small GTP proteins	Colorectal carcinoma (30%)
ERBB1/EGFR gene	Receptor tyrosine kinase (RTK)	Glioblastoma multiforme (50%), squamous cell carcinoma of skin (10–20%), melanoma, breast carcinoma, colorectal carcinoma, gastric carcinoma, lung adenocarcinoma
ERBB2 (HER2/neu) gene	Receptor tyrosine kinase	Breast carcinoma (20%), ovarian carcinoma, lung adenocarcinoma, squamous cell carcinoma of skin, salivary gland carcinoma
ERBB3 gene	Receptor tyrosine kinase	Breast carcinoma, prostatic carcinoma, urothelial carcinoma
FGF-R1	Receptor tyrosine kinase	Breast carcinoma (10%)
MDM2 gene	p53 inhibitor	Soft tissue sarcomas, osteosarcoma, breast carcinoma
AKT-1 gene	Serine/threonine kinase	Gastric carcinoma (20%)
AKT2 gene	Serine/threonine kinase	Pancreatic carcinoma, ovarian carcinomas (30%)
CCND1 gene	Cyclin D1 protein	Breast ductal carcinoma, breast lobular carcinoma, urinary bladder carcinoma, lung adenocarcinoma, squamous cell carcinoma of larynx (25–50%)
CDK4 gene	Cyclin-dependent kinase	Sarcomas (10–30%), squamous cell carcinoma in head and neck region (40%), B cell lymphomas (25%)
CDK6 gene	Cyclin-dependent kinase	Gliomas (5%)
Cyclin E gene	Cyclin	Gastric carcinomas (15%)
PIK3CA gene	Phosphoinositide 3-kinase	Squamous cell lung carcinoma, ovarian carcinoma, breast carcinoma
c-Met gene	Receptor tyrosine kinase	Gastric carcinoma (20%), esophageal carcinoma, lung carcinoma, colorectal carcinoma
K-SAM gene	Receptor tyrosine kinase	Gastric carcinoma (10–20%), breast carcinoma (10–20%)
AIB1/BTAK gene	Receptor coactivator	Breast carcinoma (15%)
c-Myc gene	Transcription factor	Leukemias, carcinomas (10–50%)

Contd...

Table 6.42 Gene amplification and associated human cancers (*Contd...*)

Name of Oncogene	Oncoprotein	Human Cancers
L-Myc/DDX1 gene	Transcription factor	Lung carcinoma (10%)
N-Myc gene	Transcription factor	Neuroblastoma (25–30%), lung carcinoma (30%), retinoblastoma, rhabdomyosarcoma, breast carcinoma
GLI gene	Transcription factor	Glioblastoma multiforme
ETS-1 gene	Transcription factor	Lymphomas
MYB gene	Transcription factor	Colon carcinoma (5–20%), leukemias

Table 6.43 Reagents and equipment required for the polymerase chain reaction (PCR)

Reagents and Equipment	Purpose
PCR target or 'template'	The segment of the nucleic acids (DNA or RNA) that is to be amplified
Nucleotides	Building blocks from which nucleic acids are constructed; adenine, guanine, cytosine and uracil
Primer	A short sequence of nucleotides complimentary to, and binding (annealing) to, known sequences of the target nucleic acid; essential for 'priming' the amplification reaction
Taq DNA polymerase	A heat-stable enzyme that makes a new copy of the target nucleic acid by adding nucleotides to annealed primer
Reverse transcriptase	An enzyme that converts RNA into a complimentary DNA sequence (used in reverse transcription PCR)
Thermocycler	The equipment in which PCR reactions occur, it is able to change rapidly to the different temperatures required for repeated PCR cycles

- Gene amplification indicates aggressive behavior of malignant tumors. Gene amplification is linked to chemotherapy resistance. Amplification of c-Myc gene is observed in small cell lung carcinoma associated with clinically aggressive malignant tumor.
- Polymerase chain reaction (PCR) is widely used method to analyze gene amplification. Reagents and equipment required for the polymerase chain reaction (PCR) are given in Table 6.43.
- Cytological analysis of gene amplification reveals homogenous staining regions (HSRs) and abnormal banding regions on chromosomes stained with special stains, and double minutes recognized as small, paired cytoplasmic bodies.

ERBB2 (HER2/neu) Gene Amplification

ERBB2 (or HER2/neu) gene amplification is most commonly detected in oncology. Upwards of 20 copies of ERBB2 genes may be demonstrated in breast carcinoma, ovarian carcinoma, lung adenocarcinoma, and squamous cell lung carcinoma, which help to drive progression of disease and associated with poor prognosis.

- ERBB2 (HER2/neu) gene encodes a member of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinase, that regulates outgrowths and stabilization of peripheral microtubules. ERBB2 gene amplification activates downstream signaling pathways (mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase) leading to unrestricted CSCs and malignant tumor growth.
- It must be noted that sometimes, proto-oncogenes only need to be overexpressed (not mutated) become oncogenes, which drive cell proliferation and render the cell unresponsive to normal growth inhibitory signals, ultimately resulting in malignant tumor growth. For example, ERBB2 (HER2/neu) gene is overexpressed in HER2/neu-positive breast carcinoma.

CCND1 Gene Amplification

The protein encoded by CCND1 belongs to the cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout cell cycle. Cyclins function as regulators of CDK kinases. CCND1 gene amplification is linked to invasive ductal/lobular breast carcinoma, urinary bladder carcinoma and lung adenocarcinoma.

The c-Met Gene Amplification

The 'c-Met' is a receptor tyrosine kinase belonging to the Met family and expressed on the surface of various cells. Hepatocyte growth factor (HGF) is a ligand for c-Met tyrosine kinase receptor.

- The binding of HGF to c-Met initiates a series of intracellular signals, that mediate embryogenesis and wound healing in normal cells. The c-Met gene amplification is linked to gastric carcinoma, esophageal carcinoma, lung carcinoma, and colorectal carcinoma.
- In CSCs, aberrant HGF/c-Met axis activation as a result of c-Met amplification promotes tumor development and progression by stimulating the PI3K/AKT, RAS/MAPK, JAK/STAT, SRC, Wnt/β-catenin and other signaling pathways. Thus, c-Met and its associated signaling pathways are clinically important therapeutic targets.

N-Myc Gene Amplification

N-Myc gene coding for protein normally functions in brain development before birth. N-Myc gene amplification is linked to **neuroblastoma** associated with poor prognosis.

EGFR Gene Amplification

Normal function of epidermal growth factor receptor (EGFR) is to regulate epithelial tissue development and homeostasis. Amplification of EGFR gene is linked to lung carcinoma, breast carcinoma, glioblastoma multiforme, gastric carcinoma, colon carcinoma, and esophageal squamous cell carcinoma.

Mouse Double Minute 2 (MDM2) Homolog Gene Amplification

Mouse double minute 2 (MDM2) homolog gene encodes E3 ubiquitin-protein ligase. MDM2 gene is important negative regulator of the TP53 (p53) tumor suppressor gene. Amplification of MDM2 gene is linked to soft tissue sarcomas, osteosarcomas and breast carcinoma.

Point Mutation in Proto-oncogene

Point mutation occurs in the genome when single nucleotide base pair in a gene sequence is substituted (altered), added, deleted. Point mutation is less serious than chromosomal alteration. Point mutation can occur during DNA replication or due to environmental mutagens, i.e. X-rays, ultraviolet rays, extreme heat or certain chemical agent (i.e. benzene).

- DNA is made up of many nucleotide base pairs (cytosine, guanine, adenine and thymine). **Codon** is a sequence of three nucleotide base pairs that codes for a certain amino acid, or to start (start codon) or stop (stop codon) the production of an amino acid chain. Point mutation changes the codon UUU to the codon UCU.
- Point mutation in proto-oncogene occurs by various mechanisms: (a) silent mutation (formation of same amino acid), (b) missense (formation of different codon but lacking stop codon) mutation, (c) nonsense mutation, i.e. substitution (formation of a stop codon but lacking codon coding for an amino acid), (d) insertion (addition of extra nucleotide base to a sequence) and (e) deletion (deletion of nucleotide base pair from a sequence). The effects of point mutations depend on how they change the genetic code. Point mutation types, examples and effects are given in Table 6.44.
- While most point mutations are benign, e.g. cystic fibrosis (deletion of three nucleotides in the cystic fibrosis transmembrane conductance regulator (CFTR) gene associated with thick mucus in lungs, salty sweat and short life expectancy), sickle cell disease (deletion of single nucleotide base pair point mutation of amino acid valine for glutamic acid in the seventh codon of β-globulin chain on chromosome 11p15) and Tay-Sachs disease (point mutation in HEXA gene associated with nerve involvement and fatal outcome before the age of four). Patients can also have functional consequences such as gene expression or alterations in encoded proteins. However, point mutation of C-RAS oncogene is linked to human cancer.

Frameshift Mutations

A frameshift mutation in a gene refers to the deletion or insertion of one or more nucleotide base pairs in numbers that are not multiple of three that changes the reading frame of the DNA sequence. Deletions remove nucleotide base pairs, and insertions add nucleotide base pairs.

- Genetic code is read in triplet nucleotides called codons. Each of these 'triplet codons' corresponds to one of 20 different amino acids used to build a

Table 6.44 Point mutation types, description, examples and effects

Point Mutation Types	Description	Point Mutation Examples	Point Mutation Effects
Silent mutation	Mutated codon codes for the same amino acid	CAA (glutamine) → CAG (glutamine)	None
Missense mutation	Mutated codon codes for a different amino acid	CAA (glutamine) → CCA (proline)	Variable effects
Nonsense mutation	Mutated codon is a remature stop codon	CAA (glutamine) → UAA (stop) usually	Serious effects

protein. Each coding sequence has three possible reading frames.
- Frameshift mutations show that genetic code is read triplets from a fixed starting point. If gene mutation disrupts the normal reading frame, then the entire gene sequence following the mutation will be incorrectly read. This can result in the addition of the wrong amino acids to the protein and/or the creation of a codon that stops the protein from growing longer leading to the synthesis of truncated proteins, that have lost their function, accelerating progression of malignant tumor.

Insertional Mutagenesis

In molecular biology, insertional mutagenesis is the creation of mutations by addition of one or more nucleotide base pairs.
- Such insertional mutagenesis can occur naturally, mediated by viruses or transposons (group of mobile genetic elements that are defined as a DNA sequence), or can be artificially created for research purposes in the laboratory to identify novel genes involved in the pathogenesis of human cancers.
- Insertional mutagenesis induces dysregulation of oncogenes or tumor suppressor genes, which can cause transformation of normal cell to CSC.
- Hepatitis B virus-related insertional mutagenesis in chronic hepatitis B patients is an early drastic genetic change leading to hepatocarcinogenesis.

CELLULAR AND MOLECULAR HALLMARKS OF CANCER

A cell is defined as the fundamental, structural and functional unit of all living organisms, which divides by two process: mitosis (somatic cell) and meiosis (gametes) and are tightly regulated by positive and negative regulators of cell cycle. Dysregulation of cell cycle is linked to human cancers of breast, colon, rectum, prostate, skin and stomach. Many cancers can be cured, if detected early by screening the patients (HPV testing and exfoliative cytology for cervical carcinoma, mammography for breast carcinoma, and colonoscopy for colon carcinoma), and treated effectively by surgery, radiotherapy and/or systemic therapy (chemotherapy, hormonal treatments, targeted biological therapies).
- The cellular and molecular hallmarks of cancer are the distinctive biologic capabilities acquired during multistep carcinogenesis by transformation of normal cell to cancer stem cell (CSC) leading to development of malignant tumor growth, invasion and distant metastasis to organ(s). Basic objective is to understand how cellular and molecular hallmarks of cancer can distinguish between normal cell and CSC.
- In early 2000, researchers Professor Hanahan and Professor Weinberg proposed that when normal cells progress towards a malignant phenotype and acquire distinctive capabilities, these have been termed '**hallmarks of cancer**', and characterized by distinctive biologic capabilities acquired by cells during multistep process of transformation of normal cell to CSC leading to tumor growth, invasion and metastasis to distant organs.
  - Writing about the complexity of the scientific literature in cancer in 2000, Professor Hanahan and Professor Weinberg brought a conceptual shift leading to the enumerate of '**six-core rules**' that orchestrate the multiple processes involved in transformation of normal cell to CSC.
  - Basic six 'Hallmarks of cancer' capabilities in the year 2000 include: (1) growth signal autonomy (unrestricted cell proliferation, (2) insensitivity to growth inhibitory (suppressor) signals, (3) evasion of apoptosis and survival CSCs even after substantial DNA damage resulting from downregulation of pro-apoptotic genes and upregulation of antiapoptotic genes), (4) enabling limitless replicative potential not limited by telomeres (immortalization), (5) induction and sustained tumor angiogenesis, and (6) activating invasion and metastasis.
  - Later in the year 2011, Professor Hanahan and Professor Weinberg included other four molecular hallmarks of cancer such as (7) reprogramming of energy metabolism (Warburg effect), (8) evasion of immune destruction, (9) genomic instability (mutated phenotype), and (10) tumor-promoting inflammation.
  - New dimensions expanding the frontiers of cancer biology in the year 2022 have been described by additional emerging hallmarks of cancer to the roster of functionally important cell types in the tumor microenvironment, which include: (11) phenotype plasticity and disrupted differentiation, (12) nonmutational epigenetic reprogramming, and (13) polymorphic microbiome. Cellular and molecular hallmarks of cancer are shown in Fig. 6.75 and given in Table 6.45.
- Cancer is caused by oncogenes resulting from activation ('**turning on**') of proto-oncogenes and/or inactivation/biallelic loss ('**turning off**') of tumor suppressor genes. Cellular and molecular hallmarks of cancer reflect alterations in cellular signaling pathways that drive cancer progression, that is achieved due to gene mutations that lead to activation of growth promoting oncogenes, inactivation/biallelic loss of tumor suppressor genes, evasion

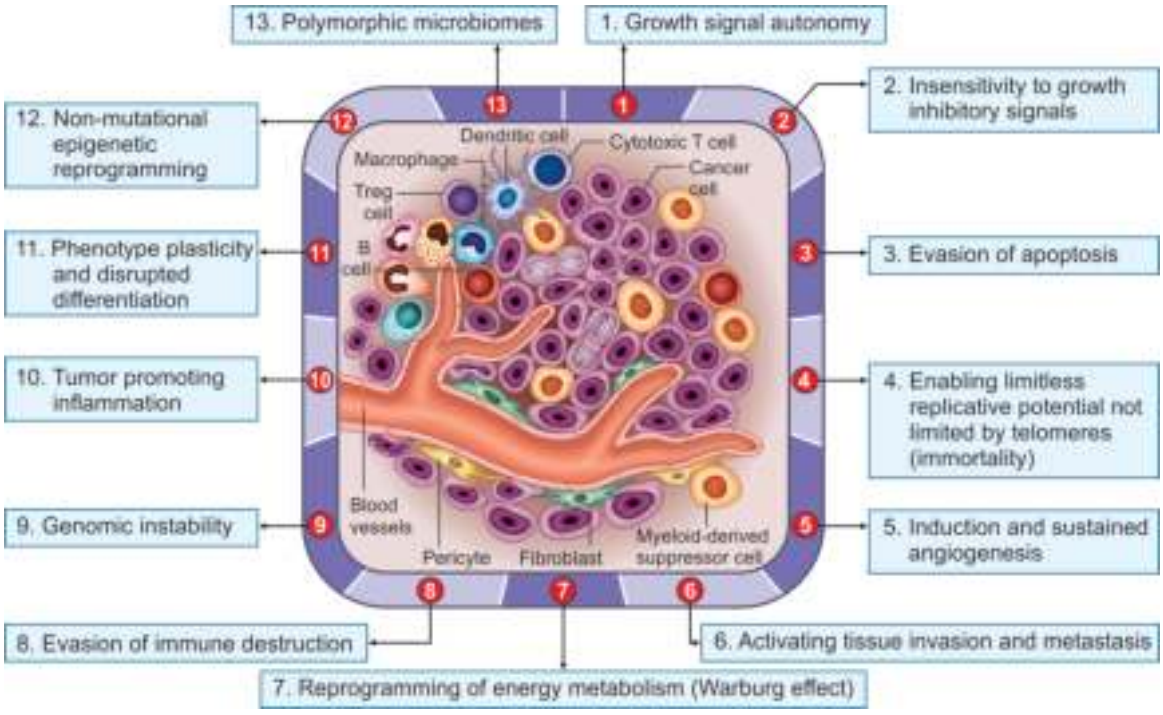


Fig. 6.75: Cellular and molecular hallmarks of cancer. These are the distinctive biologic capabilities acquired by cancer stem cells during multistep process of transformation of normal cell to cancer stem cell leading to tumor growth, invasion and metastatic dissemination.

Table 6.45 Cellular and molecular hallmarks of cancer and oncogenic activities

Oncogenic Driver Functions
▪ Growth signal autonomy (self-sufficiency of growth signal)
▪ Insensitivity to growth inhibitory signals
▪ Reprogramming of energy metabolism (Warburg effect)
Oncogenic Decreased Cell Attrition
▪ Cancer stem cells evade apoptosis
▪ Evasion of cellular senescence
▪ Cancer stem cells evade immune destruction
Oncogenic Invasion of Cancer Stem Cells
▪ Disruption of cell adhesion and cell polarity
▪ Cancer stem cells acquire motility property
▪ Extracellular matrix (ECM) degradation by matrix metalloproteinases (MMPs)
Oncogenic Fostering Functions
▪ Defective DNA repair and maintenance → Genomic instability
▪ Recruitment of normal immune cells and moulding of the tumor microenvironment → inflammation, angiogenesis and fibrosis

Pathology Pearls: Cellular and Molecular Hallmarks of Cancer

The Original Hallmarks of Cancer (2000) Proposed by Hanahan and Weinberg

1. Growth signal autonomy (self-sufficiency of growth signal)
2. Insensitivity to growth inhibitory (suppressor) signals
3. Evasion of apoptosis in cancer
4. Enabling limitless replicative potential not limited by telomeres (immortalization)
5. Induction and sustained angiogenesis
6. Activating tissue invasion and metastasis

The Next Generation (2011) updated by Hanahan and Weinberg

7. Reprogramming of energy metabolism (Warburg effect)
8. Evasion of immune destruction
9. Genomic instability
10. Tumor promoting inflammation

New Dimensions: Expanding the Frontier of Cancer Biology (2022)

11. Phenotype plasticity and disrupted differentiation
12. Non-mutational epigenetic reprogramming
13. Polymorphic microbiomes

of apoptosis and impaired DNA repair system. Genetic and epigenetic alterations are linked to carcinogenesis. Each major molecular hallmarks of cancer are antagonized by multiple tumor suppressor gene protein products.

GROWTH SIGNAL AUTONOMY (UNRESTRICTED CELL PROLIFERATION)

Normal cell remains in quiescent state (G0 phase) until stimulated by mitogenic growth signals from other cells, which enter into an active proliferative state resulting in

cell division tightly regulated by two sets of opposing functioning genes in normal cells: (a) proto-oncogenes (growth-promoting genes) and (b) tumor suppressor (growth inhibitory) genes.

- Mutated proto-oncogenes (i.e. oncogenes) can be classified according to the function of their oncoprotein products: growth factors (e.g. EGF, PDGF, VEGF, TGF-α, TGF-β, FGF, IGF, HGF, PGF, TNF-α, G-CSF, KGF, BMP), growth factor receptors (e.g. ERBB2/HER2 related to epidermal growth factor), GTP binding proteins (e.g. K-RAS, N-RAS), and nuclear binding proteins (e.g. Myc). EGFR inhibitors are therapeutic targets of this signaling pathway. Deregulated function of genes, i.e. activation of proto-oncogenes (i.e. oncogenes) and inactivation/biallelic loss of tumor suppressor genes leads to unrestricted cell division and carcinogenesis.
- Normal cell proliferation depends on external growth signals. Oncogenes are mutated form of proto-oncogenes, which code for oncoproteins (growth factors, growth factor receptors, GTP-binding proteins and transcription factor molecules), which positively regulate cell growth and differentiation. Oncogene activation results in sustained proliferation of CSCs, which can generate most of the oncogenic growth signals by themselves and induce unrestricted proliferation of CSCs.
- Oncoproteins can be produced in abnormally large quantity due to oncogene amplification (e.g. Myc oncogene in neuroblastoma) or increased transcription (Philadelphia chromosome 9/22 translocation in chronic myelogenous leukemia). Oncogenes, their mode of activation and associated cancers are given in Table 6.46.

Cell Cycle–Cell Division under Physiologic State

Cell cycle is a four-phase process: interphase (G1, S, G2) and mitotic phase (M phase). Cell cycle is tightly regulated by cell cycle positive regulators, i.e. cyclins/cyclin-dependent kinases (CDKs) and cell cycle negative regulators, i.e. RB protein, TP53 (p53 protein), INK family of CDK inhibitory proteins (p16, p15, p18, p19), and CIP/KIP (CDK interacting protein/kinase inhibitory proteins, i.e. p27, p21, p57).

Table 6.46 Oncogenes, their mode of activation and associated cancers

Proto-oncogene	Mode of Activation	Associated Malignancies
GTP-binding proteins (GTPase) involved in signal-transduction with oncogenic potential		
K-RAS (12p12.1)	Point mutation	▪ Colon carcinoma ▪ Lung carcinoma ▪ Pancreatic carcinoma
H-RAS (11p15)	Point mutation	▪ Renal cell carcinoma ▪ Urinary bladder carcinoma
N-RAS (1p13)	Point mutation	▪ Melanomas ▪ Hematologic malignancies
GNAO (16p13)	Point mutation	Uveal melanoma
GNAS (20q13)	Point mutation	▪ Pituitary adenoma ▪ Other endocrine tumors
Nonreceptor tyrosine kinase involved in signal-transduction with oncogenic potential		
ABL (Abelson mouse leukemia) (9q34.1)	▪ Translocation (9:22) ▪ Point mutation	▪ Chronic myelogenous leukemia (CML) ▪ Acute lymphoblastic leukemia (ALL)
RAS signal-transduction involved in signal-transduction with oncogenic potential		
BRAF (7q34)	Point mutation/translocation	▪ Hairy cell leukemia (100%) ▪ Melanoma (60%) ▪ Colon carcinoma ▪ Dendritic cell tumor ▪ Benign nevi
NOTCH signal-transduction involved in signal-transduction with oncogenic potential		
NOTCH1 (9q34.3)	Point mutation/translocation/gene rearrangement	▪ Leukemias ▪ Lymphomas ▪ Breast carcinoma
JAK/STAT signal transduction involved in signal-transduction with oncogenic potential		
JAK2 (Janus kinase 2) (9p24.1)	Translocation	▪ Myeloproliferative disorders ▪ Acute lymphoblastic leukemia (ALL)

- Extracellular mitogenic growth signals are transmitted to the normal cell through transmembrane tyrosine kinase receptors (RTKs) that bind to a specific class of cytoplasmic signaling transducing molecules such as additional cytoplasmic proteins and second messenger molecules, and initiate signaling transduction to the nucleus.
- The signaling pathway initiates DNA transcription of genes involved in cell growth, by binding of transcription factors to DNA regulatory proteins and recruiting chromatin remodelers to carry out gene transcription leading to cell cycle progression and cell division. In this way, mitogenic growth signals maintain cellular and tissue homeostasis. On the contrary, disruption in signaling pathways results in unrestricted cell division leading to carcinogenesis and development of malignant tumor.

Cell Cycle Deregulation and Unrestricted Proliferation of Cancer Stem Cells

Cell cycle is disrupted in human malignancies. Cancer stem cells generate their own growth signaling molecules (e.g. EGF/EFGR) and thereby reducing their dependence on external stimulation.

- CSCs respond by autocrine and paracrine mechanisms. Sustained CSC proliferative signaling occurs due to defect in negative feedback mechanisms such as insensitivity to growth inhibitory (tumor suppressor) signals, evasion of apoptosis (CSC survival even after substantial DNA damage), enabling limitless replicative potential not limited by telomeres (immortality), and genomic instability.
- Sustained proliferative signaling occurs by various mechanisms: (a) alterations in extracellular growth factor signals, (b) alterations of transmembrane receptor's growth signals, (c) alteration in GTP binding proteins, (d) aberrant signal transduction proteins, (e) aberrant nuclear transcription factors, and (f) alterations in intracellular circuits that translate those growth signals into action.

Pathology Pearls: Molecular Techniques to Assess Cell Proliferation

- Cell proliferation can be used to analyze health of normal cell, responses to toxic insult, and assess prognostic and diagnostic tool in several human cancers. The available markers typically look at the level of DNA synthesis, cellular metabolism and synthesis of specific proteins involved in cell division. A number of methods exist to assess cell proliferation and they vary in regard to which phase of cellular growth and cell division.
- The principal approaches are being used to assess cell proliferation, based on three aspects of cell division: (a) nucleoside analog (b) incorporation during DNA synthesis, and (c) cell cycle associated proteins and cytoplasmic proliferation dyes.
  - **Ki-67**, a marker of cell proliferation, is a nonhistone nuclear protein expressed throughout the active phase of cell cycle, except G0 and early G1 and used to asses cell proliferation in formalin-fixed paraffin-embedded tissue sections. It has become an important and reliable predictive marker in breast ductal carcinoma.
  - **Minichromosomal maintenance 2 (MCM2)** is also used to assess cell proliferation as a prognostic marker in certain human cancers.
  - Edu (5-Ethylene-3 deoxyuridine) is the preferred marker of choice to assess cell proliferation. DNA synthesis is the most reliable and accurate method to assess cell proliferation.
- Living cells are incubated with thymidine analogs (compounds) capable of being incorporated into newly synthesized DNA during replication and then assessed. It is important to be aware that thymidine analogs can induce gene mutations and DNA damage in some instances and thereby affect the cell cycle. Thus, method is suitable for immunohistochemistry (**IHC**), enzyme-linked immunosorbent assay (**ELISA**), flow cytometry and some multiplex assays.
- Cell proliferation can be assessed by measuring the cellular metabolic activity in culture via tetrazolium salts, which form a dye present in a metabolic active environment. The resulting color change of the media can be quantified in a spectrophotometer, giving an indication of the extent of cell proliferation. Molecular techniques to assess cell proliferation are given in Table 6.47.

INSENSITIVITY TO GROWTH INHIBITORY (SUPPRESSOR) SIGNALS (INACTIVATION/BIALLELIC LOSS OF TUMOR SUPPRESSOR GENES)

Multiple growth inhibitory (suppressor) signals maintain homeostasis in normal tissues by pushing cells out of cell cycle into temporary quiescent state, or sending them into their terminal, most differentiation state. Due to genomic instability, most cancer stem cells (CSCs) evade cleverly normal growth inhibitory (suppressor) signals in the G1 checkpoint of cell cycle by subverting the mechanisms that control cell cycle progression e.g. by disrupting TP53 (p53) and pRB signaling pathways, and over-expressing growth-stimulatory factors such as c-Myc. If carcinogenesis is to be successful, these endogenous tumor suppressors must be evaded or inactivated. Another important molecular hallmark of cancer is the lack of cell-cell contact inhibition. On contrary, in normal self-regulatory process, cells stop proliferating once cell-to-cell contact is established.

Table 6.47 Molecular techniques to assess cell proliferation

Assay Analysis	Cell Cycle Phase	Major Equipment	Recommended Applications
Nucleoside-analogues (DNA synthesis) analysis—molecular diagnostic technique			
Tritiated thymidine to label DNA	S phase	Liquid scintillation counter	Cell culture, mixed lymphocyte reactions
BrdU (5-bromo-2'-deoxy-uridine) to label DNA	S phase	▪ Flow cytometry ▪ Light microscopy ▪ Fluorescence microscopy	*In vivo* for the assessment of cell proliferation over time
Idu (idoxuridine) to label DNA	DNA replication fork progression rates, stability or origin of firing	Immunohistochemical analysis of formalin-fixed paraffin-embedded sections	Histology
Edu (5-ethylene-3 deoxy-uridine) to label DNA	G1/S and G2/M	Immunohistochemical analysis of formalin-fixed/paraffin-embedded sections	Histology
Cell cycle-associated proteins analysis—molecular diagnostic technique			
Ki-67 proliferative marker	G1, S, G2, M phases	▪ Flow cytometry ▪ Light microscopy ▪ Fluorescence microscopy	Histology
Phosphorylated-histone H3 marker analysis	M phase	▪ Flow cytometry ▪ Light microscopy ▪ Fluorescence microscopy	Histology
Proliferating cell nuclear antigen marker	S phase	▪ Flow cytometry ▪ Light microscopy ▪ Fluorescence microscopy	Histology
Cytoplasmic proliferation dye analysis—molecular diagnostic technique			
Carboxyfluorescein diacetate succinimidyl ester fluorescent dye for cell labeling	Cytokinesis	Flow cytometry	*In vivo* proliferation monitoring of transferred stained cells, mixed lymphocyte reactions
Cell trace violet for cell labeling	Cytokinesis	Flow cytometry with a 405-nm laser	*In vivo* proliferation monitoring of transferred stained cells, mixed lymphocyte reactions

Tumor Suppressor Genes Coding for Growth Inhibitory (Suppressor) Signals in Normal Cells

Within normal cells, multiple growth-inhibitory (suppressor) signals transmitted by tumor suppressor genes operate to maintain cellular quiescence and tissue homeostasis. Tumor suppressor genes code for proteins, that act to restrain inappropriate cell growth and cell division, as well as to stimulate apoptosis, before they can travel down the road to development of malignant tumor. Some of the tumor suppressor genes are involved in DNA repair processes. Inactivation/biallelic loss of these tumor suppressor genes can be disastrous.

- Binding of soluble growth inhibitory (suppressor) molecules to cell surface receptors are coupled to intracellular signaling circuits. Numerous growth inhibitory (suppressor) signals maintain the homeostasis in normal cells by pushing cells out of cell cycle into a temporary quiescent state (G0 phase), or sending normal cells into their terminal post-mitotic differentiation state.
- TP53 and RB1 tumor suppressor genes code for p53 protein and retinoblastoma protein (pRB) respectively. The p53 protein functions as a **central regulator of cell cycle**, that arrests the cell cycle upon detection of DNA damage. Retinoblastoma protein (pRB) inhibits the normal cells' passage through the restriction point in the G1 phase. The pRB protein integrates signals from diverse extracellular and intracellular sources and in response, decides whether or not a cell should proceed through its cell growth and cell division.
- Survival of normal cells is dependent on signals from extracellular growth factors, attachment of cells to solid surface, contact with other cells, and limited number of cell division before entering senescence.

Survival of Normal Cells Dependent on Signals from Extracellular Growth Factors

Survival of normal cells is dependent on signals from extracellular growth factors. On contrary, CSCs are able to survive in the absence of growth factors required by their normal counterparts. In normal cells, TGF-β

initially inhibits cell progression through G1 phase of cell cycle. However, at later stage in many cancers, TGF-β signaling is redirected away from suppression to activation of a cellular programme termed 'epithelial–mesenchymal transition' (**EMT**), which plays pivotal role in development of malignant tumor progression, invasion and metastasis to distant organ(s).

Epithelial–mesenchymal transition process is associated with reduction in the epithelial-like features of CSCs, and acquisition of mesenchymal-like features that are essential to mediate effective invasion and migration. EMT mechanism is regulated by transcription factors (i.e. SNAIL, ZEB, TWIST).

Anchorage-dependent Normal Cell Proliferation

Normal cells must be attached to a solid surface to divide, which require interaction of transmembrane proteins (integrins) with the components of extracellular matrix (ECM). Epithelial cells and connective tissue cells are anchorage-dependent cells. On the contrary, blood cells and CSCs are anchorage-independent cells. Moreover, anchor-dependent cells are mainly used in the manufacture of viral vaccines, while anchorage independent cells are required in cancer research studies. Comparison of anchorage-dependent and anchorage-independent cell proliferation is given in Table 6.48.

Cell-to-Cell Contact Inhibition

Cell-to-cell contact inhibition is defined as cessation of cellular movement, growth and cell division upon contact with other cells. When a normal cell loses cell-to-cell ability, it starts unrestricted cell division leading to development of malignant tumor. Cell-to-cell contact is mediated by transmembrane proteins called **E-cadherin**. Two mechanisms have been proposed to explain that E-cadherin maintains contact inhibition.

- **NF2 tumor suppressor gene (merlin)-mediated mechanism:** NF2 tumor suppressor gene mapped on chromosome 22 codes for '**merlin**' protein, that acts downstream of E-cadherin in signaling pathway involved in maintenance of cell-to-cell contact inhibition. NF2 gene mutation fails to maintain cell-to-cell contact inhibition. Homozygous loss of NF2 gene is linked to neural tumors. Germline mutation in NF2 gene is associated with hereditary neurofibromatosis type 2.
- **Adenomatous polyposis coli (APC) tumor suppressor gene E-cadherin/β-catenin interaction mechanism:** E-cadherin regulates cell-to-cell contact by involving its binding to β-catenin signaling protein, which is a key component of Wnt signaling pathway, that regulates the morphology and organization of epithelial cells lining the large intestine. APC gene exists in equilibrium with E-cadherin and β-catenin.
  - **Normal adenomatous polyposis coli (APC) tumor suppressor gene function:** APC gene mapped on chromosome 5 encodes APC protein, that degrades β-catenin. In addition, APC protein binding to E-cadherin, β-catenin is key component of Wnt signaling pathway involved in cell–cell adhesion.
  - **Adenomatous polyposis coli (APC) tumor suppressor gene mutation:** APC gene mutation encodes aberrant APC protein, that is linked to adenomatous polyposis coli, in which patient develops numerous adenomatous polyps in the colon, which later may undergo malignant transformation (i.e. colon carcinoma). APC gene mutation prevents β-catenin degradation that leads to sustained activation of Wnt signaling pathway, even in the absence of transcriptional factors. The β-catenin translocates and accumulates into nucleus of colonic epithelium, where it acts as a transcription activator of growth-promoting genes (i.e. cyclin D1, Myc) as well as transcriptional regulator genes (TWIST, SLUG), which repress E-cadherin expression.

Normal Somatic Cells with Limited Proliferative Activity

Normal somatic cells have a limited number of cell divisions before entering senescence. As the cells differentiate, their rate of proliferation decreases, and thus most adult normal cells are arrested in the G0 phase of the cell cycle. Some differentiated permanent

Table 6.48 Comparison of anchorage-dependent and anchorage-independent cells

Characteristics	Anchorage-Dependent Cells	Anchorage-Independent Cells
Definition	Anchorage-dependent cell survives when attached to inert surface	Anchorage-independent cell has lost the requirement of attachment to a surface
Examples of anchorage dependent/independent cells	Epithelial cells and mesenchymal cells	White blood cells and CSCs
Clinical usage	Manufacture of viral vaccines	Cancer research studies purpose
Industrial usage	Anchorage-dependent cell are used for industrial purpose in large quantities	Anchorage-dependent cells are not used for industrial purpose

cells in nervous system never divide again. Stable cells resume proliferation to replace cells that have been lost as a result of cell injury or cell death.

- Hematopoietic cells in bone marrow, and epithelial cells lining skin and digestive tract have short life span and must be replaced by continuous hematopoietic stem cell (HSC) proliferation in adult tissues/organs throughout life. Cell proliferation is thus carefully balanced with programmed cell death to maintain a constant number of cells in adult tissues and organs. On the contrary, CSCs have unlimited capacity to proliferate, which induce malignant tumors of breast, brain, lung, prostate, esophagus and colon.

Mutated Tumor Suppressors (Growth Inhibitory Signals) Linked to Human Cancers

Without functional tumor suppressor genes, there is a high-risk of dysregulated cell growth, that can result in development of malignant tumors. Mutations in tumor suppressor genes have been detected in many human cancers of ovary, breast, lung, colorectal region, pancreas, head and neck, uterus and urinary bladder. Several familial cancer syndromes including Li-Fraumeni syndrome are associated with mutations in TP53 tumor suppressor genes. Insensitivity to growth inhibitory (suppressor) signals in human cancers is given in Table 6.49.

Table 6.49 Insensitivity to growth inhibitory (suppressor) signals in human cancers

Key Marker	Gene Locus	Gene Product	Gene Function	Gene Mutations and Human Cancers
RB1 gene	13	pRB	Cell division, DNA replication and apoptosis	Biallelic loss of RB1 gene results in retinoblastoma
TP53 gene	17	p53	'Guardian of the genome' involved in cell division, DNA replication and apoptosis	TP53 gene mutation linked to several cancers including Li-Fraumeni syndrome (e.g. breast, bone, soft tissue, brain, adrenal cortex)
APC (adenomatous polyposis coli) gene	5	APC protein	APC gene is a component of Wnt/β-catenin signaling pathway	APC gene mutation linked to familial adenomatous polyposis coli that may progress to colon carcinoma
BRCA1 gene	17	BRCA1 protein	DNA repair and cell cycle	BRCA1 gene mutation linked to triple negative breast cancer (e.g. ER/PR/HER2/neu)
BRCA2 gene	13	BRCA2 protein	DNA repair and cell cycle	BRCA2 gene mutation linked to estrogen positive breast cancer
Phosphatase and tensin homolog (PTEN) gene	10	PTEN protein	PTEN gene is a component of PI3K-AKT-mTOR signaling pathway that regulates cell division	PTEN gene mutation linked to glioblastoma multiforme, lung carcinoma, breast carcinoma, prostatic carcinoma
CDKN2A (Cyclin-dependent kinase inhibitor 2A) gene	9	p16 and p14 proteins	Cell division, apoptosis	CDKN2A (INK4A) mutation linked to melanoma and many different cancers
WT1 gene	11	WT1 transcription factor	Cell division, cell survival, and angiogenesis	WT1 gene mutation linked to familial Wilms tumor
WT2 gene	11	WT1 transcription factor	Cell division, cell survival	WT2 gene mutation linked to Beckwith-Wiedemann syndrome
NF1 gene	17	Neurofibromin	RAS signaling pathway and cell division in nervous system	NF1 gene mutation linked to malignant peripheral nerve sheath tumor (MPNST), brain cancer, breast carcinoma
NF2 gene	22	Merlin or schwannomin	Cell shape, cell growth and cell-to-cell adhesion	NF2 gene mutation linked to bilateral vestibular schwannomas (acoustic neuroma) and several additional both benign and malignant tumors

Loss of Heterozygosity (LOH)

Mutant one allele of tumor suppressor gene behaves as a recessive, that is, as long as the gene contains one normal allele, which has adequate tumor suppressor activity that is known as **heterozygous state**. Inactivation/biallelic loss of tumor suppressor gene is linked to cancer, and is known as '**loss of heterozygosity**'. Retinoblastoma develops due to mutations in both alleles of RB gene. On the contrary, when one mutant allele of oncogene leads to malignant tumor formation, and it is known as **homozygous state**.

Tumor Suppressor Gene Mutations and Cancer

Caretaker tumor suppressor genes (BRCA1, BRCA2, MLH1, MSH2, MSH6, PMS2, FAMC, and Xp) maintain the integrity of the genome by repairing DNA damage. Gatekeeper genes (TP53 gene, RB and APC) inhibit the cell proliferation with damaged DNA, and also promote apoptosis of cells with DNA damage.

- Some examples of caretaker and gatekeeper tumor suppressor gene mutations associated with human cancers include: retinoblastoma (RB gene mutation), Li-Fraumeni syndrome (TP53 gene mutation), breast carcinoma and ovarian carcinoma (BRCA1 BRCA2 gene mutations), familial adenomatous polyposis/colon carcinoma (APC gene mutations), breast carcinoma (BRCA1/BRCA2, PTEN gene mutation), and hereditary nonpolyposis colon carcinoma (HNPCC) are called Lynch syndrome (MLH1, MSH2, MSH3, MSH6, PMS1, PMS2 gene mutations).

Pathology Pearls: Key Players in Cell Growth Suppression

- Key players in cell growth suppression include: (a) **TP53 gene** senses the need to inhibit cell progression and trigger apoptosis, and (b) **RB gene** is gatekeeper of cell cycle progression.
- TP53 tumor suppressor gene mutations are demonstrated in more than 50% of human cancers. **Li-Fraumeni syndrome** is genetic disorder due to germline mutation in TP53 gene that increases risk of family members to develop cancers. Females at birth, who have Li-Fraumeni syndrome have a nearly 100% risk for breast cancer.

Haploinsufficiency Susceptivity Genes: Model of Dominant Gene Actions

Haploinsufficiency in genetics describes a model of dominant gene action in diploid organisms in which a single copy of the standard **wild-type gene** is **inactivated** or **deleted**, and the remaining functional copy of the gene is not adequate to produce the needed particular gene protein product to preserve normal cell function. It is one of the major causes of certain dominant inherited diseases, as heterozygosity or hemizygosity elicits significant phenotypic impacts. Haploinsufficiency susceptibility gene mutation and associated syndromes are given in Table 6.50.

Table 6.50 Haploinsufficiency susceptibility gene mutation and associated syndromes

Haploinsufficiency Susceptibility Gene	Gene Mutation and Associated Syndrome
Deregulation of translation function	
PTEN gene	Cowden syndrome (multiple hamartomas)
LKB1 gene	Peutz-Jeghers syndrome
PTCH1 gene	Nevoid basal cell syndrome
Deregulation of cell proliferation function	
NF1 gene	Neurofibromatosis type 1 syndrome
APC gene	Familial adenomatous polyposis (FAP)
Deregulation of genetic integrity and apoptosis function	
BLM gene	Bloom syndrome
TP53 gene	Li-Fraumeni syndrome (100% risk for breast cancer)

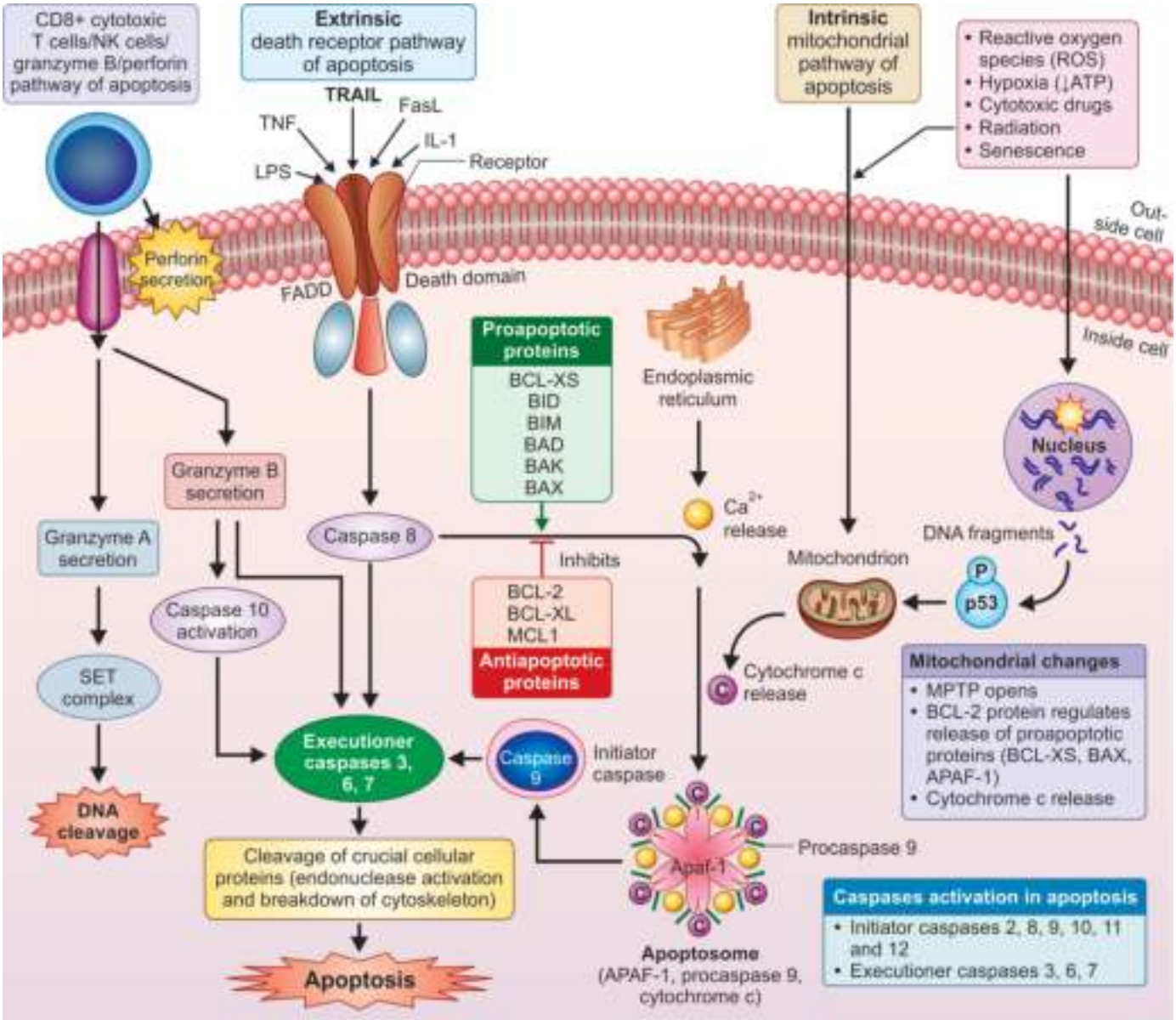
EVASION OF APOPTOSIS (PROGRAMMED CELL DEATH)

Apoptosis is the process of programmed cell death that maintains tissue homeostasis during embryogenesis to eliminate unwanted cells between fingers of developing hands. In adults, apoptosis is used to get rid of damaged cells beyond repair. Apoptosis also plays a role in preventing cancer.

- Apoptosis occurs by extrinsic (death receptor) and intrinsic (mitochondrial) pathways mediated by a family of proteases, called caspases. Both extrinsic and intrinsic pathways can flow independently until the last step of DNA degradation by **executioner caspases 3, 6** and **7**. The caspases cause proteolysis of specific substrates resulting in various morphologic and biochemical characteristics in apoptotic cells.
  - The extrinsic pathway can be engaged by activation of death receptors of the tumor necrosis factor (TNF) superfamily such as Fas (Apo/CD95) TNF receptor 1 (TNFR-1). TNF-related apoptosis inducible ligand (TRAIL) receptors and others located on the cell surface, by binding with their ligands.
  - On the contrary, intrinsic pathway of apoptosis is primarily regulated by the B cell lymphoma (BCL-2) family of proteins, that is activated by internal stress sensors in response to cellular stresses like nutrient deprivation, DNA damage and hypoxia.
  - It must be noted that perforin/granzyme apoptotic pathway is the primary signaling pathways used in activation of CD8+ cytotoxic T cells, which eliminate virus-infected and/or transformed CSCs.
- Detachment of CSCs from extracellular matrix (ECM) can also induce a form of apoptotic cell death called '**anoikis**', which acts to control the growth and attachment of detached CSCs to a different

ECM. Resistance to anoikis is an attribute of CSCs with metastatic potential. Anoikis can engage both extrinsic and intrinsic pathways of apoptosis.

- Schematic representation of apoptosis mediated by extrinsic (death receptor) and intrinsic (mitochondrial) pathways are shown in Fig. 6.76.



Morphologic changes	Apoptosis is characterized by cell shrinkage, chromatin condensation, nuclear fragmentation, plasma membrane blebbing without loss of cell integrity, organelles in the plasma membrane blebs, generation of apoptotic bodies.
Clearance	Degradation of apoptotic bodies by phagocytes (macrophages and surrounding cells).
Laboratory diagnosis	Techniques for detection of apoptotic cells include: (a) cytomorphologic changes, (b) DNA discontinuous fragmentation ladder assay on agarose gel electrophoresis, (c) TUNEL assay, executioner caspase 3 and annexin V (IHC), (d) p53, executioner caspase 3 activation, FasR, TNF-α, and TRAIL (IHC, RT-PCR, Western blot, ELISA), and (e) cytochrome c release (ELISA).

Fig. 6.76: Schematic representation of apoptosis mediated by extrinsic (death receptor) and intrinsic (mitochondrial) pathways. Both extrinsic (death receptor-mediated) and intrinsic (mitochondrial-mediated) pathways can flow independently induced by initiator caspases (2, 8, 9, 10, 11, 12) until the last step of DNA degradation by executioner caspases 3, 6, 7. The execution pathway of apoptosis results in characteristic cytomorphological features, i.e. cell shrinkage, chromatin condensation, nuclear fragmentation by endonuclease, plasma membrane blebbing, intact and preserved cytoplasmic organelles, generation and phagocytosis of apoptotic bodies by macrophages and surrounding epithelial cells and lack of inflammation. Perforin/granzyme apoptotic pathway is the primary signaling pathways used in activation of CD8+ cytotoxic T cells to eliminate virus-infected cells and/or transformed CSCs.

Pathology Pearls: Morphologic Changes in Apoptosis and Diagnostic Techniques

Cytomorphological Changes in Apoptotic Cells

- Apoptosis is characterized by several cytomorphologic features such as cell shrinkage, cell membrane blebbing, cytoplasmic organelles intact and preserved, apart from some swelling of the endoplasmic reticulum, chromosome condensation (**pyknosis**), nuclear fragmentation (karyorrhexis), DNA laddering and the eventual engulfment of the cell apoptotic bodies by phagosomes (i.e. macrophages and surrounding tissue cells).
- Phagocytic cell synthesizes cytokines (TGF-β, IL-10), which inhibit inflammation, and the apoptotic bodies are then phagocytosed by macrophages and surrounding tissue cells within an hour, rendering their appearance very transient. Kindly refer apoptosis in details in Chapter 1 'Cellular Pathology and Biology of Aging'.

Diagnostic Techniques to Detect Apoptotic Cells

- There are many diagnostic techniques available for the detection of apoptotic cells; based on cytomorphologic changes and biochemical events in apoptosis.
- Apoptosis is diagnosed to detect DNA fragmentation (DNA ladder assay, TUNNEL assay and Comet assay), phosphatidylserine (Annexin V, flow cytometric analysis), BID, p53 (RT-PCR technique, Western blot technique, ELISA analysis, flow cytometric analysis, immunohistochemistry), caspase activation (Western blot, ELISA, flow cytometric analysis), FAS, TNF, TRAIL (RT-PCR, Western blot, immunohistochemistry), and 'cytochrome c' release ELISA analysis.

Cancer Stem Cells Evade Apoptosis

Cancer stem cells (CSCs) are resistant to apoptosis, which acquire the overwhelming ability to evade apoptosis, thereby the genetically mutated CSCs become immortalized leading to carcinogenesis.

- Evasion of apoptosis allows CSCs to survive even with sustained DNA damage; resulting from various mechanisms: (a) loss of TP53 gene (p53 protein) leads to reduced function of pro-apoptotic proteins (e.g. BAX, BAK, BCL-Xs, BIK, BIM, BID, BAD), (b) reduced egress of 'cytochrome c' from mitochondria as a result of upregulation of anti-apoptotic proteins (e.g. BCL-2, BCL-XL, MCL-1), (c) loss of apoptotic peptidase activating factor 1 (APAF1), (d) upregulation of inhibitors of apoptosis, (e) reduced CD95 level in hepatocellular carcinoma, (f) inactivation of death-induced signaling complex Fas associated death domain (FADD). Mechanisms contributing to evasion of apoptosis in human cancers are shown in Fig. 6.77 and Table 6.51.
- Some cancers have high-level of anti-apoptotic proteins (e.g. BCL-2) that bind to death-inducing signaling complex Fas associated death domain (FADD) and thus, prevent the activation of caspase 8. BCL-2 activation inhibits apoptosis (programmed cell death) of atypical lymphocytes in Burkitt's lymphoma, which accumulate in the lymph nodes, bone marrow, and spill over in the blood circulation.
- Intrinsic (mitochondrial) apoptotic pathway is most often disabled in human cancers. Capspase 8, BCL-2 gene and TP53 gene products are among key apoptotic signaling proteins, that are known to be mutated in many human malignancies of breast, lung, kidney (renal cell carcinoma), ovary, endometrium, head and neck, melanoma, and hematopoietic tissue (leukemia and Burkitt's lymphoma).
- Cancer stem cells (CSCs) also evade apoptosis resulting from upregulation of phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathway and nonsignaling decoy receptor for Fas ligand.

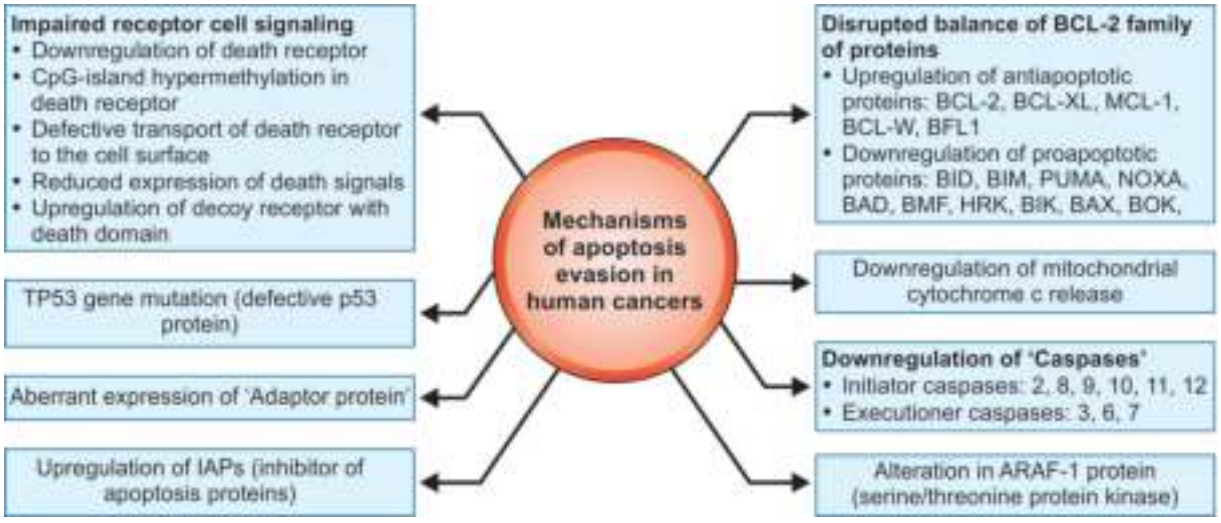


Fig. 6.77: Mechanisms contributing to evasion of apoptosis in human cancers. CSCs may escape apoptosis by downregulation of proapoptotic genes, upregulation of antiapoptotic genes respectively.

Table 6.51 Mechanisms contributing to evasion of apoptosis in human cancers

Disrupted Balance of BCL-2 Family of Proteins and Evasion of Apoptosis
▪ ↑Antiapoptotic proteins (BCL-2, BCL-XL, MCL-1, BCL-W, A1/BF1, BCLB/BCL-2-L10)
▪ ↓Proapoptotic proteins (BID, BIM, PUMA, NOXA, BAD, BMF, HRK, BIK, BAX, BOK)
Impaired Receptor Cell Signaling and Evasion of Apoptosis
▪ ↓Death cell surface receptor
▪ ↓Defective transport of death receptor ligand to the cell surface
▪ ↓Death receptor signaling
▪ ↑Decoy receptor with death domain
Dysregulation of Key Molecules and Evasion of Apoptosis
▪ ↑Inhibitor of apoptosis proteins (IAPs)
▪ Aberrant expression adaptor protein
▪ Inactivation of death-induced signaling complex FADD (Fas associated via death domain).
▪ Inactivation/biallelic loss in TP53 gene leads to reduced function of proapoptotic factors (e.g. BAX, BAK, BCL-Xs, BIK, BIM, BID, BAD)
▪ ↓Caspases expression
▪ ↓Apoptotic peptidase activating factor 1 (APAF1)
▪ ↓Egress of cytochrome C release from mitochondria resulting from upregulation of antiapoptotic factors (e.g. BCL-2, BCL-XL, MCL-1)
▪ ↓CD95 level in hepatocellular carcinoma
▪ ARAF-1 (A-RAF-1) serine/threonine-kinase protein alterations
▪ CpG-island hypermethylation in death receptors

Mutation in TP53 Tumor Suppressor Gene and Cancer Stem Cells Evading Apoptosis

Evasion of apoptosis can be acquired by the CSCs using a variety of strategies. TP53 tumor suppressor gene mapped on chromosome 17p13 codes for p53 protein, that acts as a 'guardian of cellular DNA'. The p53 protein either repairs damaged DNA before cell division by inducing cell cycle arrest at G1/S transition to allow time for DNA repair or force the cell to undergo apoptosis via activation of BAX gene and downregulation of BCL-2 antiapoptotic gene, and thus suppresses development of malignant tumor growth.

- Pathway of apoptosis-induced by normal TP53 gene involves: TP53 gene (p53 protein) phosphorylation → activation of BAX, PUMA and NOXA → cytochrome c release, and activation of apoptosome formation (α_1-apoptotic peptidase activating factor 1, cytochrome c, procaspase) → activation of caspase 9 → activation of caspase 3, 6, 7 → induction of apoptosis.
- Cancer stem cells (CSCs) evade apoptosis due to inactivation/ biallelic loss of TP53 tumor suppressor gene. Loss of apoptotic peptidase activating factor 1, and increased inhibitors of apoptosis (IAP) promote immortalization of CSCs. Upregulation of MCL-1 (BCL-2 member) prevents apoptosis of CSCs that leads to immortalization of chemoresistance.
- The p53 immunomarker may be useful as serum tumor marker, that differentiates p53 positive malignant tumors (carcinoma *in situ* and invasive carcinoma) from p53 negative reactive/metaplastic processes. The p53 immunomarker can distinguish p53 positive serous endometrial carcinoma from negative endometrioid carcinoma in formalin fixed paraffin-embedded tissue sections.

Disruption of Phosphoinositide 3-Kinase/AKT/mTOR Signaling Pathway and Cancer Stem Cells Evading Apoptosis

The phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathway is frequently activated in human cancers and represents an attractive target therapies based on small molecule inhibitors. PI3K isoforms play pivotal role in the signal transduction events activated by cell surface receptors, i.e. receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs). PI3K/AKT/mTOR signaling pathway is found to be upregulated either by extracellular factors such as GF-1 and IL-3, or by intracellular signals involving RAS protein, thus resulting in escape of CSCs from apoptosis.

Disruption of Nonsignaling Decoy Receptor for FAS Ligand and Cancer Stem Cells Evading Apoptosis

Recently, activation of nonsignaling decoy receptor for Fas ligand evades apoptosis of CSCs in lung carcinoma and colon carcinoma, by decreasing the intensity of death inducible signal and moving most of it away from Fas death receptor CSCs. It can also lead to the identification of novel targeted therapeutic agents. Purpose of chemotherapeutic agents is to induce apoptosis of CSCs. Development of multidrug chemoresistance in CSCs has been attributed to apoptosis resistance. It is therefore mandatory to understand the biologic pathways involved in imparting apoptosis resistance and to overcome of multidrug resistance development in treating cancers.

Pathology Pearls: Cell Surface Nucleolin and Evasion of Apoptosis by CSCs

- Multifunctional RNA-binding '**nucleolin**' protein encoded by NCL gene, that is primarily distributed in the nucleolus (90%), cytoplasm, and cell membrane.
- Cell surface nucleolin can bind to various ligands to affect many physiologic functions including ribosomal biogenesis, processing of ribosomal RNA (rRNA), messenger RNA

stability, downstream target of several regulation of signal transcriptional pathways and cell proliferation. Nucleolin interacts with DNA repair proteins to maintain **genomic stability** via stability of apoptosis-related mRNAs by binding to nucleolin RNA-binding domain (RBD) to the 5' and 3' UTR of mRNA and inhibit apoptosis.

- The expression and localization of nucleolin is often abnormal in human cancers, as the differential distribution of nucleolin can influence carcinogenesis, CSC proliferation, survival, angiogenesis, invasion and metastasis to distant organ(s). Thus, nucleolin may be a novel and promising target for anticancer treatment.
- Cell-surface nucleolin can bind to Fas and block the interaction of Fas/FasI, which prevents CSCs from entering Fas-induced apoptosis. The interaction of cell-surface nucleolin with ERB1 and RAS also favors unrestricted cell proliferation. Therefore, cell-surface nucleolin can facilitate anti-apoptotic phenotype and induce initiation and survival of CSCs.
- Upregulation of nucleolin can also elevate levels of anti-apoptotic BCL-2 protein in CSCs. Nucleolin binds to BCL-2, thereby increasing half-life of BCL-2. The anti-apoptotic BCL-2 protein is overexpressed in a variety of human cancers, particularly leukemias. Nucleolin also interacts with microRNAs 15, 6 which are negative regulators of BCL-2 expression.

ENABLING REPLICATIVE POTENTIAL (IMMORTALITY)

Enabling replicative potential (immortalization) of CSCs is one of the key hallmarks of cancer. CSCs have limitless replicative potential, that permit malignant neoplastic cells to avoid cellular senescence and mitotic catastrophe.

- Normal cells possess finite life span and limited number of successive **60–70 cell divisions**, which self-limit their replication at some point due to TP53 tumor suppressor gene (p53 protein), and RB tumor suppressor gene (pRB protein) dependent cell cycle arrest at the G1/S and G2/M checkpoints. This replicative limit is termed '**Hayflick limit**' after its discoverer Leonard Hayflick. Once cells go into 'cellular senescence' (G1 phase of cell cycle), and it is an irreversible change; although cells fail to grow and divide, hence the cells remain alive. Cellular senescence process occurs as a result of shortening of telomeres at the end of chromosome after each mitotic division.
- However, CSCs differ from normal cells, because they can exceed 'Hayflick limit' continue to undergo cell divisions (mitosis) to become immortal that avoid cellular senescence and mitotic catastrophe.
  - Telomeres are located at the ends of a chromosome; which have a specific sequence of nucleotide base pairs; shorten after each mitotic cell division in cell cycle process.
  - Cancer stem cells rely on the '**telomerase enzyme**' to maintain the length of telomeres at chromosomal ends above a critical threshold that allows them to undergo cell division by inhibiting cellular senescence and apoptosis. Telomerase inhibitors are therapeutic targets of this signaling pathway.
- The Wnt/β-catenin signaling pathway regulates pluripotency self-renewal of stem cells and differentiation. Abnormal activation of Wnt/β-catenin signaling pathway promotes CSCs progression and metastasis. The Hippo signaling pathway disruption is responsible for development of malignant tumor. Therefore, it is important to administer targeted therapy to maintain telomere machinery and disrupt abnormal action of Wnt/β-catenin and Hippo signaling pathways.

Telomeres

Telomeres are regions of repetitive TTAGGG sequence of DNA capping and protecting the ends of chromosome from degrading or fusing with another chromosome. Each time the cell undergoes division, telomeres become slightly shorter. Eventually, telomeres become so short that cell can no longer divide successfully, and the cell dies. This sequence occurs when the tumor suppressors p53 protein and pRB protein are intact. Progressive telomere shortening activates p53 protein and pRB protein, which leads to cell cycle arrest at the G1/S and G2/M checkpoints.

- Without telomeres, each time, the cell undergoes division, human genome progressively loses information because the chromosomes would keep on shortening. With every cell replication, 50–100 nucleotide base pairs of DNA in telomeres is lost, thus eventually causes the telomeres to lose their ability to protect the ends of chromosomal DNA.
- Left unprotected by telomeres, the exposed ends of chromosomes become damaged. The DNA damage response results in cell cycle arrest and senescence. When chromosomal ends fuse with each other, thus irreversible DNA damage results in cell crisis and apoptosis.

Pathology Pearls: Telomere Shortening → Genomic Instability

- Consequent replicative senescence triggers cell death.
- Sequence of events of senescence triggered cell death include: chromosome with capped telomeres → progressive cell mitoses → shortening of telomeres → activation of tumor suppressor p53 protein and pRB proteins → cell cycle arrest at the G1/S and G2/M checkpoints → replicative senescence → cell death.

- Nonhomologous end joining (NHEJ) DNA repair defects, homologous recombination repair of double-stranded DNA defects, DNA damage checkpoint defects and telomere dysfunction lead to double-stranded breaks (DSBs) and telomere uncapping.
- Intact p53 protein causes apoptosis of cells. Deficient p53 protein leads to genomic instability, cell survival and transformation of normal cell to CSCs.

Telomere/Telomerase Complex and Unlimited Replicative Potential Linked to Cancer

The telomere/telomerase complex is the key element that determines unlimited replicative potential, one of the hallmarks of cancer. Normal cells undergo apoptosis after 60–70 cell divisions. Telomere/telomerase complex and enabling limitless replicative potential of CSCs (immortalization) are shown in Fig. 6.78 and Table 6.52.

- Normal cells shorten their telomeres during each round of DNA replication. On the contrary, in cancer stem cells (CSCs), telomere length is stabilized by reactivation of human telomerase reverse transcriptase (hTERT) enzyme, and a multiprotein complex is called '**shelterin**'. The hTERT enzyme has a diminished capacity to repair DNA double-strands break.

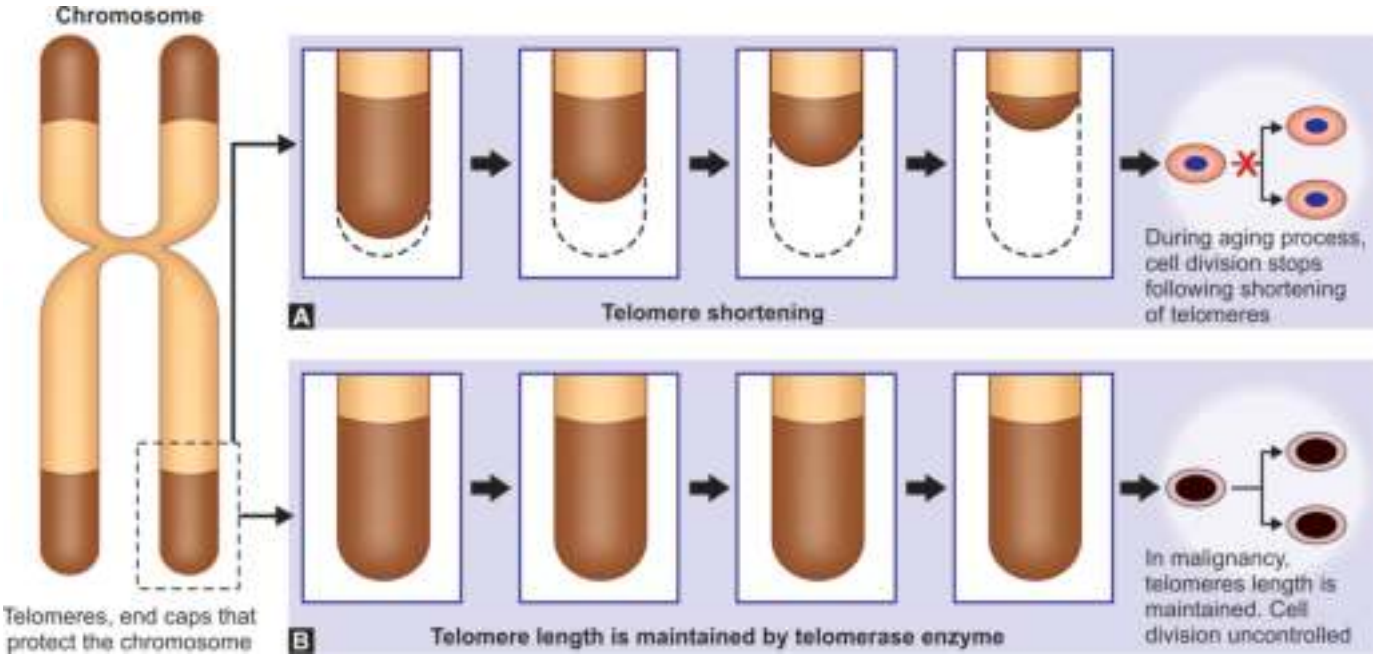


Fig. 6.78: Telomere and telomere/telomerase complex and enabling limitless replicative potential of cancer stem cells (immortality). Telomeres are the caps at the end of chromosomes that shorten as aging process continues. (A) During aging process, cell division stops following shortening of telomeres, (B) in malignancy, length of the telomeres is maintained by telomerase enzyme. Uncontrolled cell division takes place in cancers.

Table 6.52 Telomere and telomere/telomerase complex and enabling limitless replicative potential of cancer stem cells (immortalization)

Key Marker	Function
Telomere maintenance and regulation	
hTRET	▪ hTRET is the major component of telomerase activity ▪ Telomerase has been identified as a diagnostic marker for various human malignancies
TRF1/TRF2/POT1/TIN2/RAP1/TPP1 (Shelterin complex)	TRF1/TRF2/POT1/TIN2/RAP1/TPP1 (Shelterin complex) is a core of six proteins integral for telomere function
TP53 (p53) signaling pathway	
TP53 gene (p53 protein)	TP53 gene (p53 protein) is tumor suppressor gene and called 'guardian of the genome' and key regulator of gene expression
MDM2 gene	MDM2 is a proto-oncogene that inhibits transcriptional activation of TP53 gene (p53 protein)
$p14^{ARF}$ gene	$p14^{ARF}$ is a tumor suppressor gene that binds to MDM2-TP53 (p53) complex and prevents degradation of TP53 (p53)
E2F-1 transcription factor	E2F-1 is the transcription factor of p53 signaling pathway that regulates via initiating transcription of $p14^{ARF}$

- On the contrary, CSCs escape replicative limit, which undergo unrestricted cell divisions leading to carcinogenesis, invasion and metastasis.
  - CSCs become immortal and contain damaged chromosomes. The immortalization of CSCs due to maintenance of telomere length and structure by telomerase enzyme represents essential step in tumorigenesis and progression.
  - Telomerase enzyme activity is maximum in germ cells. However, somatic cells lack telomerase activity. Telomerase activity is increased in 90% of human cancers.
- Genetic and epigenetic alterations increase activity of telomerase enzyme that maintains length and structure of telomeres in chromosomes, hence germ cells limitless proliferative potential leads to initiation and progression of malignant tumors, thus making telomere/telomerase complex as an attractive therapeutic target.

INDUCTION AND SUSTAINED TUMOR ANGIOGENESIS

Angiogenesis is the formation of network of new functional blood vessels from preexisting neighboring venules, which occurs as a result of an intricate balance between endogenous proangiogenic factors and anti-angiogenic factors. '**Angiogenic switch**' involves sprouting, splitting and remodeling of the existing blood vessels. Endogenous proangiogenic factors involved in promoting angiogenesis are shown in Table 6.53. Endogenous antiangiogenic factors involved in inhibiting angiogenesis are shown in Table 6.54.

- One specific and promoter of angiogenesis is vascular endothelial growth factor (**VEGF**) in both normal and tumor angiogenesis.
  - Binding of VEGF to two structurally related membrane receptor tyrosine kinases '**switches on**' multiple signaling pathways leading to vascular endothelial cell growth, proliferation and survival.
  - Angiopoietin 1 (**Ang1**) binding to vascular endothelial cells recruits pericytes and supports cells around endothelial cells. Maturation and stabilization with remodeling of the newly formed blood vessels occurs under the influence of angiopoietin 2 (**Ang2**) and local growth factors.
- Angiogenesis plays important role in injured tissue healing, placenta formation after fertilization in the uterus in female populations, embryonic development, body growth, organ regeneration and cancers.

Table 6.53 Endogenous proangiogenic factors involved in promoting angiogenesis

Categories	Angiogenic Growth Factors
Proteins	VEGF, TGF-α, TGF-β, FGF acidic/basic, TNF-α, PDGF (A, B, C, D, EGF, PlGF, HGF, angiogenin, scatter factor, G-CSF, interleukins (IL-1, IL-6, IL-8), proliferin, leptin, angiopoietins (Ang1, Ang4), HIF-1,-2,-3
Small molecules	Adenosine, nicotinamide, 1-butyryl glycerol, prostaglandins E1 and E2
Trace element	Cu (copper)
Protease	Cathepsins, gelatinase A and B, stromelysin, urokinase (plasminogen activator)
Oncogenic proteins	c-Myc, RAS in VEGF-mediated angiogenesis, c-Src, V-Raf, c-Jun

VEGF (vascular endothelial growth factor), TGF-β (transforming growth factor-β), FGF (fibroblastic growth factor), TNF-α (tumor necrosis factor-α), PDGF-A,-B,-C,-D (platelet-derived growth factor-A,-B,-C,-D), EGF (epidermal growth factor), PlGF (placental growth factor), HGF (hepatocyte growth factor), G-CSF (granulocyte colony-stimulating factor), interleukins (IL-1, IL-6, IL-8), Ang1, Ang4 (angiopoietins) and HIF-1, 2, 3 (hypoxia-inducible factors 1, 2, 3)

Table 6.54 Endogenous anti-angiogenic factors involved in inhibiting angiogenesis

Categories	Anti-angiogenic Factors
Extracellular matrix (ECM)-derived angiogenic inhibitors	Angiostatin, endostatin, thrombospondin 1 and 2, anastellin, endorepellin, fibulin, tumastatin, canstatin, arresten
Nonmatrix-derived angiogenic inhibitors	Angiopoietins (Ang2, Ang3), angiostatin 2, vasculostatin, vasostatin, PF-4 (platelet factor 4), caveolin I and II
Protease inhibitors	TIMP-1,-2,-3 (tissue inhibitor metalloproteinase-1, 2, 3), PAI-1 (plasminogen activator inhibitor 1)
Cytokines	IL-10, IL-12, IL-18
Trace element	Zn (zinc)
Miscellaneous molecule	Prolactin and its 16-kD terminal fragment

- Cancer stem cells (CSCs), like normal cells, are unable to grow without vascular supply to bring oxygen and nutrients, and remove waste metabolic products.
- Tumor angiogenesis occurs through proliferation of circulating endothelial progenitor cells (EPCs) and differentiation of vascular endothelial cells.
  - The acquisition of new vasculature permits much degree of CSC proliferation and development of malignant growth. Hence, malignant tumors must induce angiogenesis for their progression.
  - Malignant tumor needs to trigger the formation of new vasculature by releasing proangiogenic growth factors (i.e. VEGF, FGS, TNF-α, TGF-β, and angiopoietin). It has been established that malignant tumors go through an '**angiogenic switch**' that allows them to grow from microscopic to macroscopic malignant tumor growth.
- Angiogenesis occurs through one or more of the following mechanisms: sprouting angiogenesis from mature quiescent endothelial cells (ECs), intussusceptive angiogenesis (mid-vessel lateral budding, and remodeling), vasculogenesis ('*de novo*' neovascularization from bone marrow derived endothelial progenitor cells), vascular mimicry induced by VEGF-VEGFR signaling pathway promoting epithelial–mesenchymal transition (EMT) mimicry by upregulating the expression of EMT-related genes. VEGF inhibitors are therapeutic targets of this signaling pathway.
  - **Proliferation of vascular endothelial cells:** Binding of VEGF to VEGFR can interact with Grab/Src/Gab1/Shb/PKC-γ, which activate RAS/RAF/MEK/ERK (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathways and promote the proliferation of vascular endothelial cells and formation of a capillary sprout from a pre-existing mature blood vessel.
  - **Migration, invasion and survival of vascular endothelial cells:** VEGFR can interact with three RAS-related GTP-binding proteins (GTPases) such as Rho/Rac/cdc42, that regulate the assembly and disassembly of the actin cytoskeleton in response to extracellular signals. VEGFR interacts with Rho/Rac/cdc42 that activates phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathway, that promotes vascular endothelial cell migration, invasion and survival leading to vascular tube formation.
  - **Maturation of the newly formed vessel:** Maturation of the newly formed blood vessel requires remodeling of the vascular network into large and small blood vessels, along with the recruitment of supporting pericytes and smooth muscle cells. Angiopoietin 1 binds to Tie receptors on vascular endothelial cell and results in endothelial stabilization, mesenchymal cell activation and pericyte activation. Under physiologic state, newly formed blood vessels are stabilized by pericytes.
  - **Increased vascular permeability:** VEGFR can enhance vascular permeability by activating NFAT/β-catenin/VE-cadherin, eNOS and PLC-γ signaling pathway.
  - **Vascular mimicry:** VEGFR can promote epithelial–mesenchymal transition (EMT)-induced vascular mimicry by upregulating the expression of EMT-related genes.

Angiogenesis during Tissue Healing

During tissue healing, vascular endothelial growth factor (VEGF) is produced by monocytes/macrophages, fibroblasts, mast cells and keratinocytes, that plays a role in wound healing. During acute phase of tissue healing, VEGF increases blood vessel permeability and expression of cell adhesion molecules, which assist in the recruitment of inflammatory cells. VEGF levels can influence the rate of wound closure/reepithelialization, inflammation, angiogenesis, granulation tissue formation, and scar tissue deposition.

Tumor Angiogenesis

Tumor angiogenesis is defined as the growth of newly formed blood vessels which develop due to stimulation of vascular endothelial cells within existing vascular networks near the malignant tumor, providing a supportive microenvironment rich with oxygen and nutrients to sustain optimum tumor growth. Angiogenesis plays a key role in tumor growth, invasion and metastasis.

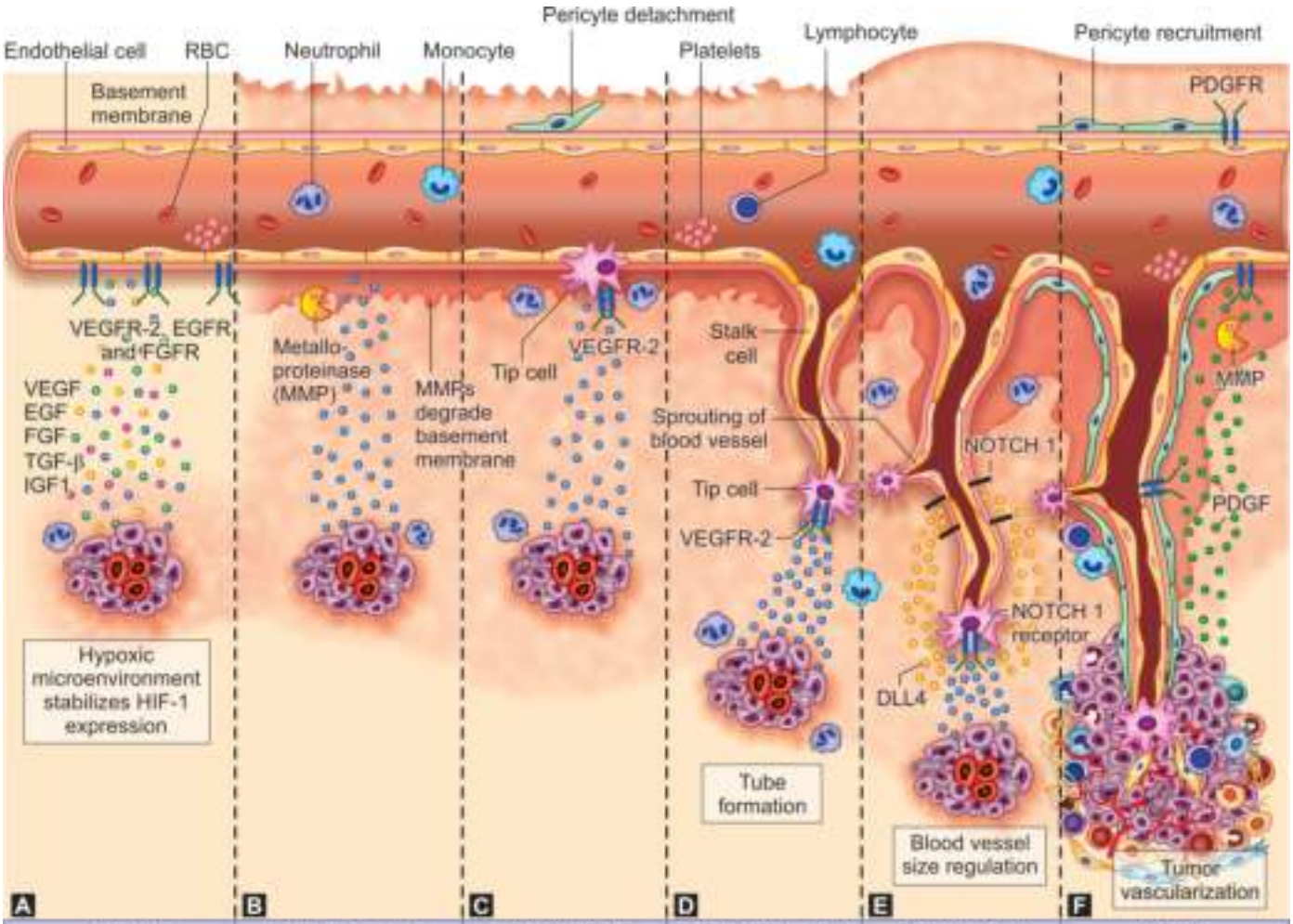
- Tumor angiogenesis plays a critical role in the growth of solid cancer beyond 1–2 mm in size microscopic cancer to attain macroscopic malignant tumor, as it requires adequate supply of oxygen and nutrients to survive, proliferate and invade in surrounding tissues. Cancer stem cells need to reside in close proximity to blood vessels to access the blood circulatory system essential for metastasis to distant organs(s).
- Tumor angiogenesis occurs through several distinct biological processes in anatomic location and within tumor microenvironment orchesterized by a range of secreted angiogenic factors and their binding to their cognate membrane-bound receptors (e.g. receptor tyrosine kinase) and transduce their signal downstream inside endothelial cells.
  - Common growth factor signaling pathways involved angiogenesis in human cancers include vascular endothelial growth factor (VEGF), platelet-

derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF) via binding to receptor tyrosine kinases (RTKs), and transforming growth factor-β (TGF-β) via binding to serine/threonine kinase receptors. TGF-β can act as stimulator and inhibitor in wound healing and carcinogenesis.
  - Signal transduction involves the communication process where signals from outside the cells are transferred to the nucleus inside the cell.
  - The existing quiescent vasculature sprouts to form new blood vessels as a result of vascular endothelial cell proliferation, migration, survival and stabilization by pericytes, that help to sustain expanding tumor growth exponentially.
- Vascular endothelial growth factor (VEGF) is the potent promoter of angiogenesis, which involves binding to membrane receptor tyrosine kinase, 'switches on' signaling pathways, stimulates the growth, survival and profanation of vascular endothelial cells and promotes malignant tumor growth, invasion and metastasis.
  - Tumor vasculature formed under the influence of VEGF is structurally and functionally abnormal, irregularly shaped, tortuous, having dead ends and not organized into venules, arterioles and capillaries, leaky and hemorrhagic due to poor stabilization by loose and incomplete pericytes around vascular endothelial cells, which result in high interstitial pressure, suboptimal blood flow and further VEGF production.
  - The central role of VEGF in the production of tumor vasculature makes it a rational target for anticancer therapy.
- Sequence of events in tumor angiogenesis is discussed as under:
  - **Hypoxic microenvironment:** Hypoxia induces HIF-1 expression and consequent release of proangiogenic factors (e.g. VEGF, EGF, FGF, TNF-α, TGF-β, IGF1), which bind to their cognate receptors on vascular endothelial cells. Vascular endothelial growth factor (VEGF) is the most important proangiogenic factor and synthesized by CSCs, and macrophages. VEGFA binds to and activates both cognate VEGFR-1 and VEGFR-2 receptor leading to mobilization and recruitment of endothelial progenitor cells (EPCs) from bone marrow, vascular endothelial cell activation, proliferation, migration, sprouting, increased vascular permeability, upregulation of anti-apoptotic genes in vascular endothelial cells, and vascular mimicry by upregulating the expression of epithelial–mesenchymal transition (EMT)-related genes. Mechanism and its regulation of tumor angiogenesis are shown in Fig. 6.79A. Vascular endothelial growth factor (VEGF) signaling pathways in the induction of angiogenesis are shown in Fig. 6.79B.
  - **Proteolytic degradation of basement membrane by matrix metalloproteinases (MMPs):** Hypoxic microenvironment also upregulates MMPs expression, leading to degradation of basement membrane and detachment of pericytes of vascular endothelium.
  - **Tip cell migration along angiogenic gradient:** Tip cells are specialized endothelial cells, which migrate along angiogenic factor gradient.
  - **Vascular endothelial cell differentiation to stalk cell and tube formation:** VEGF binds to VEGFR-1 receptor and mediates vascular endothelial cell proliferation leading to highly proliferative stalk cells, tube formation, the main body of the newly formed blood vessel.
  - **Regulation of blood vessel lumen size:** VEGF stimulates synthesis of DLL4, which binds to NOTCH-1 receptors resulting in downregulation of VEGFR and suppression of vascular endothelial cell proliferation.
  - **Vascular and stabilization:** Angiopoietins (Ang1 and Ang4) promote vascular endothelial cell migration, proliferation, sprouting and survival. Angiopoietin 1 binds to Tie2 receptors on vascular endothelial cells, recruits pericytes around vascular endothelial tubes. PDGF-β induces attachment of incomplete and loose pericytes, vascular endothelial cell proliferation and VEGF sensitivity. Maturation and stabilization with remodeling of blood vessels occur under the influence of angiopoietin 2 and local growth factors. Enhanced blood supply stimulates further malignant tumor growth.

Microvasculature in Healthy Tissue and Malignant Tumor

Tumor angiogenesis occurs as a result of an intricate balance between proangiogenic and anti-angiogenic factors. Morphologic and functional characteristics of microvasculature differ in healthy tissue, and tumor stroma.

- **Microvasculature in healthy tissue:** Microvasculature in healthy tissue displays an organized and hierarchical branching pattern of arteries, veins and capillaries, which are supported by basement membrane and complete and firm pericyte coverage and they are tightly connected by stable cell–cell junctions.



A Hypoxic microenvironment	B MMPs degrade basement membrane	C Tip endothelial cell migration	D Tube formation in angiogenesis	E Regulation of blood vessel size	F Tumor vascularization
• Hypoxia → ↑HIF-1 → synthesis of angiogenic factors (e.g. VEGF, EGF, FGF, TNF-α, TGF-β, IGF1) • Angiogenic factors bind to their receptors on endothelial cells	• Hypoxia → ↑HIF → MMPs • MMPs degrade basement membrane • Detachment of pericytes from basement membrane	• Tip cells are specialized endothelial cells • Tip cells migrate along angiogenic factor gradient	• Endothelial cells are differentiated into highly proliferative stalk cells • Stalk cells form the main body of the new blood vessel	• VEGF → ↑DLL4 • DLL4 binds to NOTCH 1 receptors → downregulation of VEGFR → suppression of proliferation of endothelial cells	• PDGF → ↑pericyte recruitment and attachment to endothelium • Reduction in endothelial cell • Reduction in VEGF sensitivity • Tumor angiogenesis → tumor growth

Fig. 6.79A: Mechanism and its regulation of tumor angiogenesis. Tumor angiogenesis is multistep process involving extensive interplay between cells, soluble factors, and extracellular matrix components.

- **Microvasculature in malignant tumor stroma:** Microvasculature in tumor stroma has dead ends and not organized into venules, arterioles and capillaries. Microvasculature in malignant tumor stroma displays vascular endothelial cell sprouting, small diameter of lumen, disruption of endothelial cell junctions, incomplete and loose pericytes coverage, irregular and chaotic branching, tortuous with blunt ends, leaky and heterogenous microvascular density resulting in increased tissue hypoxia and intravasation of cancer stem cells.
  - Moreover, microvasculature in stroma of malignant tumor exhibits abnormal vascular endothelial cell basement membrane, loose association of endothelial cells and variable thickness of endothelial cells. Leaky newly formed blood vessels cause hemorrhage, that results in high interstitial pressure leading to suboptimal blood flow and further VEGF production.

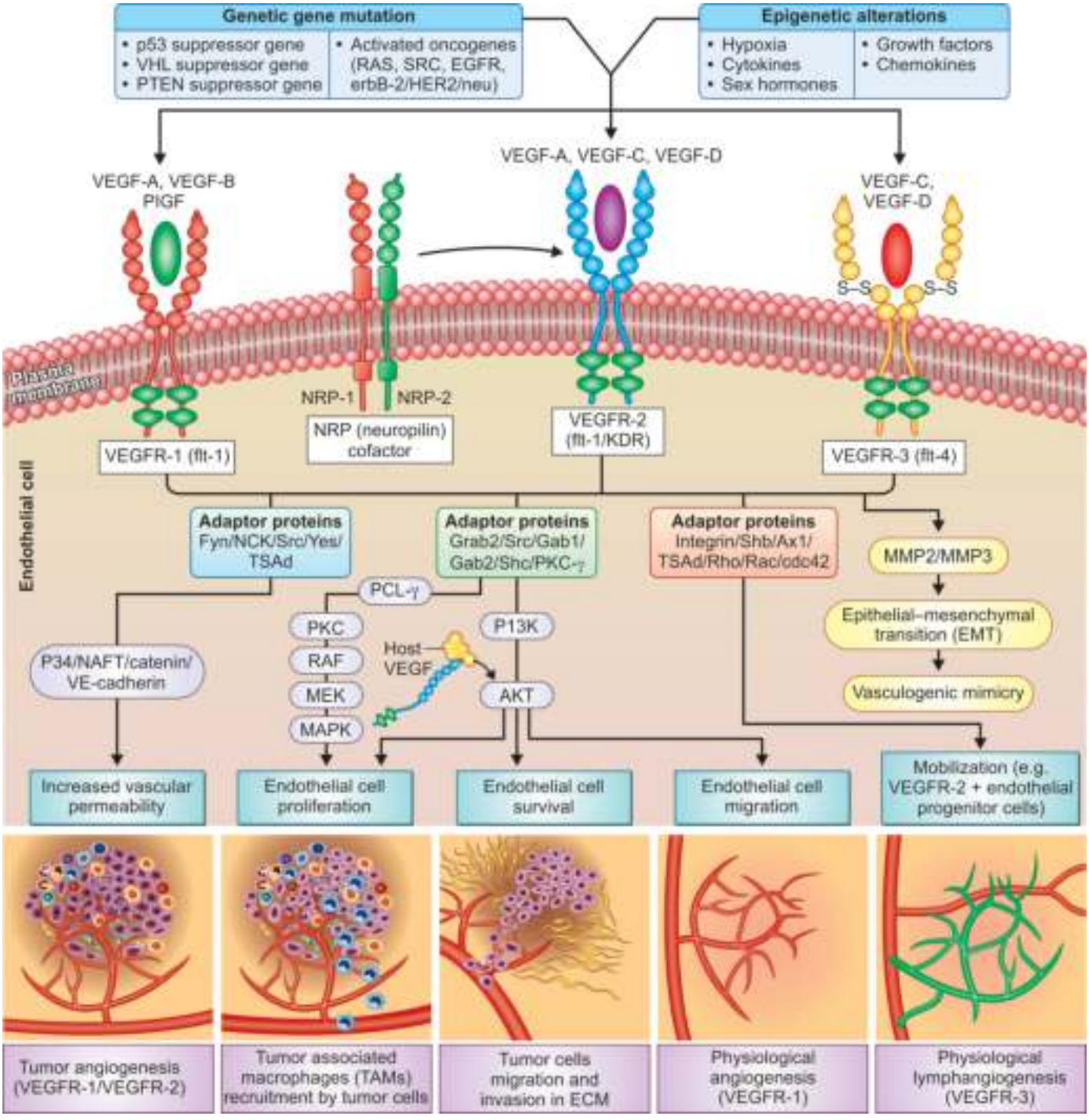


Fig. 6.79B: Vascular endothelial growth factor (VEGF) signaling pathways in the induction of angiogenesis. The vascular endothelial growth factor (VEGF) and its receptor (VEGFR) have been shown to play major roles not only in pathological angiogenesis but also most pathological angiogenesis in cancer. The VEGF-VEGFR system is an important target for anti-angiogenic therapy in cancer.

- Growth of the vascular network in malignant tumor is important for metastasis, as CSCs require a sufficient supply of nutrients and oxygen, and removal of waste metabolic products, which is achieved by angiogenesis and lymphangiogenesis, respectively. Morphologic and functional characteristics of microvasculature differ in healthy tissue and tumor stroma are given in Table 6.55.

Angiogenesis in Cancer Susceptibility Syndromes

Proangiogenic factors are also produced in human cancer susceptibility syndromes. Fumarate hydratase (FH) and von Hippel-Lindau (vHL) tumor suppressor

Table 6.55 Morphologic and functional characteristics of microvasculature in healthy tissue and tumor stroma

Characteristics	Vasculature in Normal Tissue	Vasculature in Tumor Stroma
Vascular endothelium	Continuous vascular endothelium	Discontinuous vascular endothelium
Basement membrane	Intact basement membrane	Discontinuous basement membrane
Pericyte coverage	Complete and compact	Incomplete and loose
Nerve supply	Innervation present	Innervation absent
Branching of microvasculature	Proper arborization and quasifractal branching	Abnormal and chaotic branching
Lumen diameter	Gradual changes in lumen diameter	Paradoxical changes in lumen diameter
Shunts or corkscrew structures	Absent	Present
Blunt ends and tortuosities	Absent	Present
Architecture	Organ specific architecture	Heterogenous vascular density ('hot spots')

proteins are mutated in familial cancer syndromes. Mutations in tumor suppressors such as FH and vHL cause upregulation of proangiogenic factors that induce tumor angiogenesis. Effects on the vasculature are not limited to the primary tumor microenvironment, but altered vascular function is also present in distant organs in persons with cancer.

Role of Hypoxia-inducible Factor (HIF) in Angiogenesis

Activation of signaling pathways of phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR and RAF/MEK/ERK/mitogen-activated protein kinase (MAPK) signaling pathways via receptor tyrosine kinases (RTKs), nonreceptor tyrosine kinases or G protein-coupled receptors regulate synthesis of hypoxia-inducible factor-1α (HIF1A) protein.

- **Normoxic state:** In normoxic state, HIF1A gene is constitutively transcribed to HIF1A messenger RNA, which is then translated to HIF1A protein. Degradation of HIF1A protein is regulated by O_2-dependent prolyl hydroxylation by prolyl hydroxylases (PHDs) enzymes, which targets the protein for ubiquitylation by E3 ubiquitin-protein ligases. The E3 ubiquitin-protein ligases contain the von Hippel-Lindau (vHL) tumor suppressor protein, which binds specifically to hydroxylated HIF1A. Ubiquitylated HIF1A is rapidly degraded by the proteasome.
- **Chronic hypoxic state:** During chronic hypoxic state, HIF1A no longer binds to vHL protein and accumulates, enabling HIF1A protein to move into the nucleus leading to formation of active HIF transcription complex.
  - **HIF1B expression:** HIF1B is also known as ARNT (acryl hydrocarbon receptor nuclear translocator), which is involved in expression of genes involved in metabolism such as c-Jun proto-oncogene, CREEB-cAMP responsive element binding protein 1, EP300-E1A binding protein. HIF1B activates the transcription of many genes that encodes proteins involved in angiogenesis, glucose metabolism, CSC proliferation, survival, invasion and metastasis to distant organs.
  - **HIF1A expression:** HIF1A is overexpressed in human cancers as a result of intratumoral hypoxia as well as genetic alterations, i.e. gain-of-function mutations in oncogenes (ERBB2), and loss-of-function mutations in tumor suppressor genes (i.e. vHL, PTEN). HIF1A overexpression is associated with treatment failure and mortality. Role of hypoxia-inducible factor (HIF) in angiogenesis under normoxic state and chronic hypoxic state is shown in Fig. 6.80.

Pathology Pearls: Chronic Hypoxic State and Tumor Invasion and Metastasis

- Chronic hypoxic state resulting from poor vasculature in malignant tumor stroma leads to subsequent recruitment of macrophages and neutrophils and increased vascular permeability by secretion of additional proangiogenic factors.
- Sustained hypoxia stimulates CSCs invasiveness by induction of epithelial–mesenchymal transition (EMT) and contributes to impaired therapy response.
- Tumor cell-derived cytokines and proangiogenic factors are carried throughout the body in plasma or cargo in platelets or microvesicles and can contribute to formation of premetastatic or antimetastatic niches in organs that exert sites for metastasis. Recruitment of leukocytes to the metastatic sites mediate metastatic colonization.

ACTIVATING TISSUE INVASION AND METASTASIS

A key feature that distinguishes malignant neoplastic cells, i.e. CSCs from all other cells is the capability of CSCs to disseminate throughout the body by two related mechanisms: invasion and metastasis. Hanahan

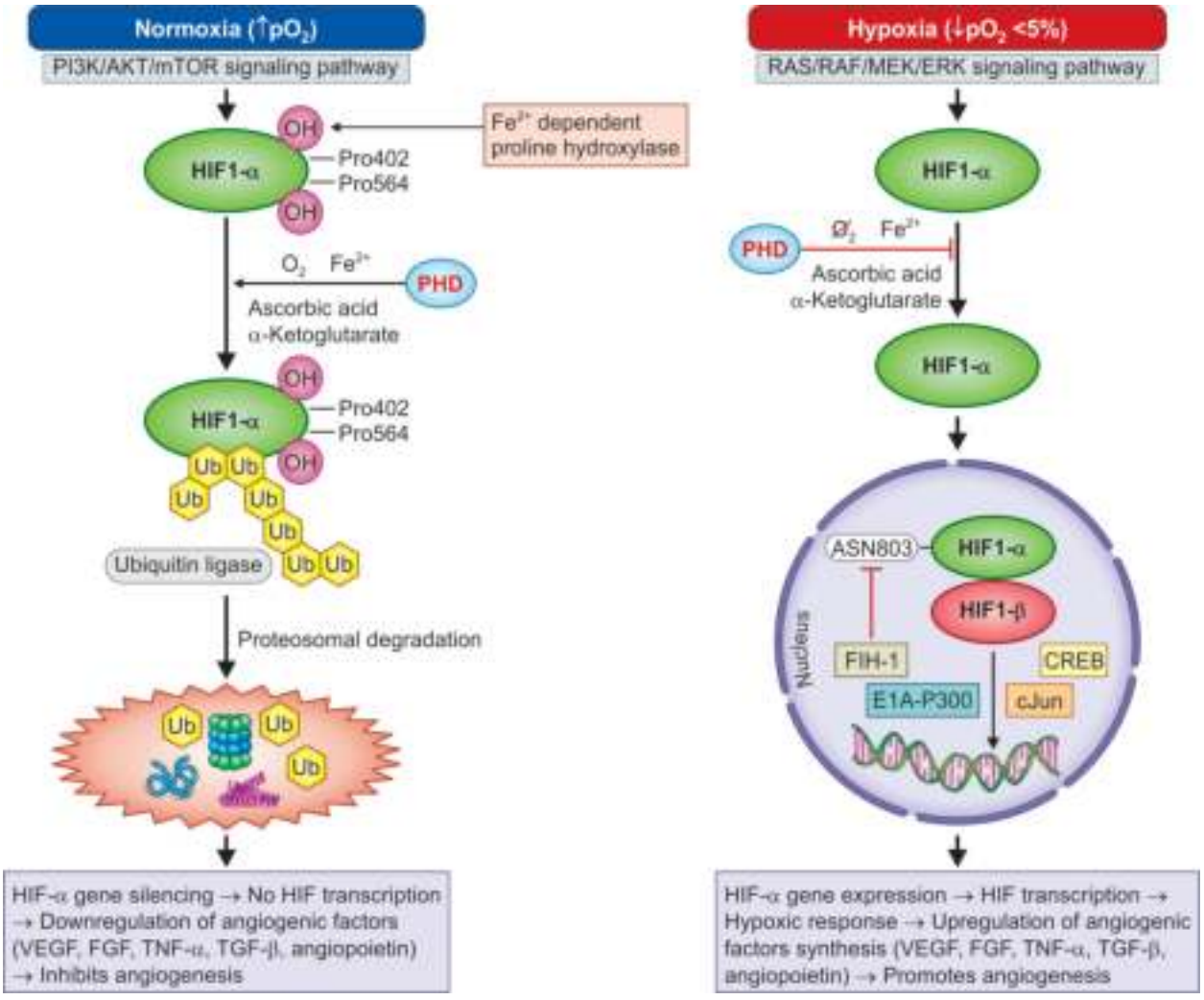


Fig. 6.80: Role of hypoxia-inducible factor (HIF) in angiogenesis under normoxic state and chronic hypoxic state. In normoxic state, HIF1A gene is constitutively transcribed to HIF1A messenger RNA, which is then translated to HIF1A protein, which HIF1A protein is rapidly hydroxylated by the prolyl hydroxylases (PHD) enzymes, which enables binding von Hippel-Lindau tumor suppressor protein (pvHL) and the ubiquitin ligase, leading to proteasomal degradation. During chronic hypoxic state, HIF1A no longer binds to pvHL and accumulates, enabling HIF1A to move into the nucleus and form the active HIF transcription complex (HIF1B also known as ARNT-aryl hydrocarbon receptor nuclear receptor, c-Jun proto-oncogene, CREB-cAMP response element binding protein 1, EP300 gene—E1A binding protein p300) and subsequent transcription.

and Weinberg recognized invasion and metastasis as hallmarks of cancer in solid malignant tumors. CSCs proliferate locally, accumulate genetic alterations, and synthesis of growth factors, chemokines and proangiogenic factors leading to development of primary malignant epithelial tumor.

- Local invasion is the direct extension and penetration of CSCs into the neighboring tissues. The proliferation of transformed CSCs and the progressive increase in malignant epithelial tumor size eventually leads to breach in the barriers (basement membrane), and invades in adjacent tissue by allowing modifications in CSC interactions with their surrounding environment through E-cadherin, integrins, and other cell adhesion molecules, synthesis of MMP, and downregulation of tissue inhibitors of metalloproteinases (TIMPs).
- Then CSCs intravasate lymphatic channel and/or blood vessel, travel the bloodstream as single cell/cluster in the form of tumor emboli, extravasate and colonize as single/multiple metastases at a secondary site in various distant organs (e.g. lungs, bones, liver, and brain).
- Local invasion is the first stage in the process that leads to the development of metastases in secondary organs/tissues. The ability of CSCs to migrate from a primary site of disease is attributed to the mutation of genes that regulate synthesis of proteins and normally tether to their surrounding tissues.

- Metastasis refers to the ability of CSCs to penetrate into lymphatic and blood vessels, circulate through these lymphovascular systems and invade normal tissues elsewhere in the body. This process proceeds in an orderly and predictable manner and termed 'metastatic cascade' that consists of multiple steps (i.e. local invasion, intravasation, and extravasation).
- Tumor microenvironment regulates tumor growth, recurrence and metastasis. Different populations of cells of innate immune system are involved in different stages of the metastatic cascade both locally and in distant organs/tissues, which regulate the survival and unrestricted proliferation of CSCs and assisting in the establishment of a permissive tissue environment enabling tumor invasion and metastasis.
- Some CSCs undergo dormancy and metabolic changes as an adaptation mechanism to new stressful tumor microenvironment in distant organs. Tumor microenvironment regulates tumor growth, recurrence and metastasis.
- Notably, both epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) are required for the process of metastasis, while EMT mobilizes CSCs in the primary malignant epithelial tumor. MET terminates the migration process and thereby resulting in the colonizing of cancer stem cells (CSCs) in distant organs as single/multiple metastases.
- Biomolecules involved in activation of invasion and metastasis are given in Table 6.56. Schematic representation of the malignant epithelial tumor invasion and metastasis is shown in Fig. 6.81.

Primary Malignant Epithelial Tumor and Local Invasion

Normal cells are transformed into CSCs, which have same genetic abnormalities in key genes coding for aberrant proteins (e.g. growth factors, growth factor receptors, signal transduction proteins, transcription factor and DNA repair system).

- Carcinoma arising from epithelial tissue usually undergoes through several stages of development: atypical hyperplasia, carcinoma *in situ*, and then invasive carcinoma, which might further metastasize to distant organs.
- As the malignant epithelial tumor develops, it undergoes further somatic gene mutations resulting in additional oncogene activation and/or inactivation/biallelic loss of tumor suppressor gene. Malignant epithelial tumor has clones with different genetic make-up. Some subclones possess ability to metastasize to distant organs, while other

Table 6.56 Biomolecules involved in activation of invasion and metastasis

Key Biomolecule of Components	Functions
Extracellular matrix (ECM) component	
Hyaluronan	▪ Hyaluronan is a glycosaminoglycan found in the extracellular matrix (ECM) ▪ Hyaluronan is dramatically upregulated in most human malignancies
Versican	Versican in ECM is either expressed by CSCs or stromal cells, which activates EGFR signaling leading to carcinogenesis progression, invasion and metastasis
Collagen IV	Collagen IV is essential for tumor angiogenesis by modulating cell growth and proliferation
Adhesion molecules	
Carcinoembryonic antigen related cell adhesion molecule 1 (CEACAM1)	▪ L-Form CEACAM1 has tumor suppressor function and found in the early carcinogenic process ▪ CEACAM1 is downregulated in several human malignancies
DCC deleted in colorectal cancer	▪ Netrin-1 transmembrane receptor DCC or colorectal suppressor is a protein encoded by the DCC gene, which is involved in development of nervous system. ▪ Mutations in DCC gene are linked to colorectal carcinoma, esophageal carcinoma and neuroblastoma
E-cadherin	▪ E-cadherin is transmembrane protein that mediates cell-cell adhesion ▪ E-cadherin loss has been observed in subsets of Merkel cell carcinoma, anaplastic carcinoma, lobular carcinoma of breast, small cell neuroendocrine tumors and urothelial carcinoma
Secreted factors	
Tenacin C	▪ Tenacin C interacts with extracellular matrix (ECM) proteoglycans ▪ Tenacin C interferes with tumor suppressor activity of fibronectin
Fibrinogen	Fibrin deposits occur in the stroma of many human malignancies and affect progression of CSCs
Periostin	Periostin is secreted adhesion-related protein expressed and periodontal ligaments and plays a role in oncogenesis

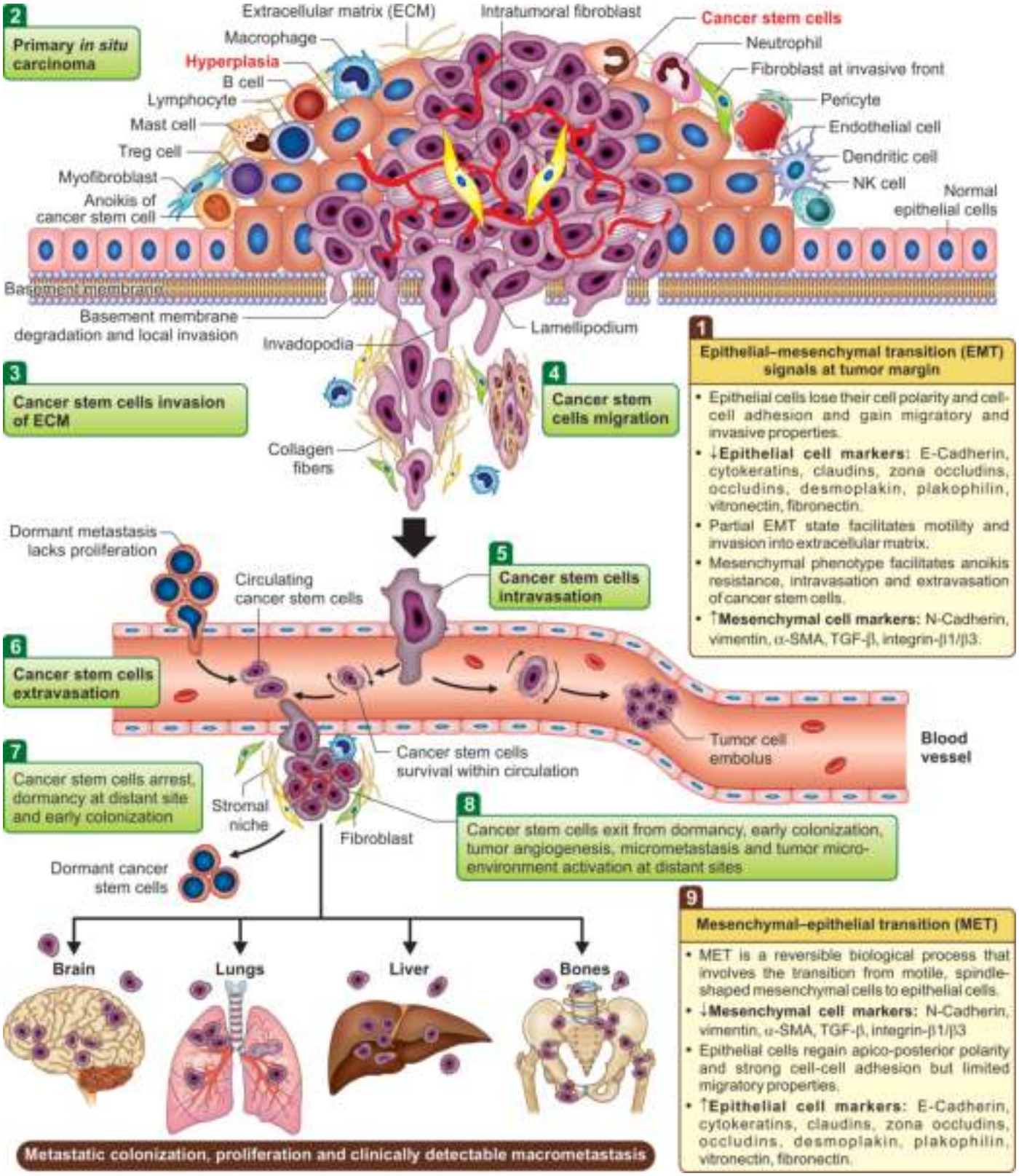


Fig. 6.81: Schematic representation of the malignant epithelial tumor invasion and metastasis. The initial transformation of normal epithelial cells to cancer stem cells results in carcinoma *in situ*. With reduced adhesiveness and enhanced migratory behavior, carcinoma *in situ* progresses to an invasive carcinoma. After enzymatic degradation of the basement membrane by secreted matrix metalloproteinases (MMPs), CSCs invade the surrounding extracellular matrix, migrate and intravasate into lymphatics and blood vessels. Circulating survived CSCs arrest in the capillaries of distant tissues/organs. There, CSCs remain in dormant state without reproliferating for considerable time. Alternatively, CSCs exist blood circulation (extravasate) proliferate and produce secondary tumors after CSC proliferation, tumor angiogenesis in tumor microenvironment and settle in distant organ(s).

subclones undergo rapid proliferation forming poorly-differentiated malignant tumor. The goal of the chemotherapy is to destroy CSCs in the malignant epithelial tumor. Cancer stem cells may develop resistance to chemotherapy. Only resistant cancer stem cells survive after chemotherapy. Cancer stem cells in recurrent malignant tumor remain resistant to chemotherapy.

Tumor Angiogenesis

Tumor angiogenesis is the process of developing new blood vessels from pre-existing blood vessels that has a key role in tumor growth, invasiveness and metastasis. Malignant epithelial tumor needs to trigger the formation of new vasculature by releasing proangiogenic growth signals. It has been established that malignant epithelial tumor undergoes through an 'angiogenic switch' that allows them to grow from microscopic to macroscopic malignant tumor growth. VEGF inhibitors are therapeutic targets of this signaling pathway.

Epithelial–Mesenchymal Transition

Epithelial–mesenchymal transition (EMT) involves the disruption of cell-to-cell adhesion (downregulation of E-cadherin), apical-basal cell polarity, remodeling of the cytoskeleton, and changes in cell-to-extracellular matrix adhesion associated with mesenchymal phenotype, enhanced motility, and invasive properties of CSCs in primary malignant epithelial tumor.

- Epithelial–mesenchymal transition is regulated by transcription factors (i.e. SNAIL, ZEB, TWIST), cell signaling, and epigenetic modification. EMT plays critical roles in metastatic cascade process.
- Notably, both EMT and MET are required for CSC metastasis, while EMT mobilizes CSCs in the primary malignant epithelial tumor. MET terminates the migration of CSCs and thereby resulting in the colonizing of CSCs in distant organs.

Expansile Tumor Growth, Detachment of Cancer Stem Cells from Primary Malignant Epithelial Tumor and their Migration

As the malignant epithelial tumor grows, it exerts mechanical pressure on the surrounding cells and tissues, which eventually undergo cell death due to blockage of blood supply to malignant tumor. Loss of mechanical resistance opens the way for the CSCs to spread along the lines of least resistance and occupy the space once filled with dead cells. CSCs detach from primary malignant epithelial tumor (carcinoma), become motile, undergo a change in their shape to acquire a polarized mesenchymal phenotype, similar to locomoting leukocytes, migrate and invade basement membrane, and then surrounding healthy stromal extracellular matrix.

Tumor Development and Acquisition of Invasive Potential through Basement Membrane

Tumor development and acquisition of invasive potential is regulated by metastatic signature genes (e.g. Ezrin, SNAIL and TWIST genes including microRNA-10b) and metastatic tumor suppressor genes (e.g. NM23, KAI-1, KiSS and NM23).

- Cancer stem cells possess receptors for laminin, a complex glycoprotein in the basement membrane. The receptors for laminin permit the CSCs to attach to the basement membrane forming a bridge-like connection.
- Cancer stem cells expand and breach barriers, i.e. basement membrane, extracellular matrix (ECM) and penetrate into nearby surrounding tissue. Cancer stem cells and stromal cells of tumor microenvironment synthesize matrix metalloproteinases (MMPs), which play important role in degradation of basement membrane and ECM.
- Extracellular matrix has many components that regulate cell migration, growth and differentiation, which include: (a) fibers (i.e. collagen, elastin, laminin, and fibronectin), (b) proteoglycans (syndecan-1 and aggrecan), (c) glycoproteins (tenacin, vitronectin and entactin), and (d) polysaccharides (hyaluronic acid).
- Extracellular matrix is a macromolecule network, composed of collagen (most abundant and can be degraded by MMPs). Triple-helical protein is highly resistant to degradation by MMPs, that gives a structural and biochemical support. Matrix-metalloproteinas (MMPs) are strictly regulated by endogenous tissue inhibitor metalloproteinases (TIMPs).

Anoikis of Cancer Stem Cells

Detachment of CSCs from ECM can also induce a form of apoptotic cell death called '**anoikis**', which acts to control the growth and attachment of the detached CSCs to a different ECM. Resistance to anoikis is an attribute of cancer stem cells with metastatic potential. Anoikis can engage both extrinsic and intrinsic pathways of apoptosis.

Increased Motility of Cancer Stem Cells

Cancer stem cells secrete chemokines that stimulate development of finger-like projections (**invadopodia**) on CSCs involved in their motility to overcome the dense scaffold of local environment. Polarity of cancer stem cells has a leading front and rear region, which depends on a distinct actin cytoskeleton and generation

of various cell junctions. **Lamellipodia** are flat, sheet-like membrane protrusions formed at the leading edge of migrating CSCs.

Matrix metalloproteinases (MMPs) Proteolytic Enzymes Synthesis

Cancer stem cells and stromal cells of tumor microenvironment produce powerful proteolytic MMPs enzymes such as collagenases and proteases, which degrade basement membrane and surrounding tissue and promote CSCs to invade lymphatic channel or blood vessel. In addition, there is downregulation of tissue inhibitor of metalloproteinases (TIMPs).

Intravasation, Transport and Arrest of CSCs

Cancer stem cells exit primary malignant epithelial tumor site through degradation of basement membrane, interstitial tissue, and extracellular matrix (ECM) by matrix metalloproteinases (MMPs) synthesized by CSCs and stromal cells, invade lymphovascular system, enter circulation, and travel as platelet-tumor emboli (single/clusters) thereby becoming circulating cancer stem cells and potential metastatic seeds.

- Only a small number of CSCs survives in the circulation due to evasion from immune system. Circulating CSCs may interact with cavity mesothelial cells and metastasize by transcoelomic route to distant organ(s). In the lymph nodes, communications between the lymphatic channels and venous tributaries allow malignant neoplastic cells, i.e. CSCs access to the systemic circulation.
- Circulating cancer stem cells are involved a cascade of events consisting of CSCs arrest on the vascular endothelium resulting in the formation of dynamic contacts that give rise to significant cytoskeletal alterations, transendothelial migration and subsequent invasion of surrounding ECM degradation by MMPs (proteolytic enzymes).

Extravasation of Cancer Stem Cells

Only a small number of CSCs survives in the circulation and then extravasate through basement membrane and surrounding tissues and remain dormant while retaining a stem-like tumor initiating capacity.

- Invadopodia are required for CSC extravasation and thus remain a therapeutic target for metastasis. CSCs extend invadopodia through the vascular endothelium into extravascular stroma prior to their extravasation at endothelial junctions and distant metastasis and reveals an opportunity for therapeutic intervention in this clinically important process.
- After extravasation, surviving CSCs are disseminated, they must reactivate their epithelial properties by means of the process of EMT, known as the mesenchymal–epithelial transition (MET). MET is a reversible process of EMT, that involves the transition from mobile multipolar or spindle-shaped mesenchymal cells to planar arrays of polarized epithelial cells with cell-to-cell junctions, apical-basal polarity and expression of epithelial markers. The seeding of migrating CSCs may occur quickly with subsequent formation of metastasis in distant tissue/organ sites and therapy resistance.

Pre-metastatic Niche for Distant Metastasis

After extravasation, CSCs undergo adaptation to local tumor microenvironment (TME), successful colonization finally establishment of clinically detectable metastatic disease. Metastasis is modulated by tumor microenvironment and immunity. Genes play pivotal role in both metastatic dormancy and reactivation of CSCs.

Colonization of CSCs in Distant Organ(s) at Secondary Site

Cancer stem cells invade and adapt to tissue microenvironment in distant organs (e.g. liver, lungs, brain, adrenal glands, and bone marrow), and build secondary tumors (micrometastasis or macrometastasis) and tumor angiogenesis or remain in dormant metastasis.

Tumor Dormancy and Reactivation at Secondary Sites

Extravasated CSCs may remain dormant over a period with subsequent reactivation involving intracellular signaling, extracellular signaling, and induction signals originating from bone marrow niche.

- Tumor dormancy can be induced through several mechanisms, such as genetic and/or epigenetic changes in the malignant tumor, tumor hypoxia, angiogenic switch, evasion of immune destruction and inflammatory switch. Extravasated CSCs interact and adapt with tissue microenvironment and induce neoangiogenesis in order to ensure sufficient vascularization of malignant tumor.
- Alteration in the tumor microenvironment can facilitate tumor growth, recurrence and metastasis, and thereby permit the CSCs to exit from dormancy through interaction with vascular endothelial cells, cancer-associated fibroblasts (CAFs), tumor-associated immune cells, inflammatory cells and the extracellular matrix (ECM).
- In the novel stromal environment, CSCs form micrometastasis with the ability to generate macrometastasis, fully malignant secondary tumors elsewhere in distant organs such as the lungs, bones, liver, brain and other tissues.

Pathology Pearls: Activating Invasion and Metastasis: Sequence of Molecular Events

Carcinoma Development and Acquisition of Invasive Potential

- Proto-oncogene mutations (oncogenes)
- Tumor suppressor gene mutations
- Epithelial mesenchymal transition—epithelial cells acquire mesenchymal traits (i.e. loss of adherent cell–cell junctions, change in cellular morphology, expression of matrix metalloproteinases (MMPs), increased motility)
  - EMT signals at primary malignant epithelial tumor margin.
  - Partial EMT state facilitates CSCs motility and invasion into tumor stroma
  - Mesenchymal phenotype CSCs facilitate intravsation and anoikis resistance during dissemination

Expansion of Tumor Growth and Invasion of Basement Membrane into Surrounding Tissue

- Enhanced activity of matrix metalloproteinases (MMPs)
- Decreased tissue inhibitor of metalloproteinase (TIMPs)
- Enhanced mesenchymal CSCs motility, interaction with surrounding tissue ECM stromal cells
- Decreased integrity strength of cell–cell contacts (E-cadherin)

Tumor Angiogenesis, Intravasation and Transport of Mesenchymal Phenotype CSCs around the Body

- Enhanced activity of matrix metalloproteinases (MMPs)
- Decreased tissue inhibitor metalloproteinases (TIMPs)
- Mesenchymal phenotype CSCs migration/interaction through ECM, interaction with vascular endothelial cells and pass-through basement membrane, invasion into the blood vessel, interaction with platelets, lymphocytes and other blood components, and survival in circulation/evade immune destruction.
- Mesenchymal phenotype CSCs interaction with cavity mesothelial cells during transcoelomic metastasis.

CSCs Arrest and Extravasation at Secondary Site

- Mesenchymal phenotype CSCs arrest interacts with vascular endothelium via with integrin and laminin cell adhesion receptors leading to extravasation, survival and invasion into distant tissues/organs.
- Mesenchymal phenotype CSCs interaction occurs with cavity mesothelial cells during transcoelomic metastasis.

Metastasis into Secondary Site and Formation of Micrometastasis and Macrometastasis

- Metastasis of mesenchymal phenotype CSCs depend on blood supply, organ tropism/adhesion and functional loss of E-cadherin.

  Mesenchymal phenotype CSCs extravasation, migration and invasion into secondary tissues, and their survival, interaction and adaptation to tissue microenvironment, MET in secondary site, colonization, CSCs proliferation and formation of micrometastasis and colonization of CSCs forming macrometastasis, establishment of new vasculature in tumor (tumor angiogenesis).
- Secondary tumor can remain in dormancy and reactivated state.

REPROGRAMMING ENERGY METABOLISM (WARBURG EFFECT)

Cellular growth and proliferation depend upon the acquisition and synthesis of specific metabolic products, which fuel the bioenergy, biosynthesis, and redox potential required for duplication of cellular biomass. Normal cells use oxygen to process glucose and produce energy. Metabolism in CSCs is different from normal cells. Cancer stem cells consume >20 times as much glucose compared to normal cells, but produce lactic acid instead of breaking it down completely into carbon dioxide (CO_2).

- Cancer stem cells can switch to aerobic glycolysis even in the presence of oxygen for generation of adenosine triphosphate (ATP) and called 'Warburg effect'. Though aerobic glycolysis is less efficient but faster, that produces several intermediate metabolic products used by CSCs as building blocks, i.e. macromolecules such as proteins and lipids to support unrestricted cell proliferation. Other CSCs use lactate as their main energy source. Aerobic glycolysis inhibitors are therapeutic targets of this signaling pathway.
- Biochemist Otto Warburg observed in 1920 that when CSCs were provided with glucose, that generated large amount of lactate regardless of whether oxygen (O_2) was provided or not. This metabolic difference is referred as the 'Warburg effect' or aerobic glycolysis, where pyruvate is diverted from the Krebs cycle to produce lactate under aerobic conditions that allow more oxygen is delivered to tissues. Comparison of cellular glucose metabolism in normal and CSCs are shown in Fig. 6.82.

Energy Bioenergetics in Normal Cells

Glycolysis is a universal pathway for catabolism of glucose in normal cells via **Embden-Meyerhof pathway** that provides energy and intermediates required for other metabolic pathways.

- Normal cells take up glucose via glucose transporters (GLUTs) and utilize aerobic respiration. Glucose is metabolized to glucose-6-phosphate to produce pyruvate if O_2 supply is limited. Pyruvate in turn is converted to lactate by lactic dehydrogenase A enzyme.

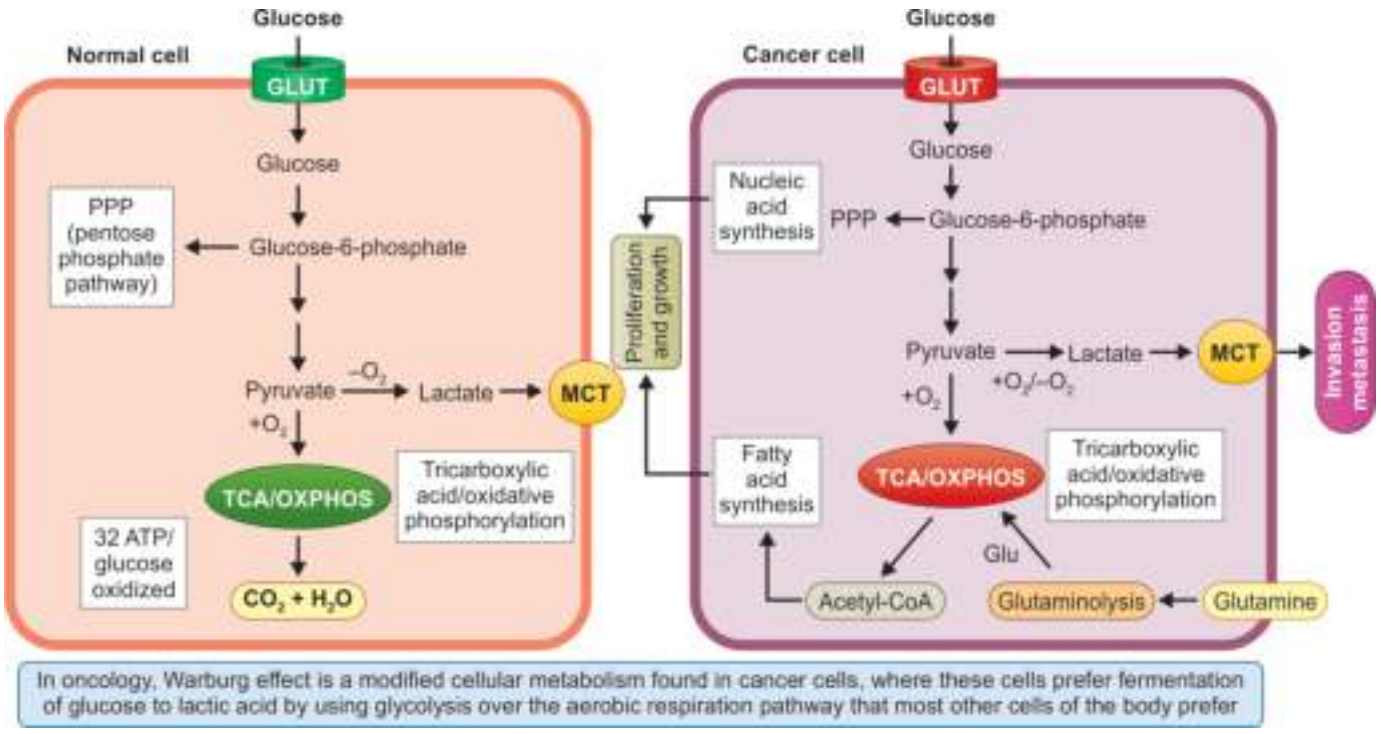


Fig. 6.82: Comparison of cellular glucose metabolism in normal and CSCs. Normal cells do not metabolize glucose to lactate when oxygen is available. When oxygen is absent, normal cells resort to anerobic glycolysis to convert glucose to lactic acid. On contrary, CSCs metabolize glucose to lactate even in the presence of oxygen and fully functioning mitochondria. This process is known as 'Warburg effect'—hallmark of cancer, which prefers fermentation of glucose to lactic acid by using glycolysis over the aerobic pathway that most normal cells of the body prefer.

- Lactate is exported from the normal cell by monocarboxylate transporter protein. Glucose is metabolized to pyruvate, which is completely oxidized to carbon dioxide (CO_2) and abundant ATPs for cellular energy through Krebs cycle, i.e. tricarboxylic acid cycle (TCA cycle) and oxidative phosphorylation process in the mitochondria.
- If O_2 is limited, pyruvate is metabolized to lactate. Altogether, mitochondria can generate as much as 36 ATP molecules per glucose molecule during aerobic glycolysis in normal cells, and 2 ATP per glucose molecule during anaerobic glycolysis. Key markers in reprogramming of energy metabolism (Warburg effect) in cancers are given in Table 6.57.

Altered Bioenergetics ('Warburg Effect') in Cancer Stem Cells

Cancer stem cells (CSCs) in malignant tumor require high levels of energy and nutrients with the ability to survive in hypoxic tumor environment,which is not completely vascularized. To meet energy and nutrients needs, RAS/RAF/MEK/ERK (MAPK) and phosphatidylinositide 3-kinases (PI3K)/AKT/mTOR signaling pathways are altered in human cancers contributing to the Warburg effect in cancer stem cells. Altered signaling pathways and their contribution to the 'Warburg effect' in CSCs are shown in Fig. 6.83.

Table 6.57 Key markers in reprogramming of energy metabolism (Warburg effect) in cancers

Hypoxia State Markers
▪ Hypoxia-inducible factor (HIF1A, HIF2B, HIF1B) is heterodimeric DNA binding transcription factor that regulates a broad range of cellular systems to hypoxia ▪ CAIX is a mediator of hypoxia-induced stress response in a CSC ▪ AP-1/c-Jun transcription factor plays an important role in tumor development and progression ▪ GLUT1 levels can be elevated in hypoxia state. GLUT1 analysis is done to assess the degree of hypoxia
Glycolysis Markers
▪ TOMM20 and GADH are essential for glutamine metabolism. Concentrations of TOMM20 and GADH are increased in various human malignancies ▪ V-ATPase expression is increased in CSCs ▪ AP-1/c-Jun transcription factor plays an important role in tumor development and progression ▪ GLUT1 levels can be elevated in hypoxia, and its analysis can be used to indicate the degree of hypoxia
Mitochondrial Metabolism Markers
▪ COX IV is a marker for assessment of inner membrane of mitochondria ▪ VDAC1/Porin is a marker for assessment of outer membrane of mitochondria ▪ ATPase-β subunit has a crucial role in the structural and functional maturation of Na^+/K^+ ATPase

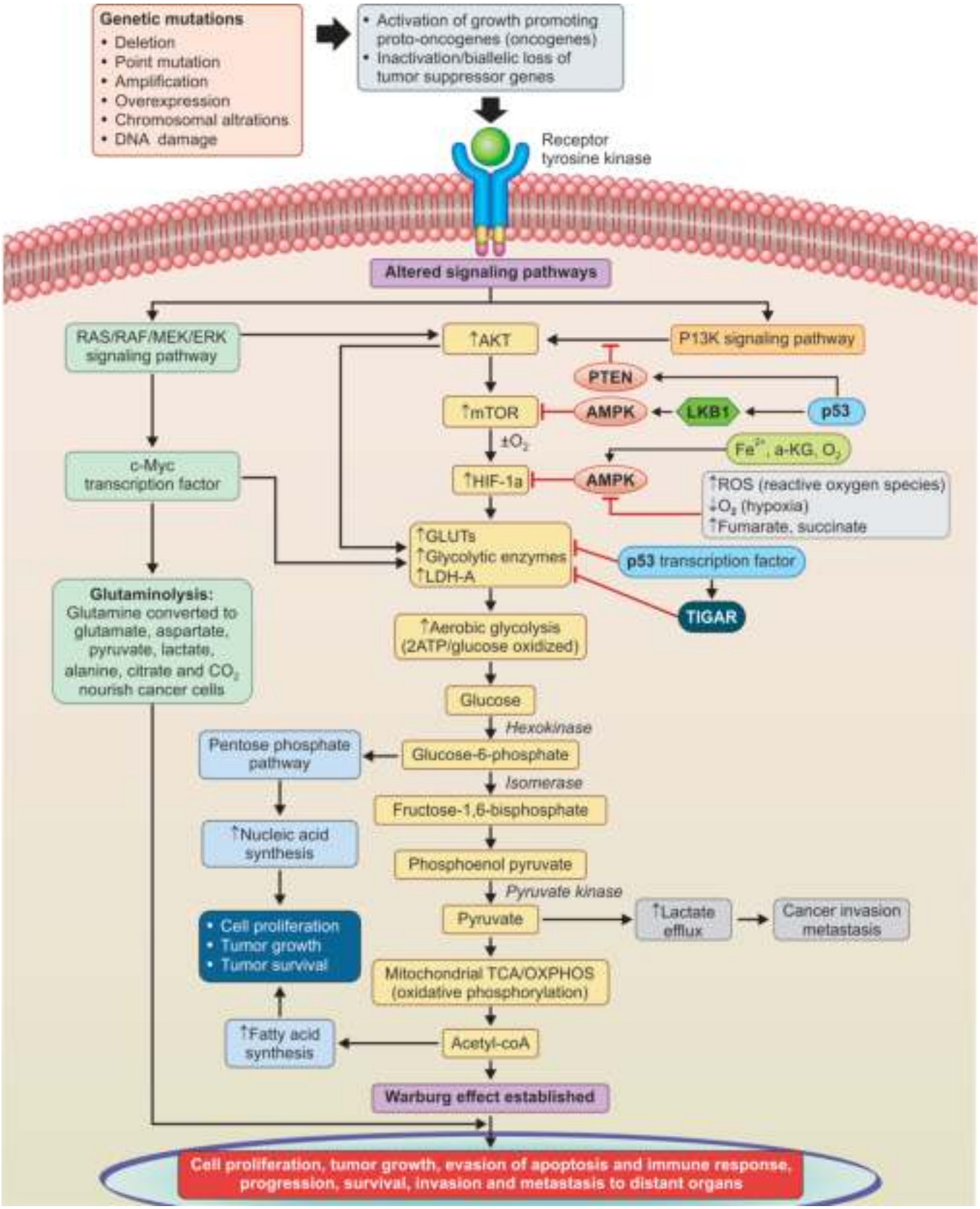


Fig. 6.83: Altered signaling pathways and their contribution to Warburg effect in CSCs. In human cancers, the rate of glucose uptake dramatically increases and lactate is produced even in the presence of oxygen and fully functional mitochondria. This process is known as Warburg effect.

- Phosphatidylinositide 3-kinase (PI3K)/AKT/mTOR signaling pathway increases synthesis of lipids as well activity of cell membrane transporters so that increased amino acids are available to support the proteosynthetic needs of CSCs. Transcription factors such as hypoxia-inducible factor-1α (HIF1A) and c-Myc play a pivotal role in glucose metabolism in CSCs via increased GLUT1 and GLUT3 glucose transporters.
- CSCs rely primarily on aerobic glycolysis 'Warburg effect' even in the presence of oxygen required for glucose metabolism to generate abundant lactate and less ATPs (i.e. 2 ATP per CSC) in mitochondria than aerobic respiration, instead of catabolism of glucose to CO_2, H_2O and ATP.
- Lactate produced from aerobic glycolysis causes acidification of tumor microenvironment (**TME**), favoring the development of a more aggressive, invasive and metastatic phenotype. Lactate can also serve as a nutrient for other cells in the malignant tumor.
- CSCs also increase glutamine metabolism to produce glutamate during glutaminolysis serves as the major substrate to refuel the tricarboxylic acid (TCA)/Krebs cycle. Citrate derived acetyl-CoA is used for lipid production. The increased synthesis of nucleic acids (DNA and RNA) and lipids promote unrestricted proliferation of CSC, and survival.

EVASION OF IMMUNE DESTRUCTION

The immune system (innate/non-specific and adaptive/specific) performs several functions such as defense against exogenous microbes or endogenous altered virally infected transformed cells, homeostasis, destruction of virally transformed cells and surveillance.

- Cytokines are naturally occurring proteins produced by the cells of immune system (macrophages and lymphocytes) that coordinate and initiate effector defense functions. Cytokines include interleukins, interferons, colony-stimulating factor and tumor necrosis factor-α, which mediate and regulate immune defense functions by acting as messengers between various cells of immune system.
- Hanahan and Weinberg discussed evidence supporting the key role played by immune system as a barrier to CSCs. Cells and tissues are under constant surveillance by immune system, that detects and tries to kill CSCs of malignant tumors.
- CD4+ helper T cells assist B cells, that recognize the processed CSC antigen and progress to become plasma cells. T cell and B cell interaction requires the major histocompatibility class II (MHC-II) molecules for presenting a processed CSC antigen, plus two additional recognition connections via CD40, specifically recognizing the MHC class II complex and the CD40 binding to CD40 ligand.
- CD8+ cytotoxic T cells must recognize the processed CSC antigen presented only by major histocompatibility class I (MHC-I). CD8+ cytotoxic T cell activation is enhanced by CD4+ helper T cell binding to processed CSC antigen. Each T cell receptor (TCR) must bind with the processed CSCs antigen presented by appropriate major histocompatibility molecules. CD4+ helper T cell and CD8+ cytotoxic T cell bind to the respective major histocompatibility molecules, i.e. MHC-II and MHC-I respectively.
- CD8+ cytotoxic T cells can only be activated by binding to dendritic cells. Once CD8+ cytotoxic T cells activated, they leave the lymph node and then are attracted by cytokines and chemokines released by the innate immune cells at the malignant tumor site. During transit time, CD8+ cytotoxic T cells synthesize specific proteins such as perforin, granzyme B and Fas ligand. Perforin causes fenestrations within the CSC membrane, allowing entry of granzyme and Fas ligand. Granzyme and Fas ligand induce apoptosis via two different mechanisms leading to CSC death.
- CSCs develop different strategies by acquisition of new gene mutations to escape immune surveillance leading to development of malignant tumor growth.

Pathology Pearls: Cancer Stem Cells Evasion of Immune Destruction

Modulation of Antigens on CSCs

- ↓Tumor immunogenicity
- ↑Maintenance of cancer stem cell clones
- ↓Immunogenic antigens

Antigen Presentation in Lymph Node

- Inhibition of dendritic cells maturation and antigen presentation
- ↑IL-10, MCSF, VEGF, prostaglandins, TGF-β, IDO
- ↑CD4+ T regulatory cells, myeloid-derived suppressor cells (MDSCs)
- ↓IL-12, IFN-γ

Priming and Activation in Lymph Nodes

- Suppression of T cell activation
  - ↑Calcium/NFAT signal
  - ↓B7.1/B7.2:CD28, 4-1BBL:4-1BB, OX40L:OX40
  - ↓CD70:CD27, GITRL:GITR

Trafficking of T Cells to Cancer

- Inhibition of T cell migration and infiltration
- ↑Cleaved CXCR3 ligands, FasL
- ↑VEGF, PGE_2, CXCL12, ECM
- ↓CXCl9, CXCl10, CXCL11

Immune Cell Cycle Regulation
▪ Normal immune cell cycle disruption ▪ Cancer stem cells evade immune destruction
Regulation of CSC by T Cell
▪ Inhibition of CSC recognition by immune cells ▪ ↑Maintenance of CSC clones without antigens, immune response of natural killer cells ▪ ↓MHC-1 (remove, reduce, transform) peptide—MHC components, NKG20 ligands ▪ ↓Antigens, proteosome components, TAP/TAP2, MHC-1 and β-microglobulin
Cancer Stem Cells Killing
▪ Immune checkpoint molecules—↑PD-1, BTLA↑, ↑LAG-3, ↑TIM-3, ↑TIGIT ▪ Immunosuppressive cells—↑M2 macrophages, ↑MDMS, ↑regulatory T cells, ↑IDO, ↑arginase, ↑IL-10, ↓arginase, ↓iNOS, ↓TGF-β, ↓perforin, granzyme.

Immune System Dysfunction and Cancer Stem Cells Survival

When the immune system loses its function of surveillance, cancer stem cells have ability to form a malignant tumor. CSCs evade immune destruction can be explained by the following proposed potential mechanisms such as CSC antigen recognition, immune system dysfunction, and T cell exhaustion.

- In addition, CSCs evade immune destruction by accumulating specific metabolites and signaling factors within tumor microenvironment (TME) or limiting nutrients available to immune cells.
- Interaction of signaling costimulatory molecules, i.e. CD155 on CSCs and CD226 on effector T cells causes reinforcing the immune response.
- Following the discovery of B7.1 (CD80) on antigen-presenting cells (APCs), several other costimulatory molecules stimulate T cell activation resulting in improving anti-tumor immunity. CSCs evade immune destruction can be explained by the following proposed potential mechanisms.

Decrease in Tumor Immunogenicity

During carcinogenesis, immunosurveillance removes cancer stem cell clones that express strong immunogenic neoantigens. At this point, cancer stem cells (CSCs) evade immune destruction due to modulation of antigens on CSCs by various mechanisms: (a) elimination of immunogenic antigens, (b) entry of neoantigens on CSC, (c) neoantigens on CSCs masked by increased glycocalyx. Modulation of antigen and poor immunogenicity limit the ability of immune system to recognize

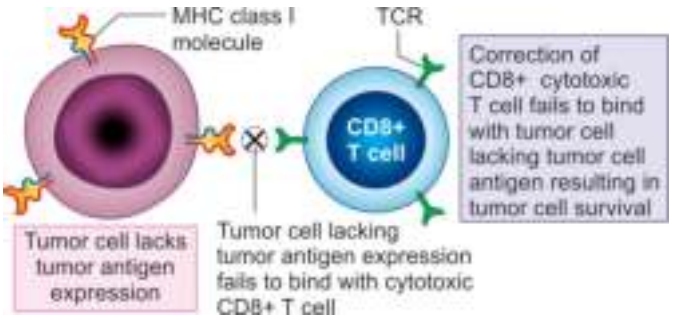


Fig. 6.84: Evasion of immune destruction of cancer stem cells due to modulation of tumor antigens and lack of recognition by CD8+ cytotoxic T cells. Many tumors may be weakly antigenic or lacking antigen. Tumor antigens are masked as a result of increased synthesis of glycocalyx on stem cells. Tumor antigen modulation limits the ability of the immune system to recognize the tumor cells and may avoid immunologic destruction by T cells.

cancer stem cells as non-self during dormancy state. Evasion of immune destruction of cancer stem cells due to modulation of tumor antigens and lack of recognition by CD8+ cytotoxic T cells is shown in Fig. 6.84. Immunogenicity of malignant tumor is shown in Fig. 6.85.

Inhibition of Dendritic Cell Maturation

Cancer stem cells do not give off inflammatory warning signals, hence evade immune destruction. In tumor-promoting inflammation, damaged-associated molecular patterns (DAMPs) are endogenous danger molecules that are released from damaged or dying normal cells into tumor microenvironment and activate dendritic cell (DC) maturation of innate immune system by interacting with pattern recognition receptors (PRRs).

- Cancer stem cell-derived factors such as IL-10, macrophage colony stimulating factor (M-CSF), vascular endothelial growth factor (VEGF), prostaglandins, TGF-β, and indoleamine 2,3-dioxygenease (IDO) inhibit maturation and function of DC.
- In addition, immunosuppressive cells in tumor microenvironment (TME) such as CD4+ regulatory T cells, and myeloid-derived suppressor cells (MDSCs) express inhibitory factors, that suppress dendritic cell maturation, reducing the expression of major histocompatibility complex molecules and co-stimulatory factors in dendritic cells, leading to reduced production of inflammatory cytokines, such as IL-12, and IFN-γ, which inhibit proliferation of T cells.

Suppression of T Cell Activity

Both antigen recognition and co-stimulatory signals are required for T cell activation. Co-stimulatory interactions between dendritic cells and CD4+ helper/

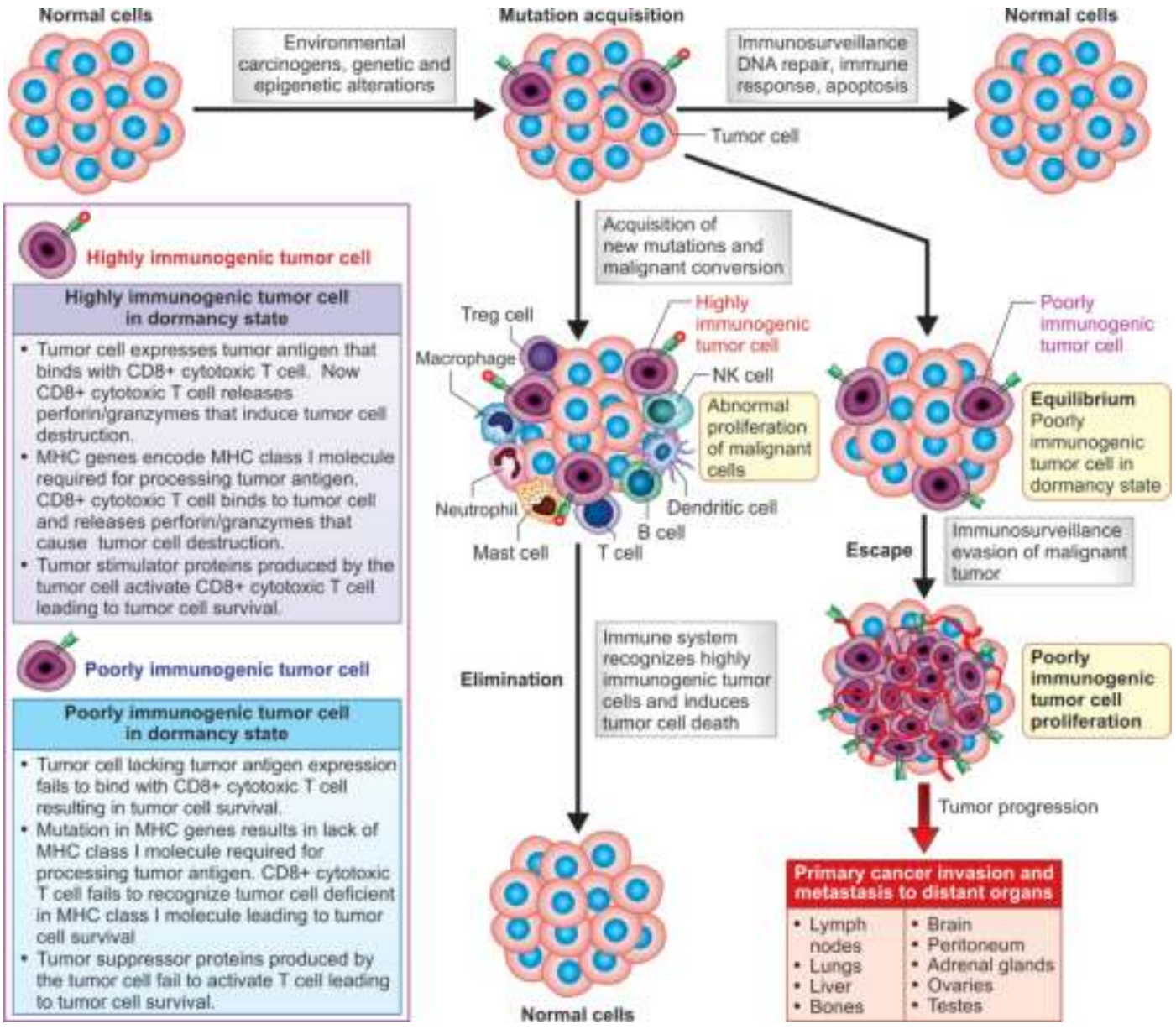


Fig. 6.85: Immunogenicity of malignant tumor. Tumor dormancy is an arrest in tumor growth, which may occur during the formation of primary tumor or after dissemination to distant organs. Primary tumor dormancy and metastatic dormancy appear to be distinct processes. Immunogenic tumors with sufficient antigens and priming elicit good CD8+ cytotoxic T cell responses, while poorly immunogenic tumors fail to generate CD8+ cytotoxic T cell responses.

CD8+ cytotoxic T cells include B7.1 or B7.2:CD28, 4-1BBL:4-1BB, OX40L:OX40, CD70:CD27, GITRL: GITR. These co-stimulatory interactions play key roles in cell proliferation, differentiation, survival, cytotoxic function, memory, and cytokine synthesis of T cells.

- Cancer stem cells inhibit priming and activation of T cells in lymph nodes by reducing the expression of co-stimulatory factors and major histocompatibility complex (MHC), thus limiting the co-stimulation required for T cells.
- When the T cell receptor (TCR) is activated without co-stimulation, excessive activity of calcium/nuclear factor of activated T cell (NFAT) signals induce the expression of negative modulating factors and T cells become unresponsive (T cell anergy).

Inhibition of T Cell Migration and Infiltration into Malignant Tumor

Malignant tumors exploit a number of mechanisms to limit T cell homing and infiltration. T cell trafficking across vascular endothelial barriers into the tissues

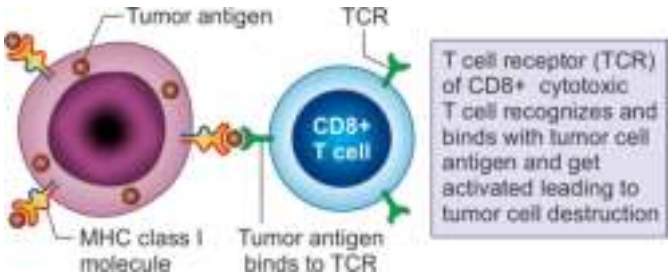


Fig. 6.86: Role of CD8+ cytotoxic T cells in anti-tumor immune response. CD8+ cytotoxic T cells recognize tumor antigen presented by antigenic presenting cell leading to destruction of cancer stem cells.

is highly regulated. In nonlymphoid tissues, resting vascular endothelial cell lining capillaries and venules are normally refractory to leukocyte entry.

- Transmigration of T cells and monocytes occurs in the tumor microenvironment (TME). Presence of CD8+ cytotoxic T cells within tumor microenvironment is a positive prognostic factor for patients towards better clinical outcomes. Role of CD8+ cytotoxic T cells in anti-tumor immune response is shown in Fig. 6.86.
- Conversely, impaired anti-tumoral immune response is a hallmark of growing malignant tumors. The concept of T cell-driven immunosurveillance against malignant tumor has led to the development of immunotherapies. Malignant tumors enriched in T cells especially CD8+ cytoxic T cells are more susceptible to be controlled by programmed cell death 1 (PD-1) blockade.
- CD4+ regulatory T cells (**Tregs**) are key immunosuppressive cells that promote malignant tumor growth by hindering the effector immune response.
  - CD4+ regulatory T cells (Tregs) utilize multiple suppressive mechanisms to inhibit proinflammatory responses within the TME by inhibition of effector function and immune cell migration, secretion of inhibitory cytokines and metabolic disruption.
  - In addition to immunosuppressive function, Tregs also serve as key interactors in the peritumoral stroma, vasculature and lymphatics to limit anti-tumor immune response.
  - CD4+ regulatory T cells promote a physical and biological barrier resulting in exclusion of immune and non-immune cells outside and within TME by various mechanisms. Within TME, CD4+ regulatory T cells (Tregs) utilize inhibitory receptors (PD1, TIM3, TIGIT and LAG3), inhibitory cytokines (IL-10, IL-35 and TGF-β), dendritic cell modulation (via CTLA-4 and LAG-3) and metabolic disruption (via CD39/CD73) to suppress the anti-tumoral T cell immune response.
  - CD4+ regulatory T cells (Tregs) derived TGF-β induce cancer-associated fibroblasts (CAFs) increase extracellular matrix (ECM) production and deposition within the peri-tumoral stroma to inhibit trafficking of effector T cells.
  - CD4+ regulatory T cells (Tregs) inhibit entry of effector T cells through preventing proper cytokine signals that promote vascular endothelial venule formation as well as production of cytokines such as IL-10 and vascular endothelial growth factor (VEGF) by CSCs to promote dysregulated angiogenesis. VEGF also reduces the expression of cell adhesion molecules on vascular endothelial cells and inhibits migration of T cells outside and within microenvironment (TME).
  - CD4+ regulatory T cells (Tregs) can modulate dendritic cell maturation in lymphoid organs and induce apoptosis of CD8+ cytotoxic T cells to prevent proper effector T cell activation.

Inhibition of Recognition of CSCs by Immune Cells

Normally, co-stimulatory signaling major histocompatibility complex class I (MHC-I) molecules play key role in CSC antigen presentation to immune cells. MHC class I molecule downregulation/allelic loss allows CSC antigen to go unrecognized by antigen-presenting cells (APCs), that facilitates CSCs to escape from CD8+ cytotoxic T cell-mediated immunosurveillance. Evasion of immune destruction of CSCs due to mutations in MHC genes normally needed for antigen processing is shown in Fig. 6.87.

- Approximately, 40–90% of human cancers express downregulation/allelic loss of major histocompatibility complex class I molecules. Downregulation/loss of MHC class I antigen expression is frequently

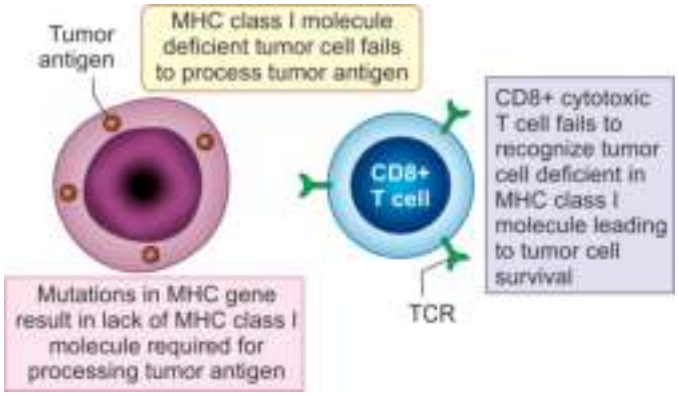


Fig. 6.87: Evasion of immune destruction of CSCs due to mutations in MHC gene normally needed for antigen processing. Silencing, downregulation, or loss of HLA alleles inhibits peptide antigen presentation and facilitates CSCs to escape from immunosurveillance.

associated with invasive and metastatic tumor phenotype. Altered MHC class I involves complete loss of expression, haplotype, and loss/downregulation of locus, alleles, and combinations.

Synthesis of Immunosuppressive Molecules by CSCs

Cancer stem cells synthesize excess immunosuppressive molecules, that inhibit the body's immune response resulting from failure of priming and activation of antigen-presenting cells (APCs), and recognition of antigen by T cells via the T cell receptor (TCR) in the lymph node, which normally attack CSCs.

Downregulation of Antiapoptotic Proteins

Downregulation of antiapoptotic proteins (BCL-2, c-FLIP, IAPs) and poor stimulation of CD8+ cytotoxic T cells limit the ability of immune system to recognize and destroy CSCs.

Aberrant Signaling Pathways

Aberrant signaling pathways, i.e. phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR, RAF/MEK/ERK mitogen-activated protein kinase (MAPK), JAK/STAT, Wnt/β-catenin, Hedgehog and NF-κB signaling pathways limit destruction of CSCs by immune system.

Synthesis of Factors by CSCs and Creation of Physical Barrier to the Immune Cells in Tumor Microenvironment

Synthesis of factors by CSCs leads to creation of physical barrier to the immune cells in tumor microenvironment (e.g. macrophages, CD4+ helper T cells, CD8+ cytotoxic T cells, B cells and natural killer cells), hence limit destruction of CSCs by immune system.

Synthesis of Co-suppressor Molecules by CSCs

Cancer stem cells synthesize co-suppressor molecules, which include: checkpoint programmed cell death ligands 1 and 2 (PD-L1, PD-L2), transforming growth factor-β (TGF-β), CD155, HVEM, MHC class II, galectin-3, lymphocyte blastogenesis inhibitory factor (LBIF), p15E, and suppressive E-receptor (SER). Co-suppressor molecules (e.g. PD-1, TIGIT, BTLA, TIM-3, LAG-3) are expressed on T cells. RAS/RAF/MEK/ERK (MAPK), phosphatidylinositide 3-kinase (PI3K)/AKT/mTOR, JAK/STAT, Wnt/β-catenin, Hedgehog and NF-κB signaling pathways promote the expression of PD-1/PD-L1 axis. Moreover, increasing understanding of co-inhibitory receptors has highlighted key additional signaling pathways that can dominantly inhibit anti-tumor T cell function.

- **Programmed cell death protein 1 (PD-1):** Programmed cell death protein 1 (PD-1) expressed on CSCs plays key role in suppression of immune response and promoting self-tolerance through modulating the activity of T cells, activating apoptosis of antigen-specific T cells, and inhibiting apoptosis of CD4+ regulatory T cells.
- **Programmed cell death ligand 1:** Programmed cell death ligand 1 (PD-L1), a transmembrane protein, is considered to be co-suppressor molecule of the immune response. PD-L1 can combine with PD-1 to reduce proliferation of PD-1 positive T cells, inhibit their cytokine secretion and induce apoptosis.
  - Programmed cell death protein 1 (PD-1) also plays an important role in various human malignancies where it attenuates the immune response to CSCs. Based on these perspectives, PD-1/PD-L1 axis is responsible for CSCs immune escape and makes a huge effect on cancer therapy.
  - On the other hand, binding of B7 on CSC to CD8+ cytotoxic T lymphocyte antigen-4 (CTLA-4) inhibits T cell activation, that prevents destruction of CSC. Blocking CTLA-4 or B7 allows T cell to kill CSCs. Some checkpoint inhibitors are used to treat human cancers.
  - Improving positive costimulation, and interfering with negative regulation, continues to represent an attractive immunotherapeutic approach for the treatment of human cancers. Immune checkpoint targets such as PD1/PD-L1, TIM3, and LAG3 are all critical checkpoint molecules that have revolutionized caner immunotherapy.
  - Cancer stem cells evading immune destruction due to synthesis of immunosuppressive proteins (TGF-β) are shown in Fig. 6.88. PD-1/PD-L1 axis in CSCs and immune checkpoint inhibitors used in cancer treatment are shown in Fig. 6.89.

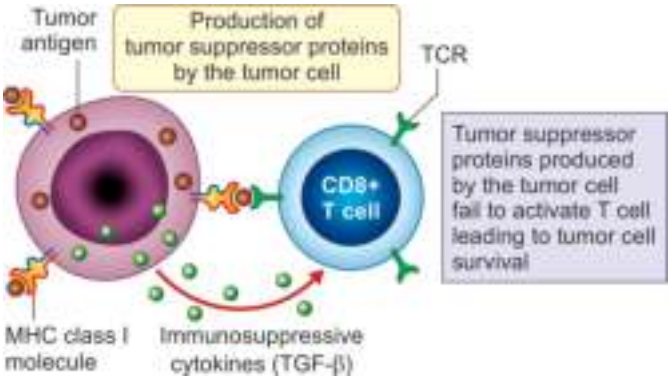


Fig. 6.88: Cancer stem cells evading immune destruction due to synthesis of immunosuppressive proteins (TGF-β). Some well-characterized immunosuppressive molecules synthesized by CSCs include transforming growth factor-β (TGF-β), checkpoint programmed cell death protein 1 (PD-1)/programmed cell death ligands 1 and 2 (PD-L1, PD-L2), galectin-3, lymphocyte blastogenesis inhibitory factor (LBIF), p15E and suppressive E-receptor (SER). Checkpoint programmed cell death protein 1 (PD-1)/programmed cell death ligands 1 and 2 (PD-L1, PD-L2) are negative immunoregulatory molecules that promote immune evasion of CSCs.

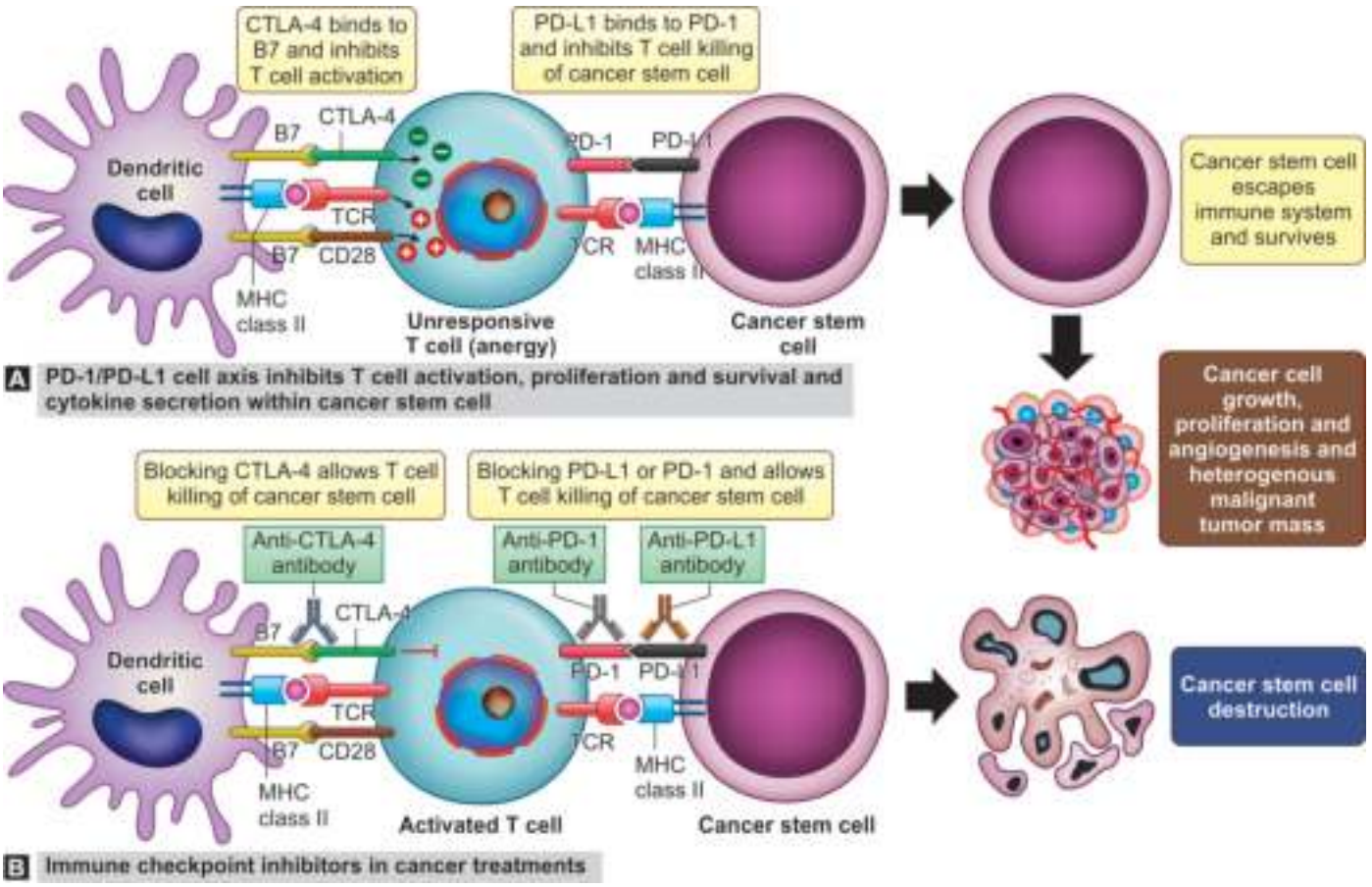


Fig. 6.89: PD-1/PD-L1 axis in CSCs and immune checkpoint inhibitors used in cancer treatment. Antigen-presenting cells (APCs) such as dendritic cells (DCs), regulate antigen-specific T cell responses to CSCs. Checkpoint proteins are found on CSCs (PD-L1 and B7) and on T cell (PD1 and CTLA-4). PD-1 (programmed cell death protein 1)/PD-L1 (programmed cell death ligand 1) cell axis inhibits T cell activation, proliferation and survival and cytokine secretion within CSCs, which lead to escape of CSCs destruction and survive. Blocking PD-L1 or PD-1 allows T cell to kill CSCs. On the other hand, binding of B7 on cancer stem cell with CTLA-4 (cytotoxic T lymphocyte antigen 4) on T cell inhibits T cell activation, that prevents destruction of cancer stem cell. Blocking CTLA-4 on T cell or B7 on cancer stem cell allows T cell to kill CSC. Some checkpoint inhibitors are used to treat cancers.

Pathology Pearls: Cancer Stem Cells Destruction by Immune System

- Tumor antigens are presented and processed by major histocompatibility complex (MHC) class II molecule on antigen-presenting cells (APCs) in the lymph node, that leads to priming and activation of APCs.
- Recognition of tumor antigens on the MHC class I molecule by T cell receptor (TCR) especially CD8+ cytotoxic T cells leads to priming and activation of CD8+ cytotoxic T cells in the lymph node.
- Trafficking of activated CD8+ cytotoxic T lymphocytes (CTLs) occurs around cancer stem cells (CSCs) in tumor microenvironment (TME).
- Activated CD8+ cytotoxic T cells infiltration occurs into TME.
- Recognition of tumor antigens on the MHC class I molecules by CD8+ cytotoxic T cells within tumor microenvironment and leads to destruction of CSCs.
- CD8+ cytotoxic T cells directly destroy CSCs.

GENOMIC INSTABILITY (MUTATED PHENOTYPE)

Normal eukaryotic cells have 23 pairs of chromosomes per cell, stored in the nucleus. The maintenance of genomic stability is essential for cellular genomic integrity to prevent errors from DNA replication, endogenous genotoxic stress, i.e. reactive oxygen species (ROS), reactive nitrogen intermediates (RNI) derived from cellular metabolism, and exogenous genotoxic carcinogenic insults such as ultraviolet light, ionizing radiation and chemical agents. Genomic stability and instability mechanisms are shown in Fig. 6.90. Genomic instability due to defective DNA repair machinery is given in Table 6.58.

- **Genomic stability:** Genomic stability (integrity) is closely monitored by several surveillance mechanisms: DNA repair machinery and mitotic checkpoints and telomere maintenance during cell division. The response to DNA damage can result in two major physiologic consequences during cell

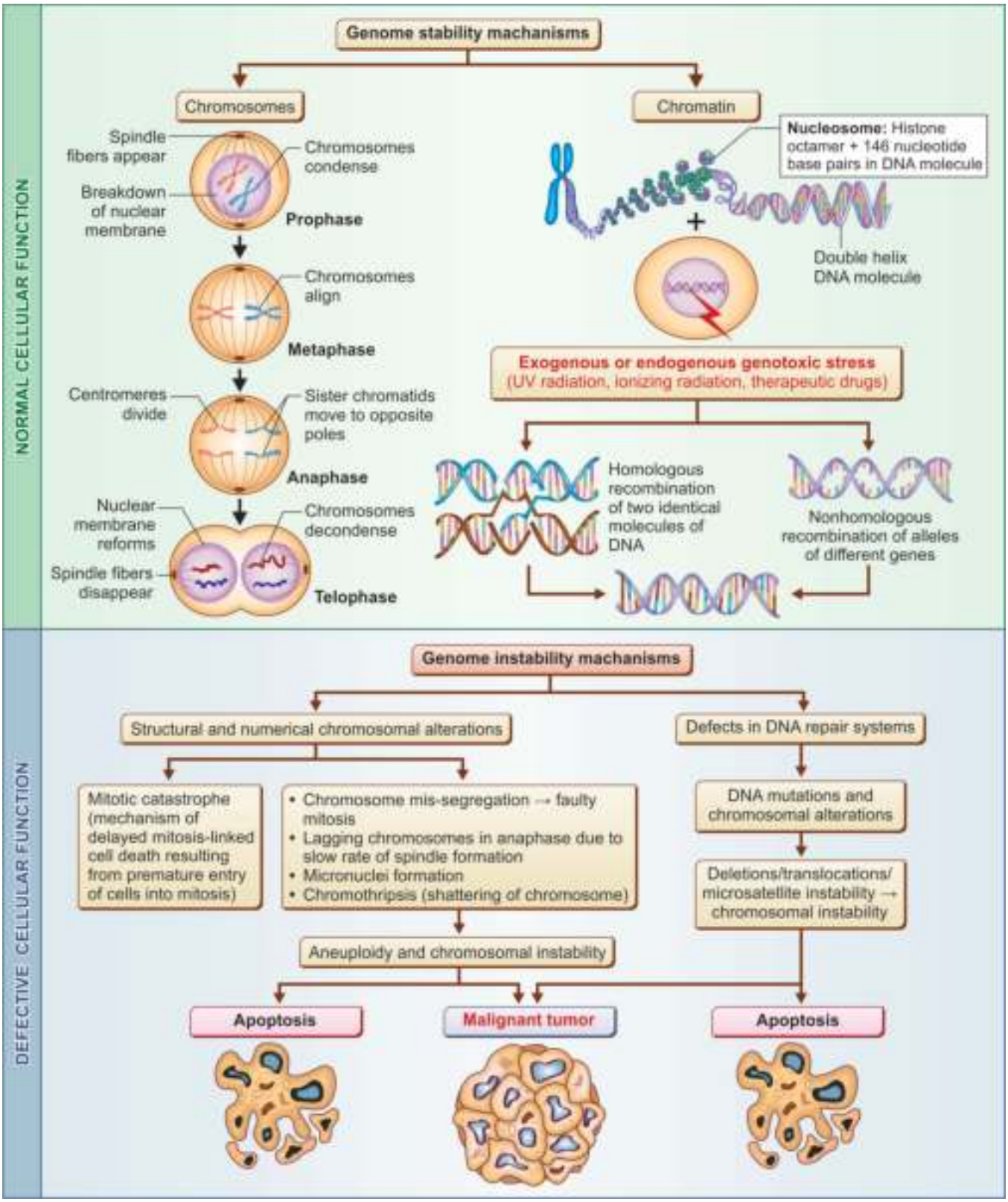


Fig. 6.90: Genomic stability and instability mechanisms. Genomic stability is critical for cell survival and cell growth. Genomic chromosomal rearrangements and defective repair systems result in gene mutations that underlie many human diseases, ongoing genomic instability likely contributes to the development of many cancers.

Table 6.58 Genomic instability due to defective DNA repair machinery

Key Molecule	Function
Nucleotide excision repair of DNA	
ERCC-XPF (endonuclease)	DNA damage repair (DNA interstrand crosslinks and double-strand break repair)
XPA (zinc finger protein)	DNA damage repair
TFIID complex (transcription factor IID)	TFIID complex binds to the TATA box in the core promoter of the gene
Nucleotides base excision repair of DNA	
APEX1/APEX2 (endonuclease)	DNA repair
PNKP (polynucleotide kinase 3-phosphatase), DNA processing enzyme	Catalyzes 5'-kinase and 3'-phosphatases activity
FEN1 (flap endonuclease 1)	Removes 5' overhanging flaps in DNA repair
Double-strand break (DSB) repair of DNA	
Gamma H2AX (component of histone octamer in the nucleosome)	Gamma H2AX is phosphorylated in DNA damage
XRCC4 (XRCC4 + DNA ligase IV + DNA dependent protein kinase)	DNA double-strand break (DSB) repair
BRCA1 gene	DNA repair and cell cycle regulation
53bp1 (53bp1 + damaged chromatin)	DNA repair
KAP1 gene product	DNA repair
Mismatch nucleotide base repair of DNA	
MSH1 and MSH2 gene products	DNA mismatch nucleotide base repair
MSH2 and MSH3 gene products	DNA repair by insertion/deletion loop repair
PMS2	DNA mismatch nucleotide base repair
KAP1 gene product	Key regulator of normal development and differentiation

division: (a) cell can undergo cell cycle arrest to repair damaged DNA, and (b) if damaged DNA is not repaired, the cell undergoes apoptosis.

- **Genomic instability:** Genomic instability is a characteristic of most human cancers. DNA damage in a normal cell results in cell cycle arrest followed by either DNA repair and restoration of normal functions, or apoptosis of cell unable to repair DNA damage. Interference in DNA repair may occur either due to loss of recognition and repair of damaged DNA cell or abnormal gatekeeping of cell cycle. Genomic instability and mutation act as enabling molecular hallmark of cancer. Poly (ADP-ribose) polymerase (PARP) inhibitors are used in clinical practice.
  - Genomic instability occurs due to high frequency of genomic alterations such as nucleic acid sequences, DNA strand breaks, crosslinks, mismatches, microsatellite instability, chromosome rearrangements or aneuploidy, or telomere stability and/or DNA damage response during cell division.
  - Genomic instability occurs in 90% of human cancers (e.g. breast carcinoma, prostatic carcinoma, small cell lung carcinoma, neuroblastoma, leukemia). Changes in genome may arise through direct DNA genetic mutations or through epigenetic modifications that can change protein expression levels and adversely affect genomic stability (integrity). Cancer stem cells generally have several chromosomal abnormalities.
  - Based on the level of disruption, genomic instability is of two types: (a) nucleotide instability occurs due to nucleotide substitution, deletion or insertion (e.g. xeroderma pigmentosum, MYH associated polyposis), and (b) microsatellite instability occurs due to defect in mismatch repair (e.g. Lynch syndrome).
  - Chromosomal instability is most prominent form of genomic instability. About 90% of human cancers exhibit chromosomal aneuploidy, gene amplification, deletions, translocations and inversion in the settings of breast carcinoma, prostatic carcinoma, non-small cell lung carcinoma and chronic myelogenous leukemia (CML). Karyotyping is performed to asses genomic instability.
  - Defects in the caretaker genes result in DNA damage, and inactivation of DNA repair machinery. Inability to inactivate genome mutagenic molecules results in DNA damage and genomic instability.

Comparative genomic hybridization (CGH) is a molecular genetic analysis technique to compare patient DNA to reference DNA to check the gains and losses of gene copies in the patients' cell genome by using fluorescent dye.

Tumor Suppressor Genes: Genome Stability Enablers

Tumor suppressor genes regulate different critical cell processes, i.e. apoptosis, cell division, cell differentiation, signal transduction, cell adhesion, maintenance of genomic integrity and DNA (deoxyribonucleic acid) damage repair.

- Tumor suppressor genes can be divided into two major categories: (a) caretaker genes [e.g. BRCA1, BRCA2, DNA mismatch repair genes (MLH1, MSH2, MSH3, MSH6, PMS1, PMS2), Fanconi anemia DNA repair genes, DNA nucleotide excision repair genes, and (b) gatekeeper genes (e.g. TP53, RB1, APC, BRCA, APC/β-catenin).
- Caretaker genes regulate the maintenance of the genome stability, while gatekeeper genes inhibit cell growth or induce apoptosis. Inactivation of caretaker and gatekeeper tumor suppressor genes contributes to development of malignant tumor and its progression.

Adenomatous Polyposis Coli Tumor Suppressor Gene

Adenomatous polyposis coli (APC) tumor suppressor gatekeeper gene mapped on chromosome 5q22.2 codes for APC protein product involved in regulation of Wnt/β-catenin signaling pathway.

- Adenomatous polyposis coli gene mutation is linked to familial tumors (FAP—familial adenomatous polyposis) and sporadic tumors (gastric carcinoma, thyroid carcinoma and melanoma).
- Classic FAP is diagnosed when a person develops >100 adenomatous polyps in colon. An adenomatous polyp is an area where normal cells that line the inside of colon form a mass on the inside of the intestinal tract. Surgical excision of adenomatous polyps in the colon is the standard treatment to reduce the risk of development of colon carcinoma.

Retinoblastoma Tumor Suppressor Gene

Retinoblastoma (RB) tumor suppressor gene mapped on chromosome 13p14 encodes RB protein (pRB) product, which acts as master brake on cell cycle and inhibits G1/S transition during cell cycle. RB protein (pRB) inhibits transcription factor. RB gene germline mutation is linked to hereditary tumors (retinoblastoma, osteosarcoma), and acquired (somatic) gene mutation linked sporadic tumors (retinoblastoma, osteosarcoma, breast carcinoma, colon carcinoma, prostatic carcinoma, urinary bladder carcinoma and lung carcinoma).

CDKN2A Tumor Suppressor Gene

CDKN2A tumor suppressor gene mapped on chromosome 9p21 codes for CDK inhibitor proteins such as $p16^{INK4a}$ and $p14^{ARF}$ indirectly activates TP53 tumor suppressor gene. Germline mutation in CDKN2A gene is linked to familial melanoma (40%) and acquired (somatic) mutation linked sporadic human cancers (e.g. breast carcinoma, pancreatic carcinoma, esophageal carcinoma).

TP53 Tumor Suppressor Gene

TP53 tumor suppressor gene mapped on chromosome 17p13.1 encodes p53 protein, which is important genome stability enabler (known as guardian of genome). TP53 gene mutation promotes carcinogenesis, invasion and metastasis.

- TP53 gene product (p53) sensors DNA damage by two protein kinases such as ATM and ATR (ataxia-telangiectasia RAD-3 related), which halts cell cycle in G1/S transition for DNA repair by inhibiting MDM2 transcription factor, until the damaged DNA gets repaired. The p53 protein activates proapoptotic BAX gene resulting in apoptosis. The p53 protein also interacts with at least 17 cellular and viral proteins. It must be noted that MDM2 transcription factor degrades p53 protein.
- TP53 gene product (p53 protein) is inactivated by viral oncoproteins like E6 protein of HPV. More than 50% of human cancers show mutations in TP53 gene. Germline mutation in TP53 gene is linked to familial Li-Fraumeni syndrome characterized by breast carcinoma (100%), adrenal cortex cancer, sarcoma, leukemia, brain tumors, and many acquired (somatic) mutation linked sporadic tumors.

BRCA1 and BRCA2 Genes

BRCA1 (17p21) and BRCA2 (13q12-13) tumor suppressor genes code for BRCA1 and BRCA2 proteins respectively, which move to the site of DNA damage and bind RAD51 molecule that mediates repair of double-stranded breaks in DNA.

- BRCA1 protein also activates TP53 gene product (p53 protein) involved in activating apoptosis in response to irreparable DNA damage. The essential roles of BRCA1 and BRCA2 gene products in DNA repair help in understanding their functions as molecular caretakers in our genome surveillance system. Both BRCA1 and BRCA2 are high-penetrance caretaker tumor suppressor genes responsible for breast carcinoma and ovarian carcinoma.
- Mutations in either of BRCA1 or BRCA2 genes result in failure to produce DNA repair complexes.

Therefore, DNA damaged cells become hypersensitive to ionizing radiation, radiomimetic chemical carcinogens and mechanical stress on chromosomes, that cause DNA damage. Thus, BRCA1 and BRCA2 proteins are crucial for the activation of cell cycle checkpoints.

- BRCA1 gene mutation is responsible for germline mutation related familial breast carcinomas (52%), and acquired (somatic) mutation related breast carcinomas (1–2%). On the other hand, BRCA2 gene germline mutation is responsible for familial breast carcinomas (32%), and acquired (somatic) mutation related breast carcinomas (≤2%).
- BRCA1 gene germline mutation is linked to familial tumors (breast carcinoma, ovarian carcinoma, prostatic carcinoma, pancreatic carcinoma and fallopian tube carcinoma), and acquired (somatic) mutation linked sporadic breast carcinomas (breast medullary carcinoma and rarely breast metaplastic carcinoma).

Poly (ADP-ribose) Polymerase 1 (PARP1) Gene

Poly (ADP-ribose) polymerase 1 (PARP1) gene encodes PARP 1 enzyme, that regulates several proteins involved in DNA repair, and maintenance of genome integrity by modulating chromatin structure. Inhibitors of PARP1 enzyme comprise a new type of anticancer chemotherapeutic drug that selectively destroy CSCs by targeting homologous recombination in DNA repair defects.

Mismatch DNA Repair Genes

Mismatch repair genes such as MLH1 gene (3p21), MSH2 gene (2p15), MSH3 gene (5q11.12), MSH6 gene (2p16), PMS1 gene (2p32) and PMS2 gene (7p22) belong to tumor suppressor caretaker genes. Their gene products ensure the integrity of the cellular genome by replication and mismatch DNA repairs. These are also known as mutator genes, which increase the rate of mutation of one or more other genes.

- Mutation of DNA mismatch repair genes are unable to correct errors in nucleotide pairing resulting in accumulation of numerous mismatched errors in DNA replication. DNA blood tests are now available for evaluation of all these genes.
- DNA repair gene mutations are linked to familial tumors (hereditary nonpolyposis colorectal cancer—HNPCC inherited syndrome), known as Lynch syndrome, characterized by increased risk for endometrial carcinoma, ovarian carcinoma, right colon carcinoma, and sporadic tumors (colorectal carcinoma, endometrial carcinoma, Muir-Torre syndrome, hepatobiliary carcinoma, genitourinary carcinoma, glioblastoma multiforme). Extensive history of screening of early development of these cancers is required in the management of these patients with hereditary nonpolyposis colorectal cancer (HNPCC).

Pathology Pearls: Hereditary Nonpolyposis Colon Cancer: Lynch Syndrome

- Hereditary nonpolyposis colon cancer (HNPCC) also called Lynch syndrome occurs due to inactivation of DNA mismatch repair genes principally MLH1 gene (3p21), MSH2 gene (2p15), MSH3 gene (5q11.12), MSH6 gene (2p16), PMS1 gene (2p32) and PMS2 gene (7p22). These mutator genes (MLH1, MSH2, MSH3, MSH6, PMS2) are unable to correct errors in nucleotide base pairing in DNA.
- In patients with HNPCC, there is increased risk for human cancers in decreased frequency (e.g. endometrial carcinoma, ovarian carcinoma and right colon carcinoma, urothelial carcinoma, gastric carcinoma and brain tumors). Right colon carcinoma is often preceded by serrated adenomas, rather than tubular adenomas seen in the traditional APC tumor pathway.
- Microsatellite instability is observed in 90% of the tumors that develop in HNPCC patients. A variant that involves a propensity for sebaceous tumors of the skin is known as Muir-Torre syndrome.

DNA Damage by Endogenous and Exogenous Genotoxic Agents

Prolonged exposure to endogenous and exogenous genotoxic agents can cause DNA damage including nucleotide base modifications, DNA strand breaks, crosslinks and mismatches.

- Spontaneous DNA damage occurs when DNA nucleotide base pairs react with environmental mutagenic agents, which hydrolyze a nucleotide base pair causing it to pair with an incorrect nucleotide base pair by two mechanisms: (a) deamination of adenine, cytosine and guanine, and (b) depurination (loss of purine bases) resulting from cleavage of the bond between the purine bases and deoxyribose, leaving an apurinic (AP) site in DNA. DNA replication errors are minimized by proofreading. Sometimes mismatched nucleotide base pairs escape proofreading. Chemical agents modify nucleotide base pairs and interfere with DNA replication.
  - Nitrosamines, which are found in products such as pickled foods and beer can cause alkylation of DNA nucleotide bases (addition of alkyl group such as methyl or ethyl at O6 position of guanine results in the formation of O6-methylguanine).
  - Ultraviolet (UV) rays can cause production of oxygen-derived free radicals, which fuse adjacent pyrimidines creating pyrimidine dimers that prevent DNA replication.
  - Oxidizing agents and ionizing radiation create oxygen-derived free radicals in the cells that oxidize

nucleotide base pairs, especially guanine (G). Ionizing radiation and certain chemotherapeutic agents such as bleomycin, can block DNA replication by creating double-stand breaks (DSBs) in DNA. These agents can also cause breaks in single-strand DNA, though this form of damage is most often easier for the cells to overcome.
  - Base analogs and intercalating agents can cause abnormal insertions and deletions in the DNA sequence.
- Many carcinogens (e.g. benzo-(a)pyrene) react with DNA nucleotide bases, resulting in the addition of large bulky chemical groups to the DNA molecule.
- DNA repair comes from the double helix structure of DNA, that carries two separate copies of all the genetic information in each of its two strands. When one strand of DNA is damaged, another strand is used as a template to restore the DNA nucleotide base pairs sequence to the damaged strand. When both strands of the double helix DNA molecule are broken, however, leaving no template strand for repairs, the cells may use one of two distinct alternative mechanisms to address repairs. Enzymes involved in DNA nucleotide excision repair are given in Table 6.59. Defective DNA repair syndromes are given in Table 6.60.

TUMOR-PROMOTING INFLAMMATION

Chronic inflammation can contribute to multiple hallmarks of cancer capabilities by supplying bioactive molecules for initiation, promotion, transformation of normal cell to CSC, and favorable tumor microenvironment, which include: (a) growth factors that induce proliferative signaling in CSCs, (b) survival factors that limit CSCs apoptosis, (c) synthesis of proangiogenic factors that induce tumor angiogenesis, and (d) extracellular matrix (ECM) modification that facilitate angiogenesis, invasion, metastasis, and chemoresistance, through gene mutations, genomic instability, and epigenetic modifications.

- Chronic inflammatory cells infiltrate within and around most developing solid malignant tumors and secrete diverse factors that facilitate invasion and metastasis.
- Chronic inflammation linked to carcinogenesis, progression and metastasis is shown in Fig. 6.91. Chronic inflammation linked to carcinogenesis, invasion, metastasis and chemoresistance is given in Table 6.61. Dysregulation of signaling pathways and tumor-associated macrophages in chronic inflammation linked to carcinogenesis is given in Table 6.62.
- Cancer stem cells, surrounding stromal cells and inflammatory cells in malignant tumor, engage in well-orchesterized reciprocal interactions to form an inflammatory tumor microenvironment (TME). Cells within the tumor microenvironment are highly plastic, continuously changing their phenotypic and functional characteristics. Tumor-promoting inflammation closely resembles inflammatory

Table 6.59 Enzymes involved in DNA nucleotide–excision repair

Human DNA Repair Protein	Yeast Functional Homolog of Human DNA Repair Protein	Function
XPA	RAD14 (zinc metalloproteinase)	Recognition of DNA damage by ultraviolet light
XPB	RAD25	ATP-dependent helicase
XPC	RAD4	DNA binding
XPD	RAD3	Helicase
XPF	RAD1	5'-Endonuclease
XPG	RAD2	3'-Endonuclease
ERCC1 (DNA repair excision protein)	RAD10	Dimer with XPF

XP: Xeroderma pigmentosum

Table 6.60 Defective DNA repair syndromes

Syndrome	Inheritance Pattern	Gene Mutation	Associated Neoplasm(s)
Xeroderma pigmentosum	Autosomal recessive	One of several nucleotides' excision repairs	Skin cancer
Ataxia-telangiectasia	Autosomal recessive	ATM (ataxia-telangiectasia)	Lymphoid malignancies
Hereditary nonpolyposis colon cancer (HNPCC)	Autosomal dominant	MSH2, MLH1, MSH6	Colon adenocarcinoma

Ataxia-telangiectasia due to ATM gene mutation is also associated with cerebellar ataxia.

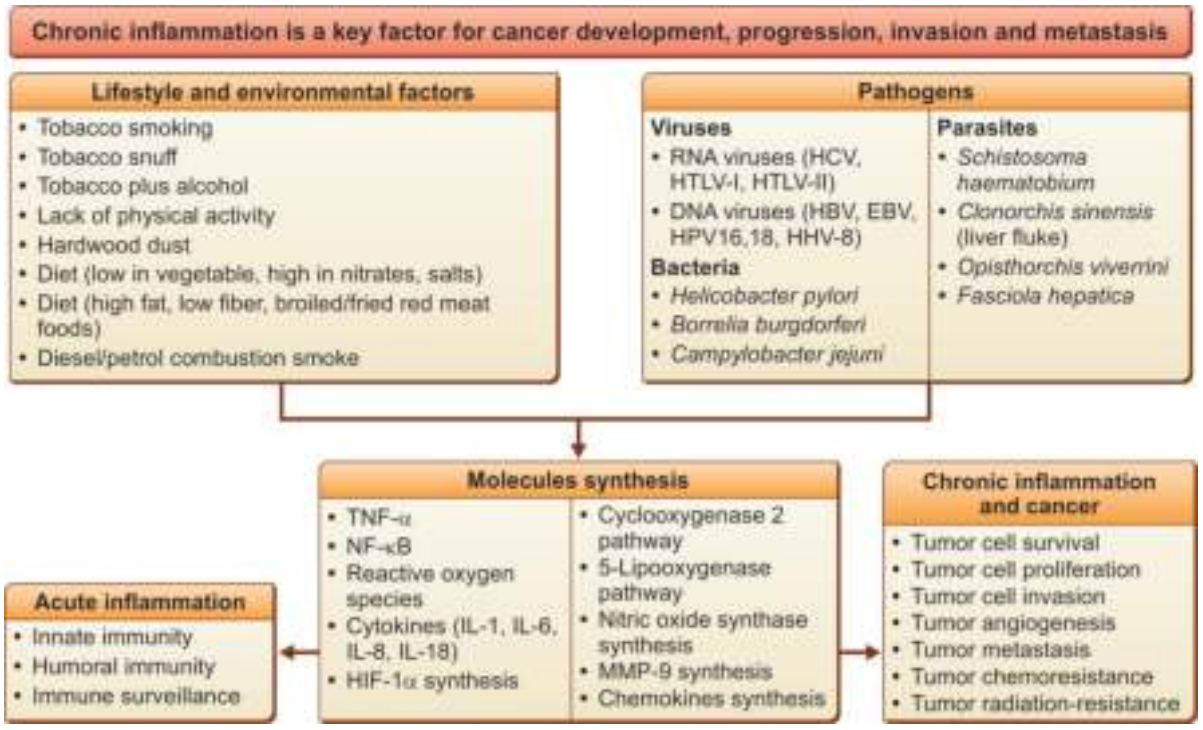


Fig. 6.91: Chronic inflammation linked to carcinogenesis, progression and metastasis. Chronic inflammation induces gene mutation and creates an environment that is conductive to transformation of normal cell to CSC, malignant tumor growth, invasion and metastasis.

Table 6.61 Chronic inflammation linked to carcinogenesis, invasion, metastasis and chemoresistance

Gene mutations
Genomic instability
Immunosuppression
Tumor promotion, tumor cell growth and survival
Tumor angiogenesis
Metastasis
Tumor re-emergence
Tumor chemoresistance

response observed during innate/adaptive immunity, and maintenance of tissue hemostasis.

- Additionally, tumor-promoting inflammation occurs because CSCs cause necrosis of normal cells which release cell contents into tumor microenvironment, triggering the release of proinflammatory chemical mediators, notably reactive oxygen species (ROS), that are actively mutagenic for nearby stem cells, accelerating their genetic evolution toward states of heightened malignancy.
- Several types of inflammation can promote tumor development and progression. Persistent *Helicobacter*

Table 6.62 Dysregulation of signaling pathways and tumor-associated macrophages in chronic inflammation linked to carcinogenesis

Key Marker	Function
Signaling pathways	
NF-κB signaling pathway	▪ NF-κB is a transcription factor that plays an important role in the regulation of cytokines ▪ Dysregulation of NF-κB is linked to inflammatory autoimmune diseases and human malignancies
IKK-β signaling pathway	IKK-β is a part of the IKK complex which is a negative regulator of nuclear factor kappa-B (NF-κB) transcription factor
Tumor-associated macrophages (TAMs)	
CD68 marker	CD68 is a key marker to recognize both M1 and M2 macrophages in tumor microenvironment
CD163 marker	CD163 is a scavenger receptor upregulated in macrophages in an anti-inflammatory environment
iNOS marker	iNOS is one of the major markers of M1 tumor-associated macrophages (TAMs)

pylori infection is linked to gastric carcinoma and MALT (mucosa-associated lymphoid tissue) lymphoma. Infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) increase the risk for hepatocellular carcinoma (HCC). Infection with *Schistosoma haematobium* is linked to urinary bladder carcinoma. Bacteroides species infection is linked to colon carcinoma. Selective anti-inflammatory drugs are used in clinical practice.

PHENOTYPIC PLASTICITY AND DISRUPTED DIFFERENTIATION

Terminal differentiation in normal cells is associated with a permanent cell proliferation arrest. Stem cell phenotype plasticity is the ability of adult tissue-specific stem cells to switch to new identities, i.e. different phenotypes (traits), that are not associated with their cell of origin, and depending on environment that is more inclined to proliferate.

- Cancer stem cells evade differentiation and unlock what is known as **phenotype plasticity**, which continue to grow and change their identity into different phenotypes depending on environment that is more inclined to 'proliferate'.
- Hanahan observed that phenotype plasticity is not a novel invention of CSCs, but rather a 'malignant twist' of normal cells.
- Main strategies for targeting therapy include: inhibiting EMT, selectively killing mesenchymal phenotype and reverting the cells.

Phenotypic Plasticity in Normal Cells

During the process of organogenesis, cells undergo terminal differentiation, and are organized into tissues/organs and assume homeostatic functions.

- During terminal differentiation, most cells exit cell cycle and enter into a permanent G0 phase of cell cycle. Hence, cell cycle arrest prevents cell proliferation upon terminal differentiation.
- Cell cycle arrest is usually initiated through the repression of cell cycle gene expression by forming a transcriptional repressor complex, called DREAM complex (RB/E2F/MuvB).
- The cells change their phenotypic characteristics as they migrate to the surface and undergo terminal differentiation. For examples, keratinocytes migrate to the surface of epidermis and become keratinized and ultimately die.

Phenotypic Plasticity in Cancer Stem Cells

In contrast, epithelial cell-derived CSCs evade terminal differentiation and unlock what is called '**phenotypic plasticity**', and continue to proliferate and survive in the face of environmental change.

- Phenotype plasticity represents the most relevant molecular 'hallmark of cancer' that alters morphologic and functional features of epithelial-derived CSCs.
- The unlocking of terminal differentiation can be done in different ways: (a) de-differentiation from mature states to progenitor states, (b) block of differentiation (terminal or maturation arrest), and (c) transdifferentiation into different lineages.

De-differentiation of Cells Linked to Colon Carcinoma

De-differentiation is a cellular process by which cells grow in reverse, from a partially or terminally-differentiated mature cell stage to less-differentiated progenitor cell-like stage within their own lineage. Colon carcinogenesis exemplifies disrupted cellular differentiation. The transition of an intestinal epithelial cell into fully transformed metastatic intestinal cancer follows a series of activating (Wnt/β-catenin signaling pathway in APC), and inactivating mutations in numerous oncogenes and tumor suppressor genes, respectively. Initially, expansion and transformation of the stem cell compartment leads to the development of adenomatous polyps in colon. Acquiring additional mutations in K-RAS oncogene and TP53 tumor suppressor gene, patient develops colon carcinoma.

Block of Differentiation (Terminal Maturation Arrest) of Cancer Stem Cells in Acute Leukemias and Melanoma

Block of differentiation (terminal differentiation block, or maturation arrest) occurs in acute promyelocytic leukemia (APL) and acute myelogenous leukemia (AML-M1), in which leukemia cells proliferate and/or prevents the terminal differentiation into granulocytes and apoptosis seen in normal myeloid-derived white blood cells. Melanoma is other example of blocked differentiation (terminal maturation arrest).

- **Block of differentiation in acute promyelocytic leukemia:** Acute promyelocytic leukemia (APL) has been documented to result from chromosomal translocation that fuses with PML locus on gene encoding the retinoic acid receptor (RAR). Myeloid progenitor cells bearing such chromosomal translocations are unable to continue their usual terminal differentiation into granulocytes, resulting in cells trapped in a proliferative promyelocytic progenitor stage.
- **Block of differentiation in acute myelogenous leukemia:** Another example of block of differentiation (terminal maturation arrest) is acute myelogenous leukemia (AML-M1) carrying chromosomal

translocation t(8;21), which produces AML1-ETO fusion protein in its own can transform myeloid progenitors, at least in part by blocking their differentiation.

- **Block of differentiation in melanoma:** Another example of block of differentiation (maturation arrest) is melanoma, that involves a developmental transcription factor SOX10, which is normally downregulated during melanocyte differentiation. Gain- and loss-of-function BRAF-induced melanoma maintains aberrantly expression of SOX10, that blocks differentiation of neural progenitor cells into melanocytes.

Transdifferentiation of Cancer Stem Cells in Adenocarcinomas of Esophagus and Pancreas

Transdifferentiation is defined as the conversion of one type stem cell switches to different cell lineage. The concept of transdifferentiation has been recognized in the form of tissue metaplasia, wherein cells of a particular differentiated phenotype markedly change their morphology to become clearly recognizable as elements of another tissue.

- Transdifferentiation of stratified to columnar epithelium inducing Barrett's esophagus → esophageal adenocarcinoma: One example of transdifferentiation is Barrett's esophagus, where chronic inflammation of the stratified squamous epithelium of the distal esophagus induces transdifferentiation into a simple columnar epithelium that is characteristic of the intestine, thereby facilitating the subsequent development of esophageal adenocarcinoma, and not squamous cell carcinoma.
- Acinar-to-ductal cell transdifferentiation → pancreatic ductal adenocarcinoma: one example of transdifferentiation is pancreatic ductal adenocarcinoma, in which acinar-to-ductal cell transdifferentiation that encompasses cellular reprogramming and phenotypic switch-over making in a cardinal event during carcinogenesis and development of highly aggressive malignant tumor associated with extremely poor prognosis.
  - The CSCs of pancreatic ductal adenocarcinoma have acquired a mesenchymal phenotype during the process of epithelial–mesenchymal transition (EMT). Epithelial–mesenchymal transition is driven by master transcription factors, i.e. SNAIL1 and SNAIL2/SLUG, encoded by the SNAI1 and SNAI2 genes, respectively resulting in reduced membrane expression of the epithelial marker E-cadherin and upregulation of mesenchymal markers such as vimentin.
  - In many epithelial tissues, the EMT programme is associated with generation of CSCs, which in pancreatic ductal adenocarcinoma promote growth metastatic spread, chemoresistance and tumor relapse after chemotherapy.

NON-MUTATIONAL EPIGENETIC REPROGRAMMING IN CANCER STEM CELLS

In the eukaryotic nucleus, deoxyribonucleic acid (DNA) is a double helical structure composed of nucleotide base pairs, i.e. adenine (A) and thymine (T) pair, cytosine (C) and guanine (G) pair. Two strands of DNA run in opposite directions to each other and are thus antiparallel. Two helices are joined together by hydrogen bonds. DNA also bears a sugar-phosphate backbone.

- Deoxyribonucleic acid (**DNA**) is organized into chromatin structure with the nucleosome as the basic unit, in which histone octamer is surrounded by the nucleotide base pairs of DNA. The histone octamer consists of two elements of the histone octamer proteins (H3, H4, H2A and H2B). The packaging of DNA into chromatin structure presents a potential barrier to factors that require DNA as their template.
- Human genome contains about 3.2 billion DNA nucleotide base pairs. Within the genome, there are about 20,000–25,000 protein encoding genes comprising only 1.5% of the genome. DNA performs several functions: chromosome duplication in DNA replication using telomerase enzyme before cell division, transcription, and translation to make proteins.
- Genetic and epigenetic modifications are interrelated that contribute to altered gene expression in human cancers. The link between genetics and epigenetics in cancer is further demonstrated by existence of aberrant metabolism, and biochemical pathways involving aberrant gene function and altered patterns of gene expression.
- Epigenetic modifications are heritable changes in gene expression (i.e. 'turning on' or 'turning off') without alterations in DNA sequences in different organs, through DNA methylation, histone octamer modifications and microRNAs, but they can change how body reads the DNA sequence. Epigenetic modifications may be caused by environmental agents (chemical agents, tobacco smoke and infection with specific microbes), cytotoxic/pharmaceutical drugs, dietary factors, obesity, physical activity, alcohol consumption, psychological stress and advancing age.
  - External and internal signaling pathways may induce epigenetic modifications and altered gene expression, that provide a permissive

microenvironment for transformation of normal cell to CSC involving different driver and passenger gene regulatory pathways. In cancer research, epigenetics is vital in early-stage cancer diagnosis and novel therapeutic design.

DNA Methylation

DNA methylation is a widespread epigenetic modification of DNA during DNA replication, and considered as a stable gene silencing mechanism. DNA methylation is a heritable epigenetic mechanism of DNA, that occurs by the addition of a methyl (CH3) group to the C-5 position of the cytosine at CpG dinucleotide islands present in the promoter regions in the DNA sequence by DNA methyltransferases encoded by family of DNMTs genes (i.e., DNMT3A, DNMT3B and DNMT1), thereby often modifying the function of genes during embryogenesis and postnatal life.

- Human genome DNA constitutes 70–75% of methylated DNA. Global changes in DNA methylation occur in both normal cells and CSCs. DNA methylation is important for establishing and maintaining cell identity and genomic stability.
- Carcinogenesis is accompanied by a global loss in DNA methylation, which facilitates the transformation of normal cell to CSC resulting from activation of previously silent proto-oncogenes (i.e. DLX1, POU3F3) after hypermethylation, and silencing tumor suppressor genes (e.g. TP53, BRCA1, p16), after methylation of gene in the promoter region.
- Global (genome-wide) hypomethylation may also cause genomic instability through interference with protective function of telomeres and centromeres, and activation of growth promoting genes (proto-oncogenes), thereby 'turning on' mitotic signals. DNA hypomethylation can also result in loss of imprinting.
- DNA methylation inhibitors and histone acetylase inhibitors have been under clinical trials with the focus on DNA methylation and histone octamer modifications in the epigenetics field in cancer patients.

CpG Dinucleotide Islands Hypermethylation

In normal cells, CpG dinucleotide islands are present in the promoter regions in the DNA sequence, which comprise cytosine followed by guanine (i.e. phosphate-linked cytosine-guanine pairs) from 5′ to 3′ direction. CpG dinucleotide islands are usually unmethylated/hypomethylated to maintain euchromatin structure and activate gene expression, thus allowing post-translational activation. Hypermethylation of CpG dinucleotide islands linked to human malignancies is given in Table 6.63.

- Hypermethylated CpG dinucleotide islands down-regulate DNA repair genes, induce epithelial–mesenchymal transition (EMT), unrestricted CSC proliferation, development of malignant tumor growth, invasion, and metastasis.
- Hypermethylation of CpG dinucleotide islands in promoter region of the mismatch repair gene MLH1 has been observed in 13% of sporadic cases of human colorectal cancers, that show microsatellite instability.
- Hypermethylation of CpG dinucleotide islands in promoter region of the mismatch repair genes (i.e. hMLH1 and hMSH2) are linked to hereditary nonpolyposis (HNPCC), also called Lynch syndrome.

Table 6.63 Hypermethylation of CpG dinucleotide islands linked to human malignancies

Methyl-CpG Binding Proteins	Human Malignancies
MeCP2	Breast, colorectal region, gastric region, and prostate
MBD1	Pancreas and prostate
MBD2	Breast, colon, pancreas, and lung
MBD4	Breast, colon, prostate, lung, and chronic myelogenous leukemia (CML)
ZBTB33 (KAISO)	Breast, colon, cervix, ovary
ZBTB38	Urinary bladder
UHR1	Esophagus, gastric region, colon, pancreas, liver, hepatic ducts, thyroid, skin (squamous cell carcinoma, melanoma), kidney, and retinoblastoma
UHR2	Colon, liver and hepatic ducts

Dysregulated methyl-CpG binding proteins promote epithelial–mesenchymal transition (EMT), cell proliferation, invasion, and metastasis.

DNA Methyltransferases (DNMTs) Gene Mutations induce DNA Hypermethylation

Aberrant expression of DNMT1, DNMT3A and DNMT3B genes induce DNA hypermethylation, oncogenic activation, genomic instability, transformation of normal cell to CSCs and development of various human malignancies of colon, breast, ovary, and cervix.

- **Mutation in DNMT1 gene-linked cancers:** Mutation in DNMT1 gene is linked to solid malignant tumors of thyroid, breast, gastric region, colorectal region, pancreas, liver, cervix, prostate, and ovary associated with lymph node metastasis and poor prognosis in these patients. Overexpression of DNMT1 protein in gastric carcinoma is significantly related to decreased E-cadherin expression, which indicates that an increase in DNMT1 expression will enhance the migration ability of the CSCs.

- **Mutation in DNMT3A gene-linked cancers:** DNMT3A mutation is linked to lung carcinoma, melanoma, vulva, squamous cell carcinoma and acute myelogenous leukemia (AML) associated with poor prognosis. DNMT3A can act both as an oncogene and as a tumor suppressor gene in malignant tumor.
- **Mutation in DNMT3B gene-linked cancers:** DNMT3B *de novo* methyltransferase is related to abnormal methylation of tumor suppressor genes. DNMT3B overexpression is linked to breast carcinoma, hepatocellular carcinoma, colon carcinoma, urinary bladder carcinoma and ovarian carcinoma.

Histone Octamer Protein Modifications

DNA exists in the condensed chromatin form to fit into the nucleus. Histones are chromosomal proteins wrapped by DNA that form a nucleosome ('beads on string'), the fundamental unit of chromatin. A tight packaging of histone octamer proteins prevents the access of gene expression-regulating proteins to DNA, thus inhibiting gene expression. A loose packing of histone octamer proteins enhances the access of gene expression-regulating proteins to DNA thus turning on gene expression.

- Histone octamer proteins modification refers to addition or removal of chemical groups from histone to regulate the tight or loose packaging and spacing of nucleosomes. Histone octamer consists of two copies each of four histone proteins; H2A, H2B, H3 and H4. Each one of these proteins has a globular C-terminal tail extension, and tails are the targets of nucleosome modification.
- Linker H1 histone octamer protein binds to the 'linker DNA' (about 20–80 nucleotides in length) region between nucleosomes, helping in stabilization of the zigzagged 30 nm chromatin fibers into high-order chromatin structure.
- Histone octamer remodeling and modification regulate the DNA replication and transcription. During DNA replication, polymerase only accesses the DNA, which is not bound by histones.
- Phosphate group give histone octamer a negative charge. Lysine and arginine give histone octamer a positive charge. Global levels of lysine methylations are quite different between cell types and corelated in various human malignancies.
  - H3K4me1 is an epigenetic modification of the DNA packaging histone octamer protein H3, which indicates mono-methylation at the 4th lysine residue of the histone H3 protein and associated with gene enhancers.
  - H3K4me2 is an epigenetic modification of the DNA packaging histone octamer protein that di-methylation at the 4th lysine residue of the histone octamer protein H3 and associated with accessible chromatin structure and gene activation.
  - H3K4me3 is an epigenetic modification of the DNA packaging histone octamer protein, that indicates tri-methylation at the 4th lysine residue of the histone octamer protein H3 around transcription start sites and associated with gene activation and active transcription.
  - Dysregulated H3K4me1, H3K4me2, and H3K4me3 histone octamer proteins are linked to many human cancers.
- In addition, lysine methyltransferases and demethylases are deregulated on various cancers. These molecular alterations lead to permanent changes in the patterns of gene expression that regulate the malignant phenotype such as unrestricted cellular growth, and invasiveness. Histone demethylases are enzymes that remove the methyl marks on lysine in nucleosome's histone octamer tails. These alterations in methylation mark regulate gene expression during transformation of normal cell to CSC and development of malignant tumor.
- Global histone lysine methylation patterns in human cancers are given in Table 6.64. Histone lysine methyltransferases linked to human cancers are given in Table 6.65. Global histone lysine demethylases linked to human cancers are given in Table 6.66.

Table 6.64 Global histone lysine methylation patterns in human cancers

Histone Octamer Modification	Histone Octamer Modifications and Expression Compared to Normal Tissues	Associated Human Cancers
H3K4me1 (mono-methylation at the 4th lysine residue of H3) protein	▪ Downregulation ▪ Upregulation upon progression	Prostatic carcinoma and urinary bladder carcinoma
H3K4me2 (di-methylation at the 4th lysine residue of H3) protein	▪ Downregulation ▪ Upregulation upon progression	▪ Small cell lung carcinoma, renal cell carcinoma, hepatocellular carcinoma, pancreatic carcinoma and breast carcinoma ▪ Prostatic carcinoma
H3K4me3 (tri-methylating at the 4th lysine residue of H3) protein	▪ Downregulation ▪ Upregulation	▪ Urinary bladder carcinoma ▪ Prostatic carcinoma and renal cell carcinoma

Table 6.65 Histone lysine methyltransferases linked to human cancers

Writer	Alterations in Human Cancers	Associated Human Cancers
MLL1 (new name: KMT2A) gene	Translocation, amplification, tandem duplication	Hematolymphoid neoplasms and myelodysplastic syndrome
Menin gene	▪ Gene mutation ▪ Reduced expression ▪ Increased expression	▪ Multiple endocrine neoplasia type 1 (MEN1) ▪ Lung adenocarcinoma ▪ Prostatic carcinoma
Ash2L gene	Increased expression	Squamous cell carcinomas of cervix and larynx, melanoma, rhabdomyosarcoma, carcinomas of breast and colon, pancreatic carcinoma and neuroendocrine carcinomas

Table 6.66 Global histone lysine demethylases linked to human cancers

Gene Name	Alterations in Cancer	Associated Human Cancers
KDM1	▪ Overexpression ▪ Downregulation	▪ Prostatic carcinoma, breast carcinoma, neuroblastoma ▪ Hepatocellular carcinoma
KDM2B	▪ Gene mutation ▪ Downregulation	▪ Lymphoma ▪ Glioblastoma multiforme
KDM4C	Overexpression	Prostatic carcinoma, esophageal squamous cell carcinoma, desmoplastic medulloblastoma, MALT lymphoma
KDM5A	Overexpression	Gastric carcinoma
KDM5B	Overexpression	Breast carcinoma, testicular and ovarian cancers, lung carcinoma, urinary bladder carcinoma
KDM6A	Gene mutation	Multiple myeloma, renal cell carcinoma and several malignancies
KDM6B	Overexpression	Prostatic carcinoma, pancreatic carcinoma, lymphoma

Pathology Pearls: Histone Octamer Protein Modifications—Mechanisms

Histone octamer protein modifications can occur by various mechanisms: methylation, acetylation/deacetylation, phosphorylation, ubiquitination, and sumoylation.

- **Histone octamer proteins methylation:** Histone octamer proteins methylation can activate oncogene (‘**turning on**’) or repress (‘**turning off**’) tumor suppressor gene expression, and favor the acquisition of molecular hallmark of cancer capabilities such as cell cycle dysregulation, evasion of apoptosis, invasion, and metastasis. Dysregulation of histone octamer proteins methylation can lead to inappropriate turning off (silencing) of tumor suppressor genes or ‘turning on’ (activation) of oncogenes, thus contributing to the development of cancers (gliomas, sarcomas, and phenotypic mixed lineage leukemia).
- **Histone octamer proteins acetylation/deacetylation:** Histone octamer proteins acetylation/deacetylation affects nucleosome spacing and gene expression.
  - Histone octamer proteins acetylation results in loose packaging and spacing of nucleosomes, hence transcription factors can bind the DNA and recruit acetylating enzymes resulting in ‘turning on’ gene expression, which is essential for cell cycle progression and differentiation. Histone octamer proteins acetylation with enhanced activity of HDACs can lead to development of malignant phenotype.
  - On the contrary, histone octamer proteins deacetylation results in compact packaging and spacing of nucleosomes, hence transcription factors cannot bind the DNA and recruit deacetylating enzymes resulting in ‘turning off’ tumor suppressor gene expression (repression/silencing), and transcription repression of various genes.
- **Histone octamer proteins phosphorylation:** Histone octamer proteins phosphorylation occurs during cellular response to DNA damage. Phosphorylation of serine and threonine residues facilitates chromatin condensation during mitosis and transcriptional activation and expression of immediate-early targeted genes.
- **Histone octamer proteins ubiquitination:** Histone octamer proteins ubiquitination plays key roles in DNA transcription, maintenance of chromatin structure and DNA repair.
- **Histone octamer proteins sumoylation:** Histone octamer proteins sumoylation regulates the functional properties of many proteins and thereby plays key role in the normal functions of cells.

MicroRNAs

MicroRNAs are regulators of chromatin modifying machinery, and gene expression, which play pivotal roles in numerous biological processes to maintain cell growth, and differentiation. DNA sequences are transcribed to coding (generating proteins), and

noncoding RNAs (lacking building blocks of proteins). Noncoding RNAs are comprised of transfer RNAs, ribosomal RNAs, small RNAs (<200 nucleotides) and long RNAs (>200 nucleotides) based on their length. Both small and long noncoding RNAs are transcribed by RNA polymerase II or polymerase III and endogenously processed by endonuclease. Schematic representation of epigenetic related small and long non-coding RNAs is shown in Fig. 6.92.

- **Biogenesis of MicroRNAs and regulation of gene expression:** Human genome contains about 1000 noncoding microRNAs constituting 5% of total genome. MicroRNAs regulate the expression of multiple proteins in the post-translation process. MicroRNAs are transcribed from DNA sequences by RNA polymerases II and III, generating primary microRNAs and processed into precursor miRNAs, and finally mature miRNAs. Biogenesis and function of noncoding microRNA are shown in Fig. 6.93.
  - **Ribonuclease DROSHA** enzyme processes the primary miRNA to generate double-stranded premiRNA in the nucleus, which is then exported to cytoplasm via **Exportin-5 (EXPO5)**. Once the premiRNA is in cytoplasm, an enzyme called '**DICER**' trims the pre-miRNA by removal of its hairpin loop and cleaves the molecule into two strands, i.e. functional leading strand and passenger strand undergoing degradation.
  - The aspartylglucosaminidase (AGA) proteins bind to the leading strand of miRNA to form the RNA-induced silencing complex 'RISC' functional unit, which facilitates the binding of miRNA into the targeted messenger RNA (mRNA) resulting in either translation repression or target degradation of the Poly-A tail via deadenylation process. In most cases, miRNAs interact with the 3′ untranslated regions (3′ UTRs) of target mRNAs to induce mRNA degradation and translation repression. Noncoding miRNAs participate in silencing posttranscriptional genes by inhibiting messenger RNA (mRNA) translation.
  - Dysregulation of microRNA biogenesis can occur because of aberrant expression of DROSHA, DICER, DGCR8, EXPO-5, AGO1/AGO2 and TRBP, which lead to carcinogenesis, invasion and metastasis.
- **Functions of microRNAs:** MicroRNAs regulate gene expression through various mechanisms at the post-transcriptional level mainly by binding with messenger RNA (mRNA) in the cell cytoplasm and play key roles in cell differentiation, cell cycle regulation, growth, proliferation, differentiation, migration, metabolism, and apoptosis. MicroRNAs

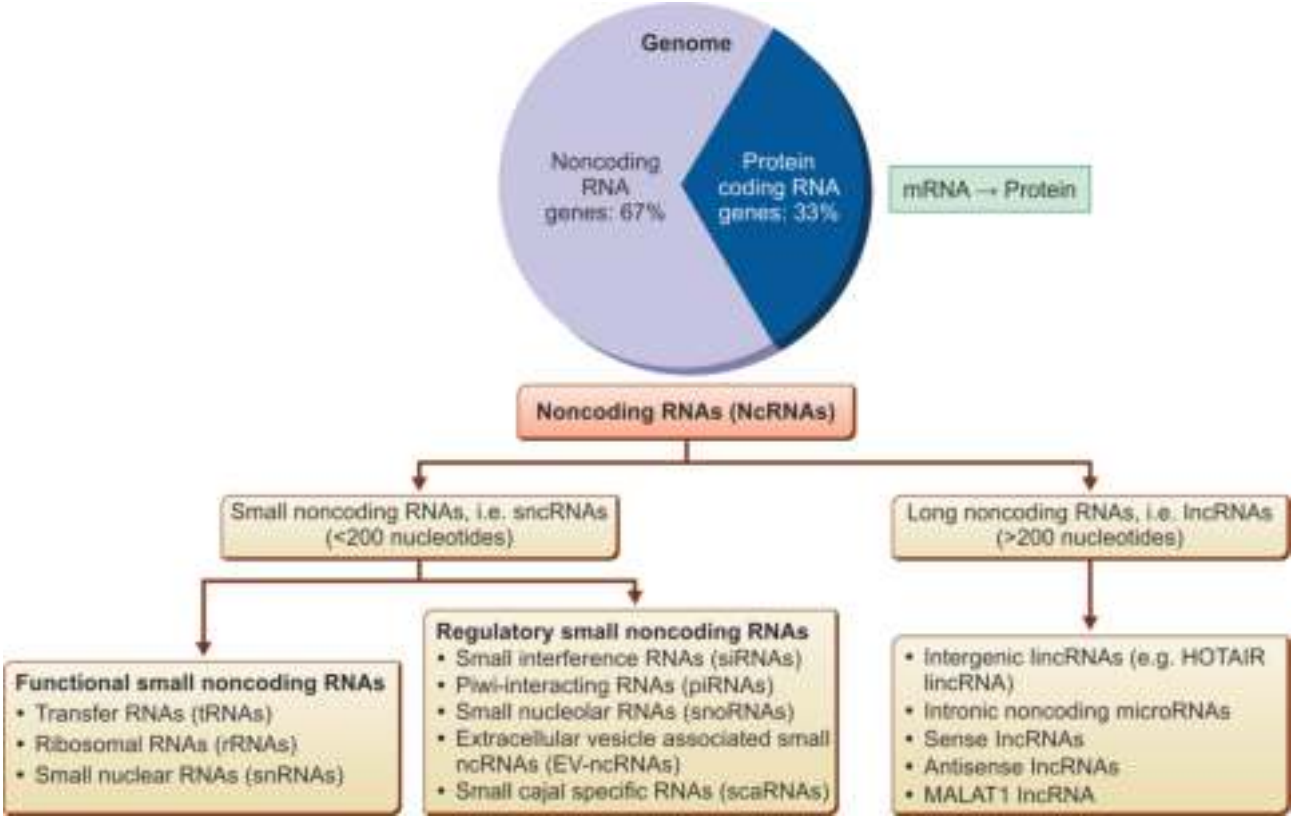


Fig. 6.92: Schematic representation of epigenetic-related small and long noncoding RNAs.

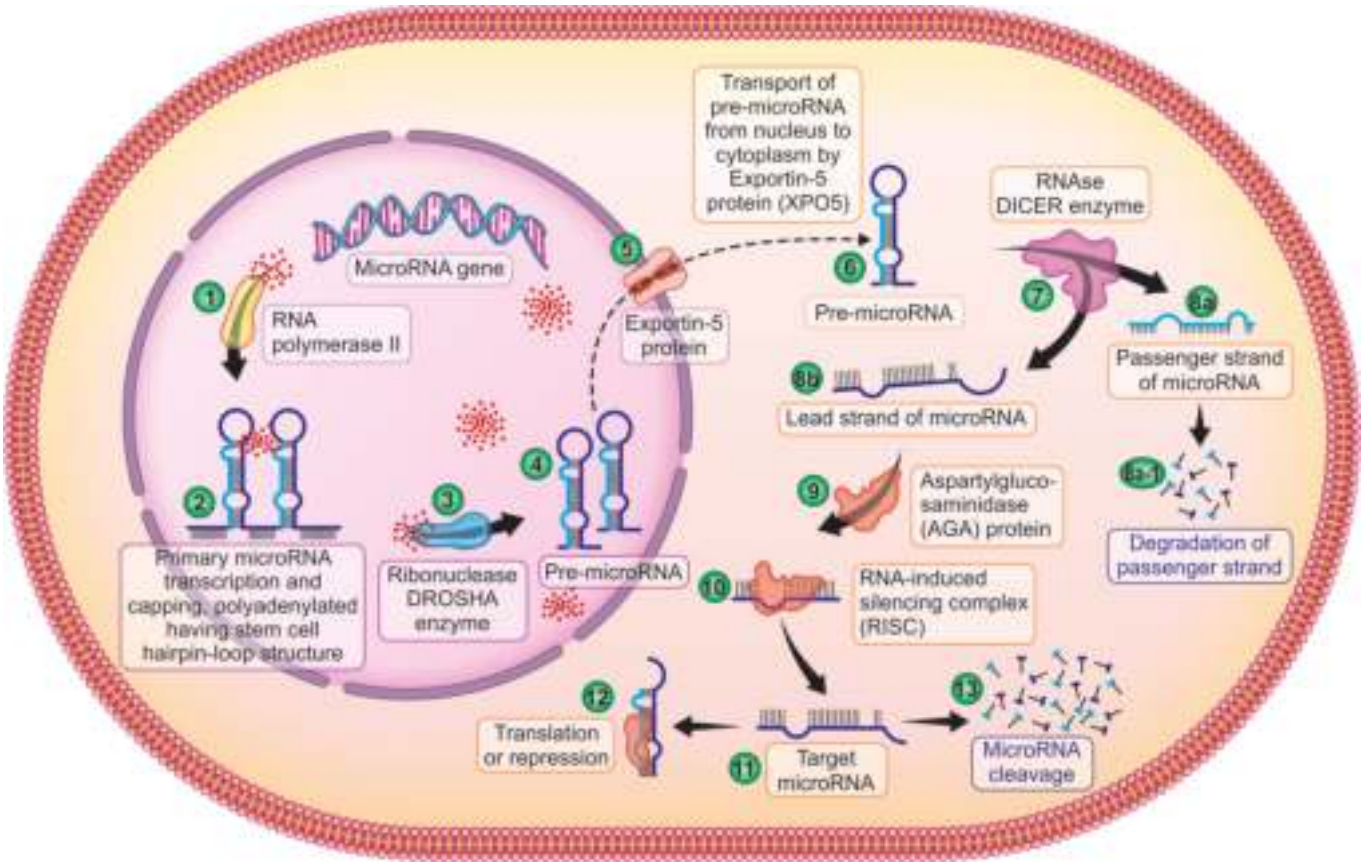


Fig. 6.93: Biogenesis of microRNA. MicroRNA is transcribed as longer precursors by RNA polymerase I and II, that undergoes series of cleavage events to form mature microRNA. MicroRNAs are transcribed as longer precursors by RNA polymerase II and III, that undergo series of cleavage events to form mature microRNA. The conventional biogenesis pathway consists of two cleavage events, one nuclear and one cytoplasmic. The pre-miRNA is exported from the nucleus by Exportin-5-Ran-GTP. In the cytoplasm, RNase DICER in complex with the double-stranded RNA-binding protein (DRBP) cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Agp2) proteins into the RNA-induced silencing complex (RISC), where its guides the RISC to target mRNA through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand undergoes degradation. Noncoding RNAs can be secreted into the extracellular space, stabilized in vesicles or protein-binding partners.

are involved in epigenetic processes such as heterochromatin formation.

- **Dysregulation of microRNAs:** Majority of microRNA genes are present in cancer-associated regions in genome. Recent studies revealed that different microRNAs regulate the interaction between CSCs and their surrounding cells within tumor microenvironment, and can function as oncogenic microRNAs or tumor suppressor microRNAs based on their modulating effect on the expression of their target post-translational gene silencing.
  - Oncogenic RNAs play a pivotal role in initiating carcinogenesis and development of malignant tumor by inhibiting tumor suppressor microRNAs and apoptosis.
  - Tumor suppressor microRNAs regulate the expression of their target genes post-transcriptionally by degrading messenger RNA (mRNA) transcripts or by inhibiting mRNA translation.
  - MetastamicroRNAs play crucial role in the metastatic process. MicroRNAs that act as oncogenes and tumor suppressors linked to human cancers are given in Table 6.67. Dysregulation of microRNAs casually related to epigenetic alterations linked to human cancers is given in Table 6.68.
- **Mechanisms of dysregulation of microRNAs:** MicroRNAs have been implicated in the regulation of virtually all signaling circuits within cell, and their dysregulation has been shown to play an essential role in the development and progression of cancer. Dysregulation of microRNAs expression in human cancers occurs through various mechanisms: (a) amplification of oncogenic microRNAs, (b) deletion of chromosomes harboring microRNAs, (c) abnormal transcriptional regulation, (d) defects in the biogenesis machinery, and (e) epigenetic alteration and gene silencing.

Table 6.67 MicroRNAs that act as oncogenes and tumor suppressors linked to human cancers

MicroRNAs	Human Malignancies
Oncogenic microRNAs	
MiR-9	Neuroblastoma
MiR-10b	Breast
MiRs-15a, 15b, 16, 16-1	Leukemia, pituitary adenoma
MiRs-17-5p, 17-92, 20a	Lung, lymphoma
MiR-21	Breast, esophagus, colorectal region, hepatic ducts, lung, pancreas, cervix, prostate and CLL
MiR-23	Breast, urinary bladder, and CLL
MiR-29, 29a	Hepatic ducts, leukemia
MiRs-31, 133b, 135, 183	Colorectal region
MiR-143	Colorectal region, cervix
MiR-146	Thyroid
MiR-155	Breast, pancreas, leukemia
MiR-221	Thyroid, liver, pancreas, prostate, glioblastoma multiforme, AML, and CLL
MiRs-17-92 cluster includes structurally homologous mircoRNAs 17-30, 17-5p,18a,19a,19b, 20a and 93a-1	Colorectal region, lung, ovary, AML, and CLL
MiR-155	Hodgkin's disease, diffuse large B cell lymphoma, Burkitt lymphoma, and CLL
MiRs-181, 181a, 181b, 181c	Glioblastoma multiforme, breast, thyroid, and CLL
Tumor suppressor microRNAs	
MiR-143	Breast, colorectal region, lung, prostate, and CLL
MicroRNA-let group (let7-1-a1 through-a3-b through-g,-1 and microRNA-98)	Breast, colorectal region, lung, prostate, AML, and CLL
MiR-145	Urinary bladder, breast, colorectal region, lung, ovary, and prostate
Multifunctional role of microRNAs that act as both oncogenic and tumor suppressors	
MiR-23 group	▪ Oncogenic MiR: breast, urinary bladder, and CLL ▪ Tumor suppressor MiR: prostate
MiR-34s	▪ Oncogenic MiR: kidney, liver and colon ▪ Tumor suppressor MiR: lung, pancreas, and neuroblastoma
MiR-181 group	▪ Oncogenic MiR: breast, urinary bladder, and CLL ▪ Tumor suppressor MiR: prostate
MiR-125 group	▪ Oncogenic MiR: pancreas, prostate, and ALL ▪ Tumor suppressor MiR: breast, thyroid, liver, ovary, prostate, glioblastoma, AML, and CLL
Let-7	▪ Oncogenic MiR: breast, colorectal region, lung, prostate, AML, and CLL ▪ Tumor suppressor MiR: breast, lung, prostate

AML (acute myelogenous leukemia), ALL (acute lymphoblastic leukemia), CLL (chronic lymphocytic leukemia)

Table 6.68 Dysregulation of microRNAs casually related to epigenetic alterations linked to human cancers

MicroRNAs	Negative Regulation of Target Gene	Effect in Cancer
MicroRNA-29 family (29a, 29b, 29c)	▪ DNMT3A ▪ DNMT3B	Aberrant DNA methylation due to DNMT3A in acute myelogenous leukemia
MicroRNAs (152, 148a, 185, 342)	DNMT1	Aberrant DNA methylation in colorectal carcinoma
MicroRNAs (101, 26A, 98, 124, 214, let-7)	EZH2	Silencing of target genes

DNMT3A, DNMT3B and DNMT1 genes encode DNA methyltransferase involved in DNA methylation. EZH2 encodes histone methyltransferase enzyme involved in histone methylation

- **Amplification of oncogenic microRNAs:** Amplification of oncogenic microRNAs can downregulate tumor suppressor genes and lead to uncontrolled cell growth, cell proliferation, epithelial–mesenchymal transition (EMT), invasion and metastasis to distant organs. Amplification of microRNA-200 is linked to ovarian carcinogenesis, EMT, invasion and metastasis. Amplification of microRNA-17/92 cluster gene results in B cell lymphoma and lung carcinoma. Amplification of microRNA-221 is linked to triple negative primary breast carcinoma.
- **Deletion of chromosomes harboring microRNAs:** Deletion of chromosome 13q14 region harboring microRNAs 15-α/16-1 cluster gene upregulates antiapoptotic BCL-2 protein resulting in chronic lymphocytic leukemia (CLL). Deletion of chromosome 5q33 region harboring microRNAs-143/145 is linked to lung carcinoma. Germline defects in DICER enzyme are linked to tumors of testes and ovaries. DICER is a microRNA processing factor, that codes for endonuclease required for synthesis of functional microRNAs.
- **Aberrant expression of transcription factors:** Normally, Myc proto-oncogene and TP53 tumor suppressor gene code for transcription factors, which tightly regulate microRNAs. Aberrant expression of transcription factors is linked to many human malignancies. The c-Myc oncogene encodes transcription factor that induces carcinogenesis, unrestricted CSCs proliferation, angiogenesis, epithelial–mesenchymal transition (EMT), invasion and metastasis. The c-Myc oncogene encoding transcription factor can enhance or repress transcription activity of microRNAs. TP53 tumor suppressor gene encodes p53 protein, that plays a central role in tumor suppression through transcriptional regulation of many target genes. TP53 gene mutation is linked to many human cancers. Recent studies have demonstrated that some microRNAs regulate the activity of TP53 gene through directly targeting p53 protein, and indirectly targeting through MDM2/MDM4 proteins. These findings have demonstrated that microRNAs are key regulators and mediators of TP53 gene signaling pathway, which highlights a pivotal role of miRNAs in the TP53 network and development of human cancers.
- **Defects in biogenesis process of mircoRNAs:** Dysregulation of microRNA biogenesis can occur because of aberrant expression of DROSHA, DICER, DGCR8, EXPO-5, AGO1/AGO2 and TRBP has been linked to carcinogenesis, malignant tumor development, invasion, and metastasis.
- **Epigenetic alterations and gene silencing:** Epigenetic alterations can affect gene expression by '**turning on**' or '**turning off**' without changes in DNA sequence through various mechanisms, which include DNA methylation, histone covalent modification and mircoRNAs. Thus, microRNAs are modulators of chromatin machinery, gene expression and play pivotal roles in numerous biological processes. Recent studies have revealed that DNA methylation and histone modifications are the major causes of microRNAs dysregulation resulting in human cancers.

Oncogenic MircoRNAs and Cancer: Promoting Mechanisms

Oncogenic microRNAs play a pivotal role in initiation and progression of human cancers by inhibiting the expression of tumor suppressor genes involved in different biological processes. Deregulation of oncogenic microRNAs is associated with genetic and/or epigenetic alterations, including deletion, amplification, point mutation and aberrant DNA methylation. Their expression profiling of human malignancies has identified signatures involving in initiation, progression, diagnosis and prognosis.

- **Oncogenic microRNAs and cell cycle progression:** Cell cycle is a series of events that takes place in a cell as it grows and divides into two identical daughter cells.
  - Oncogenic microRNAs contribute to cell cycle progression by targeting CDK inhibitors and tumor suppressor proteins such as p53, RB1, INK family of proteins (p15, p16, p18, p19), CIP family of proteins (p21, p27, p29), BRCA1, BRCA2, DNA repair proteins, ATM protein involved in cell cycle progression and unrestricted CSCs proliferation leading to human cancers. TP53 is a master regulator of human genome.
  - Oncogenic microRNA 504 acts as negative regulator of p53 protein by binding to promoter region of TP53 gene resulting in reduction in p53 protein-mediated apoptosis as well as cell cycle arrest in human cancers. Depletion of oncogenic microRNAs increases the expression of p53 protein.
  - Oncogenic mircoRNA-21 targets numerous tumor suppression genes associated with invasion and metastasis of breast carcinoma, colorectal carcinoma, ovarian carcinoma, and lung carcinoma. Oncogenic microRNAs involved in cell cycle progression and associated human malignancies are given in Table 6.69.
- **Oncogenic microRNAs as regulators and mediators of Myc proto-oncogene:** Regulation of Myc proto-oncogenes (c-Myc, N-Myc and L-Myc) is connected to microRNAs.

Table 6.69 Oncogenic microRNAs involved in cell cycle progression and associated human malignancies

Oncogenic MicroRNAs	Target Cell Cycle Negative Regulators	Human Malignancies
MiR-132,-212	↓RB1	Pancreatic carcinoma
MiRs-17–92	↓RB2	Lung carcinoma
MiRs-195,-128a, -155,-516a-3p,-372	↓Wee1*	Skin (melanoma), pituitary adenomas
MiRs-504, 25, 30d, 125b,-214,-1285	↓p53	Breast carcinoma, lung carcinoma, colorectal carcinoma, ovarian carcinoma, hepatoblastoma, osteosarcoma, neuroblastoma
MiRs-24,-31	↓$p16^{INK4A}$	Cervical carcinoma, human diploid fibroblasts
MiRs-221,-222	↓$p27^{KIP1}$	Breast carcinoma, gastric carcinoma, prostatic carcinoma, pancreatic carcinoma, thyroid papillary carcinoma, glioblastoma multiforme, CLL
MiRs-21,-221/222 cluster,-25	↓$p57^{KIP1}$	Gastric carcinoma, pancreatic carcinoma
MiRs-520 g,-106b family,-128-2	↓p21	Breast carcinoma, gastric carcinoma, colorectal carcinoma, Barrett's esophagus, non-small cell lung carcinoma, renal cell carcinoma, prostatic carcinoma
MiRs-18a,-181a/-181b,-421	↓ATM	Breast carcinoma, colorectal carcinoma, neuroblastoma

**Wee1 is nuclear serine/threonine kinase protein, a negative regulator of G2/M checkpoint acting by phosphorylating cyclin B/CDK1 at position tyrosine 15, determining protein complex inactivation*

- Myc proto-oncogenes code for oncogenic transcription factor, that regulates transcription activity of microRNA-17-92 cluster, MiR-9, MiR15a/16-1 and MiR-34a involved in numerous cellular processes, i.e. cell cycle progression, metabolism, angiogenesis, development of malignant tumor growth, epithelial–mesenchymal transition (EMT), invasion and metastasis.
- Moreover, microRNAs regulate the expression and activity of c-Myc. N-Myc amplification induces expression of numerous microRNAs, e.g. 92, 106a, let-7b, 17-5p, 93, 99 and 221 in **neuroblastoma**. Both c-Myc and N-Myc can directly bind to the promoter of microRNA-17/92 cluster and initiates transcription. N-Myc upregulates microRNA-17/92 in neuroblastoma.

- **Oncogenic microRNAs as regulators and mediators of RAS proteins:** MicroRNAs regulate gene expression by modulating the translation of protein-coding RNAs. Their aberrant expression is linked to many human cancers.
  - Dysregulated microRNA expression can lead to RAS (H-RAS, K-RAS or N-RAS) activation irrespective of their oncogenic mutations at codons 12, 13 and 61.
  - Activation of RAS proteins is linked to many human cancers. RAS belongs to a family of membrane associated GTPases that function as intracellular switches to regulate cell growth, proliferation, survival and motility to extracellular signals by initiating multiple mitogenic signal transduction pathways and its co-expression with Myc family proteins is responsible for cell proliferation.
- **DNA damage response machinery and oncogenic microRNAs:** DNA damage response (DDR) is a network of cellular pathways that contain three major components (some with overlapping functions): sensors, signal transducers and effectors.
  - Alterations in the components of the DNA repair response machinery is associated with tumorigenesis and chemoresistance.
  - DDR requires core sensors such as ATM (ataxia-telangiectasia mutated) or ATR (ATM-RAD3-related) proteins that have ability to bind to damaged DNA and to activate TP53 tumor suppressor gene product (p53) that in turn leads to DNA repair, cell cycle arrest or apoptosis, depending on the entity of the damage.
  - Surveillance ATR and ATM proteins that monitor DNA integrity can activate cell cycle checkpoints and DNA repair response machinery in response to DNA damage to prevent the generation of potentially deleterious gene mutations.
  - ATM or ATR proteins also trigger a phosphorylation cascade that activates two kinases CHK1 and CHK2. These two kinases (CHK1 and CHK2) stimulate the expression of DNA repair enzymes known as cyclin-dependent kinase inhibitors (CDKIs). Oncogenic microRNAs downregulate the expression of ATM protein resulting in tumorigenesis and progression.
- **Apoptotic pathway and oncogenic microRNAs:** In eukaryotes, numerous microRNAs perform

basic cellular functions such as cell proliferation, differentiation and apoptosis.

- Abnormal expression of microRNAs modifies/weakens various apoptotic pathways leading to development of human cancers.
- Apoptosis is mediated by three apoptosis pathways: (a) extrinsic (death receptors anchored at the cell membrane through binding of FasL TNF and TRAIL ligands), (b) intrinsic (mitochondrial), and (c) perforin/granzyme pathways.
- Several studies revealed that some oncogenic noncoding microRNAs alter apoptotic response by downregulating proapoptotic proteins (i.e. TRAIL, BAX, BAK, PUMA, BIM, caspase-3,-7,-9), and alter their functions at different levels in both extrinsic and intrinsic pathways leading to carcinogenesis, invasion, metastasis and chemotherapy resistance.
- Caspase-3,-7,-9 are downregulated by mir-106b, MiR-363, MiR-582-5p and let-7.
- Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) involved in extrinsic pathway is downregulated by MiR-22 and MiR-222. Oncogenic microRNAs involved in apoptotic pathway and associated human malignancies are given in Table 6.70.

- **Modulation of p53 protein by oncogenic microRNAs:** TP53 tumor suppressor gene product (p53) plays a crucial role in maintaining genomic stability and tumor suppression.
  - Mutant TP53 gene gain-of-function induces epithelial–mesenchymal transition (EMT) and tumor metastasis through modulation of the microRNA's expression. Thus, microRNAs could be considered attractive targets for new chemotherapeutic agent therapies.
  - Oncogenic MiR-504 acts as a negative regulator of TP53 gene product (p53) through its binding to two sites in promoter region. Oncogenic MiR-504 reduces TP53 gene product (p53) mediated apoptosis leading to osteosarcoma and lung carcinoma. Oncogenic MiR-504 reduces p53-mediated cell cycle arrest resulting in **colon carcinoma**.
- **Oncogenic microRNAs as metastamiR:** Metastasis is a major obstacle to the efficient and successful treatment of cancer. MetastamiRs are specific family of microRNAs, that can promote or inhibit metastasis. Metastasis involves multiple steps including local invasion, intravasation in lymphatic and blood vessels, circulation, extravasation, and colonization in distant organs. Oncogenic microRNAs as pro-metastamiR in human malignancies are given in Table 6.71.
  - **MiRs-126,-141,-21:** MiRs-126,-141,-21 overexpression leads to early-stage **colorectal carcinoma** metastasis to liver.
  - **MiR-9:** MiR-9 downregulates metastasis suppressor E-cadherin that leads to enhanced metastasis of breast carcinoma.
  - **MiR-373,-520c:** Prometastatic MiR-373,-520c promote migration of nonmetastatic MCF-7 breast cancer stem cells potentially through suppression of adhesion molecule CD44. MiR-373 drives the epithelial-to-mesenchymal transition (EMT) and metastasis via the MiR-TXNIP—HIF1A TWIST signaling axis in breast carcinoma.
  - **MiR-21:** MiR-21 promotes growth, invasion and metastasis by targeting multiple tumor/metastasis suppressor genes such as TPM1, PDCD4 and maspin in metastatic breast carcinoma.

Table 6.70 Oncogenic microRNAs involved in apoptotic pathway and associated human malignancies

Oncogenic MicroRNAs	Target Pro-apoptotic Proteins	Human Malignancies
MiRs-221,-222	↓TRAIL ↓BMF ↓PUMA	Non-small cell lung carcinoma, hepatocellular carcinoma Hepatocellular carcinoma Epithelial malignant tumors
MiR-221,-222,-21,-18a,-144,-32,-216a,-217	↓PTEN	Non-small cell lung carcinoma, hepatocellular carcinoma, gastric carcinoma, colorectal carcinoma, nasopharyngeal carcinoma
MiR-886-5p	↓BAX	Cervical carcinoma
MiR-125b	↓BAK	Breast carcinoma, prostatic carcinoma
MiRs-181a, 17-5p-92,-32,-106b-25, -582-5p,-263 polycistron	↓BIM	Esophageal carcinoma, prostatic carcinoma, NHL, neuroblastoma, glioblastoma multiforme
MiR-106b-25 cluster	↓Caspase 7	Prostatic carcinoma
MiRs-582-5p,-363,-let-7a	↓Caspase 3	Glioblastoma multiforme, squamous cell carcinoma
MiRs-582-5p, 363	↓Caspase 9	Glioblastoma multiforme

Table 6.71 Oncogenic microRNAs as pro-metastamiR in human malignancies

Oncogenic MicroRNAs (Pro-metastamiR)	Mechanism	Human Malignancies
MiR-126,-141,-21	Upregulation of oncogenic MiRs	Colorectal carcinoma metastasizing to liver
MiR-9	Downregulation of metastasis suppressor E-cadherin	Breast carcinoma
MiR-373,-520c	Upregulation of MCF-7 commonly used as breast cancer cell line are CD44 cell adhesion molecule	Breast carcinoma
MiR-21	Targeting metastasis suppressor proteins (e.g. TPM1, PDCD4 and maspin)	Breast carcinoma
MiR-10b	▪ MiR-10b induced by TWIST transcription factor ▪ MiR-10b upregulation RhoC and AKT phosphorylation through targeting HOXD10 ▪ MiR-10b modulates expression of invasive factors MMP14 and uPAR via target HOXD gene	▪ Breast carcinoma ▪ Gastric carcinoma ▪ Glioblastoma multiforme
MiR-885-5p	Downregulation of SMAD4	Colorectal carcinoma metastasizing to liver

All these microRNAs metastatic biomarkers need further exploration

- **MiR-10b:** Tumor invasion and metastasis is initiated by overexpressed MiR-10b in breast carcinoma. Expression of MiR-10b is induced by the transcription factor TWIST, which binds directly to the putative promoter of MiR-10b. High level of MiR-10b expression is linked to early progression of breast carcinoma. High MiR-10b modulates invasive factors MMP14 and uPAR expression via target HOXD gene that is associated with poor prognosis in glioblastoma multiforme patients. The aberrant expression of MiR-10-mediated downregulation of HOXD10 leads to increased RhoC expression and AKT phosphorylation resulting in rapid progression of gastric carcinoma.
- **MiR-885-5p:** Recent studies revealed that MiR-885-5p is upregulated in colorectal carcinoma that leads to early metastasis to liver.

▪ **Oncogenic microRNAs and chemoresistance:** Recent studies revealed that oncogenic microRNAs are responsible for chemoresistance by interfering with apoptotic pathway or DNA-repair pathway. All these lines of evidence have highlighted a pivotal role of oncogenic microRNAs in cancer cell treatment response. Oncogenic microRNAs and chemoresistance by interfering with apoptotic pathway or DNA repair pathway are given in Table 6.72. Oncogenic microRNAs and chemoresistance by interfering with apoptotic pathway or DNA repair pathway are given in Table 6.73.

Table 6.72 Oncogenic microRNAs and chemoresistance by interfering with apoptotic pathway or DNA-repair pathway

Oncogenic MicroRNAs	Mechanism	Human Cancer	Human Cancer Chemoresistance
MiR-125b	▪ ↓Apoptotic p53 and BAK proteins ▪ ↓Apoptotic BAK protein	▪ Ewing sarcoma/primitive neuroectodermal tumor ▪ Prostatic carcinoma ▪ Breast carcinoma	▪ Vincristine, doxorubicin, etoposide ▪ Paclitaxel
MiR-21	▪ ↓hMSH2 and hMSH6, the core mismatch repair (MMR) recognition protein complex ▪ ↓MicroRNA-21 binds to PTEN 3' UTR, determining PTEN depletion	▪ Colorectal carcinoma ▪ Non-small cell lung carcinoma ▪ Cholangiocarcinoma ▪ Gastric carcinoma	▪ 5-Fluorouracil ▪ Cisplatin, doxorubicin, 5-Fluorouracil ▪ Gemcitabine ▪ Cisplatin
MiR-128-2	↓Apoptotic PTEN protein	Lung carcinoma	Cisplatin, doxorubicin, 5-fluorouracil
MiR-17-5p	↓Modulation of apoptotic PTEN signaling pathway	Ovarian carcinoma	Cisplatin
MiR-217	↓Caspases 3 and 7 activities	Breast carcinoma	Cyclophosphamide
MiR-let-7a	↓Caspases 3 activity	▪ Hepatocellular carcinoma ▪ Oral squamous cell carcinoma	Interferon-gamma (INF-γ), doxorubicin

Table 6.73 Oncogenic microRNAs and chemoresistance by interfering with apoptotic pathway or DNA-repair pathway

Oncogenic MicroRNAs	Mechanism	Human Cancer	Human Cancer Chemoresistance
↑Oncogenic microRNA-125b	▪ ↓Apoptotic p53 and BAK proteins ▪ ↓Apoptotic BAK protein	▪ Ewing sarcoma/primitive neuroectodermal tumor ▪ Prostatic carcinoma and breast carcinoma	▪ Vincristine ▪ Doxorubicin ▪ Etoposide ▪ Paclitaxel
↑Oncogenic microRNA-21	↓ hMSH2 and hMSH6, the core mismatch repair (MMR) recognition protein complex	Colorectal carcinoma	5-Fluorouracil
↑Oncogenic microRNA-128-2	↓Apoptotic PTEN protein	Lung carcinoma	▪ Cisplatin ▪ Doxorubicin ▪ 5-fluorouracil
↑Oncogenic microRNA-21	↓MicroRNA-21 binds to PTEN 3' UTR, determining PTEN depletion	▪ Non-small cell lung carcinoma ▪ Cholangiocarcinoma ▪ Gastric carcinoma	▪ Cisplatin ▪ Doxorubicin ▪ 5-Fluorouracil ▪ Gemcitabine ▪ Cisplatin
↑Oncogenic microRNA-17-5p	↓Modulation of apoptotic PTEN signaling pathway	Ovarian carcinoma	Cisplatin
↑Oncogenic microRNA-217	↓Caspases 3 and 7 activities	Breast carcinoma	Cyclophosphamide
↑Oncogenic microRNA-let-7a	↓Caspase 3 activity	Hepatocellular carcinoma and squamous cell carcinoma	▪ Interferon-gamma (INF-γ) ▪ Doxorubicin

- **MiR-125b:** MiR-125b downregulates apoptotic pathway mediators (i.e. p53 and BAK protein) that leads to chemoresistance to vincristine, doxorubicin and etoposide in Ewing sarcoma/primitive neuroectodermal tumor. MiR-125b also downregulates apoptotic mediator (i.e. BAK protein) that leads to chemoresistance to paclitaxel in prostatic carcinoma and breast carcinoma.
- **MiR-21:** MiR-21 increases chemoresistance to 5-fluorouracil in colorectal carcinoma by downregulation of hMSH2 and hMSH6, the core mismatch repair (MMR) recognition protein complex.
- **MiR-128-2:** MiR-128-2 increases chemoresistance to cisplatin, doxorubicin and 5-fluorouracil in **lung carcinoma** by downregulation of PTEN. PTEN plays a pivotal role in the apoptotic pathway and its inactivation often determines chemoresistance.
- **MiR-21:** Recent study revealed that MiR-21 binds to PTEN 3' UTR, determining PTEN depletion and inducing chemoresistance in non-small cell lung carcinoma. MiR-21 also increases chemoresistance to gemcitabine in cholangiocarcinoma and to cisplatin in gastric carcinoma, through modulation of PTEN and AKT proteins.
- **MiR-17-5p:** MiR-17-5p induces chemoresistance in **ovarian carcinoma** by modulation of PTEN signaling.
- **MiR-217:** MiR-217 increases chemoresistance in **breast carcinoma** by inhibiting the activities of caspases 3 and 7.
- **MiR-let-7a:** MiR-let-7a induces chemoresistance to IFN-γ and doxorubicin in **hepatocellular carcinoma** and squamous cell carcinoma by downregulating caspase-3 activity.

Tumor Suppressor MircoRNAs and Cancer-inhibiting Mechanisms

MicroRNAs regulate the expression of their target genes post-transcriptionally by degrading mRNA transcripts or by inhibiting mRNA translation. Normally, microRNAs regulate of cell cycle, cell proliferation, apoptosis, energy metabolism and immune response. MicroRNAs have been identified to act as tumor suppressors or oncogenes based on their modulating effect on the expression of their target genes. Upregulation of oncogenic microRNAs blocks tumor suppressor genes resulting in development of malignant tumors. In contrast, downregulation of tumor suppressor function increases translation of oncogenes. Recent studies revealed that several microRNAs exhibiting tumor suppressor properties regulate the interaction between CSCs and their surrounding cells within tumor microenvironment by targeting genes either by oncogenes or tumor suppressor genes under certain circumstances. Carcinogenesis is a stepwise accumulation of genetic alterations (e.g. gene

amplification, aberrant oncoproteins as well as biallelic loss or inactivation of tumor suppressor genes) leading to unrestricted proliferation of CSCs, angiogenesis, invasion, epithelial-to-mesenchymal transition (EMT) and metastasis to distant organ(s).

- **Tumor suppressor microRNAs downregulation in CSCs and targets:** Tumor suppressor microRNAs (e.g. let-7, -15/16, MiR-34 cluster and MiR-200) prevent initiation of carcinogenesis through downregulation of target gene expression encoding oncoproteins. Tumor suppressor microRNAs promote apoptosis and inhibit cell proliferation, epithelial–mesenchymal transition (EMT), and oncogene expression.
- **Tumor suppressor microRNAs promote apoptosis of CSCs:** MiRs-15/16 cluster function as tumor suppressor microRNAs inhibits cell proliferation, promotes apoptosis of CSCs and suppresses tumorigenesis. MiRs-15/16 cluster is most often deleted or downregulated in various malignant tumors such as chronic, lymphocytic leukemia (CLL), prostatic carcinoma, colorectal carcinoma, melanoma, malignant pleural mesothelioma and pituitary adenomas.
  - The main target of MiRs-15/16 cluster is BCL-2 that triggers apoptosis by suppressing the expression of BCL-2 and also targets CCND1, MCL1, WNT3A, CDC2, ETS and Jun involved in genomic instability, unrestricted CSC proliferation, tumor angiogenesis, apoptosis resistance, immunologic escape, invasion and metastasis.
  - Recent gene profiling studies of patients with chronic lymphocytic leukemia [B cell with different immunoglobulin heavy chain variable (IgVH)] has revealed increased expression of ROR1, an oncoembryonic surface receptor tyrosine kinase protein for WNT5a on leukemic cells, that promote leukemic cell proliferation and survival. ROR1 serves as a putative target for therapy.
- **Tumor suppressor microRNAs inhibit epithelial–mesenchymal transition (EMT):** Epithelial–mesenchymal transition (EMT) is associated with reduction in the epithelial-like features of CSCs and acquisition of mesenchymal-like features that are essential to mediate effective invasion and migration.
  - EMT mechanism is regulated by transcription factors (i.e. SNAIL, ZEB, TWIST), which are repressed by some tumor suppressor MiRs (i.e. MiR-29b, MiR-30a, MiR-200 family members, MiR-205) leading to reversal of EMT and CSCs invasion. MiR-29b and MiR-30a repress translation of SNAIL-1 transcription factor.
  - Members of the MiR-200 family and MiR-205 repress translation of ZEB1 and ZEB2 transcription factors. These tumor suppressor MiRs are downregulated in various cancers, which increase the translation of transcription factors and play a crucial role in the regulation of EMT mechanism.
- **Tumor suppressor microRNAs inhibit CSC proliferation:** Tumor suppressor MiRs inhibit CSC proliferation and carcinogenesis. It is well known that Wnt/β-catenin signaling pathway drives carcinogenesis and progression of malignant tumor growth.
  - Wnt/β-catenin signaling pathway gives rise to epithelial–mesenchymal transition, which characterized by nuclear translocation of β-catenin and E-cadherin suppression.
  - MiR-340 and MiR-200 suppress Wnt/β-catenin signaling pathway by targeting transcription factor and binding to β-catenin mRNA and thus inhibits CSC proliferation. Downregulation of these MiRs is linked to human cancers.
- **Tumor suppressor microRNAs inhibit oncogene expression:** MiR-34 family (MiRs-34a,-34b,-34c) is a master regulator of tumor suppressor. Downregulation of MiR-34 family has been observed in breast carcinoma, colorectal carcinoma, lung carcinoma and prostatic carcinoma. MiR-34 inhibits numerous CSC types and suggests that it function at the core of carcinogenesis processes shared among CSCs (i.e. cell cycle, cell proliferation, antiapoptosis, cancer stemness, metastasis, oncogenic transcription and chemoresistance). Aberrant expression of MiR-34 is linked to many cancers.

MicroRNAs and Epigenetics in Cancer Diagnosis

Early cancer detection is the major step towards successful cancer treatment. Targeted sequencing is promising approach that works by detecting abnormal methylation patterns associated with cancer development. The detection of promoter hypermethylation has gained considerable attention in cancer diagnosis.

- Histone octamer modification is another potential epigenetic biomarker used in cancer diagnosis. Detection of abnormal post-translational modifications is an emerging approach for cancer diagnosis and outcome predictions. In lung carcinoma, specific histone octamer modifications, such as lower H3 and H3 methylation levels, have been found to associated with poor prognosis and mortality.
- Aberration expression of microRNAs are linked to many human cancers and therefore can be excellent diagnostic/prognostic markers in cancers. They may be targeted by therapeutic agents. MicroRNA methylation is novel epigenetic biomarker in different cancers such as lung carcinoma. Hypermethylation of microRNA-124 and microRNA-219 has been found to be associated with poor prognosis in lung carcinoma.

Table 6.74 MicroRNAs targeting the hallmarks of cancer

Noncoding MicroRNAs Therapeutics	Targets Hallmark of Cancer
↑MiR-21, ↑MiR-17-92, ↓MiR-34	Reduction of unlimited stem cell proliferation
↑MiR-9, ↑MiR-10b, ↑MiR-21, ↑MiR-103/107, ↓MiR-200	Limit tissue invasion and metastasis
↑MiR-17-92, ↑MiR-210	Limit sustained angiogenesis
↑MiR-21, ↓MiR-15-16, ↓MiR-34	Antagonize evasion of apoptosis

MicroRNAs and Epigenetic Therapy in Cancer Treatment and Future Challenge

Combined epigenetic therapies have shown promising outcomes in treating cancer patients, as tumorigenesis is associated with many epigenetic changes. Specific genetic and epigenetic changes associated with tumorigenesis should be assessed in each patient to get the highest benefits from epigenetic therapy.

- In urinary bladder carcinoma, the expression of tumor suppressor genes is inhibited by the polycomb repressive complex or *de novo* DNA methylation, which can be treated with inhibitors of histone methyltransferase enzyme. Similarly, *de novo* methylation can be blocked by inhibitors of DNA methylation.
- MicroRNAs may function as either oncogenes or tumor suppressors.
  - Oncogenic ncRNA-155 and tumor suppressor ncRNA-let may be therapeutic targets in cancer. Thus, ncRNAs could augment current cancer therapies.
  - RNA sequencing is an approach to transcriptome profiling that uses deep-sequencing molecular diagnostics to detect accurately quantity RNA molecules originating from genome by quantitative real-time polymerase chain reaction (RT-PCR) at a given moment in time. MicroRNAs targeting the hallmarks of cancer are given in Table 6.74.

POLYMORPHIC MICROBIOMES AND CANCER

Human body is colonized by a vast array of micro-organisms—microbiota that reside on and inside body surface epithelial barriers (skin, gastrointestinal tract, respiratory tract, genitourinary tract), that influence cancer susceptibility, in part, via their tremendous metabolic capacity, and their profound influence on immune cell function. Their profound influence to health and disease is now appreciated. Some of polymorphic microbiomes can exert deleterious effects on cancer development, progression, and favor the acquisition of hallmark capabilities. Non-antibiotics gut microbiome targeted therapeutic drugs, i.e. THIP hydrochloride, methenamine and mesna are used in clinical oncology.

- The microbiota most often exhibits commensalism with the host; however, when intestinal ecology undergoes disruption (dysbiosis), certain commensal bacteria (i.e. *Clostridium difficile* or vancomycin resistant enterococci) may expand and induce carcinogenesis. Metagenomic sequencing analysis revealed that large number of human malignancies have altered composition of commensal microbiota (**dysbiosis**).
- Microbiota are abundant in the lower intestinal tract. The abundant microbiota present on the gastrointestinal mucosa, translocates bacterial products and metabolites, that adversely affect local mucosal homeostasis functions and immunity, and systemic physiologic hemostasis functions.
- Altered composition of gut commensal microbiota (dysbiosis) induce pathologic local effects (i.e. inflammatory bowel disease, *Helicobacter pylori*-related chronic atrophic gastritis and gastric adenocarcinoma, *Fusobacterium* species-related colorectal carcinoma, *Salmonella enterica* or *Salmonella typhi*-related gallbladder carcinoma in 15–20% of cancer cases) and systemic effects (MALT lymphoma).
- Microbiota can alter cancer susceptibility and progression by different mechanisms, i.e. modulating inflammation, inducing DNA damage, and producing metabolites involved in the oncogenesis or tumor suppression. In addition to its association with carcinogenesis, metabolic active tumor microbiota can alter chemical structure of some chemotherapeutic agents, and modulate the efficacy and toxicity of cancer chemotherapy.
- Microbiomes selectively target malignant tumors that have rich vascular networks and chemotactic magnetism, which enhance cancer progression and chemoresistance. Anaerobic and/or facultative bacteria can survive in hypoxic tumor environments. Intratumoral gammaproteobacteria in pancreatic carcinoma express the enzyme cytidine deaminase (**CDD**), whose long form (CDDL) has been shown to metabolize gemcitabine into its inactive CDDL resulting in chemoresistance used in treating gastrointestinal malignancies.
- Non-antibiotic drugs such as THIP hydrochloride, methenamine and mesna are administered to modulate gut microbiome. Drug–microbiome interaction

has laid the foundation for a new era of more precise gut microbiome through drug repurposing, aimed at targeting specific dysbiotic events.

Helicobacter pylori-induced GIT Cancers

Helicobacter pylori is a gram-negative microaerophilic bacterium and the most crucial etiologic agent for gastric adenocarcinoma that is involved in 90% of all gastric malignancies.

- *Helicobacter pylori* contains virulence factors such as cagA (cytotoxin-associated gene A), island (cag PAI), and vacA (vacuolated cytotoxin A), which efficiently trigger atrophic gastritis (associated with hypochlorhydria/achlorhydria), which progresses to intestinal metaplasia, dysplasia, early gastric carcinoma, and advanced intestinal-type gastric adenocarcinoma.
- Environmental factors such as high-salt and tobacco smoking may also play a role in gastric carcinogenesis.

Pathophysiology of Helicobacter pylori-induced Gastrointestinal Disorders

The cag PAI encodes a type IV secretion system (T4SS) that injects at least 18 proteins including cagA pore-forming protein into host cells. CagA binds to epithelium via interaction with protein-tyrosine phosphatases induces morphologic changes of the cell, and influences various intracellular signal transduction pathways such as the RAS/mitogen-activated protein kinase/extracellular signal-regulated tyrosine kinase pathway, nuclear factor κB (NK-κB) pathway and β-catenin pathway resulting in malignant phenotype.

- The nature of the inflammatory response to *Helicobacter pylori* is also determined by proinflammatory IL-1 gene cluster polymorphisms in several host genes encoding cytokines and cytokine receptors, which induce intense inflammatory response leading to hypochlorhydria/achlorhydria and high risk for gastric carcinoma.
- The clinical manifestations of *Helicobacter pylori* include gastric chronic gastritis, duodenal ulcer (10–15%), gastric ulcer (1–3%), gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma (MALToma, 01%).

Prevention of Gastrointestinal Disorders via Helicobacter pylori Eradication

Peptic ulcer disease, MALToma and endoscopic treatment of early gastric carcinoma are well-known indications of *Helicobacter pylori* eradication. Prevention via *Helicobacter pylori* eradication and controlling environment factors such as diet and tobacco smoking is an important strategy to inhibit *Helicobacter pylori*-induced gastric carcinogenesis.

Gut Microbiome-induced Colorectal Carcinoma

The gut microbiota is critical in maintenance of host homeostasis that provides essential health benefits to its host by converting carbohydrates into short-chain fatty acids, which are an energy source.

- Goblet cells secrete mucus that serves as a physical barrier keeping gut bacteria separated from epithelial cells.
- Paneth cells eliminate opportunistic pathogens by the synthesizing antimicrobial peptides (AMPs).
- Microfold cells are present in the gut-associated lymphoid tissue of the Peyer's patches and can translocate B cells and T cells to the gut lumen in order to eliminate bacteria. Microfold cells can also present bacterial antigens to dendritic cells and elicit an IgA-specific immune response.

Dysbiosis of Gut Microbiota: Mechanism

Alteration in the gut microbiota can result from exposure to various environmental factors, including diet, toxins, antibiotics and pathogens. When the gut microbiota hemostasis is disrupted, dysbiosis occurs. Three types of dysbiosis include: (a) loss of beneficial bacteria, (b) overgrowth of potentially pathogenic bacteria and (c) loss of local microbiota bactericidal diversity. The environmental factors reduce oxygen consumption in the colonic epithelium, which drives dysbiosis by increasing the diffusion of oxygen into the intestinal lumen. Dysbiosis of the gut microbiota has been linked to inflammatory bowel disease (IBH), irritable bowel syndrome, celiac disease and colorectal cancer (CRC).

Dysbiosis of Gut Microbiota and Colorectal Carcinogenesis

Recently several studies revealed that gut microbiota can alter colorectal carcinoma susceptibility and progression by modulating mechanisms, i.e. inflammation and DNA damage. CRC is caused by combination of environmental, genetic and epigenetic factors. Several mechanisms have been identified how bacteria induce cellular transformation and promote tumor progression.

- Unlike viruses, which express consequently active viral mimics of cellular proto-oncogenes, tumor initiation and progression associated with dysbiosis of gut microbiota is a multifactorial event arises following '**multiple hits**' involving genes. Genetic heterogenicity in the gut microbiota as well as the host, in addition to environmental factors, determines colorectal carcinoma prevalence and severity.
- Dysbiosis of gut microbiota (PAMPs—pathogen-associated molecular patterns) activates the innate

immune system through pattern recognition receptors (PPRs) resulting in an inflammatory response in intestinal epithelial cells. Toxic metabolites or genotoxins (Colibactin, cytolethal distending toxin, hydroxyl radicals) produced by altered gut microbiota, damage DNA in intestinal epithelial cells resulting in initiation and progression of malignant tumor.

- *Fusobacterium nucleatum* plays important role in colorectal carcinogenesis through toll-like receptor 2 (TLR2)/toll-like receptor 4 (TLR4) signaling and microRNA-21 expression on cancer stem cells. In addition, *Fusobacterium nucleatum* increases colorectal carcinoma recurrence along with chemoresistance by mediating a molecular network of microRNA-18a and autophagy. Intratumoral bacteria further modulate the immune system.
- Dysregulated microbiota can interact directly with intestinal epithelial cells and immune cells, and can activate colorectal carcinoma-related signaling pathways such as phosphatidylinositide 3-kinase (PI3K)/AKT/mTOR signaling pathway and Wnt/β-catenin signaling pathway involved in unrestricted CSC proliferation and survival.
- Additionally, some genotoxins can also enhance signal transduction and activation of transcription 3 (STAT3) signaling pathway, which leads to cell proliferation, and T cell activation and can thereby elicit a Th17 cell mediated immune response. Th17 cells are proinflammatory CD4+ helper cells that secrete IL-17A, IL-17F, IL-21, IL-22 and provide immunity to several extracellular pathogens but have been described as uniquely pathogenic in multiple inflammatory diseases.

Dysbiosis of Host Gut Microbiota and Therapeutic Modalities of Colorectal Carcinoma

The gut microbiota has also been implicated in the metabolism of conventional chemotherapeutic agents as well as in the modulation of therapy responses and targeted immunotherapy. These findings suggest that the efficacy of a given anti-cancer therapy depends on the composition of the host' gut microbiota and may therefore vary from patient to patient.

- *Fusobacterium nucleatum* outer membrane FAP2 protein prevents the activation of natural killer (NK) cells protecting colorectal adenocarcinoma cell lines from NK cell antitumor immunity.
- Cancer treatment can be improved by manipulating microbiota by incorporating probiotics as adjuvants for checkpoint immunotherapy or designing small molecules that target microbial enzymes.

CARCINOGENIC AGENTS: CELLULAR INTERACTION AND CARCINOGENESIS

Cellular proliferation is tightly regulated by two sets of opposite functioning genes, i.e. proto-oncogenes (growth-promoting genes that stimulate cell division) and tumor suppressor genes (growth suppressor genes that halt cell division), which participate in cell growth and cell proliferation during G1, S, G2, M and G0 phases of cell cycle.

- **Carcinogenesis:** Carcinogenesis is a multistep molecular process driven by genetic and epigenetic alterations of multiple genes in the target cells involving activation of proto-oncogene (oncogene tumor promoting) and inactivation/biallelic loss of tumor suppressor (growth-inhibiting) genes, apoptosis regulatory genes, and DNA repair genes by which normal cell is transformed to CSC either spontaneously or induced by environmental mutagenic agents leading to unrestricted rapid proliferation of clonal somatic CSCs, which are genetically different from each other but part of the same malignant tumor that is known as the genetic heterogenicity. The clonal CSCs become resistant to chemotherapy, and will survive and persist. Multistep molecular carcinogenesis is shown in Fig. 6.94.
- **Carcinogen-linked malignancies:** Carcinogens are capable of causing human cancers, which may occur spontaneously or induced by environmental mutagenic agents, which include: (a) chemical carcinogens—asbestos, components of tobacco smoke, alcohol consumption, aflatoxin B1 synthesized by *Aspergillus flavus* (food contaminant in stored grains) and arsenic in drinking water contaminant, (b) physical carcinogens—ultraviolet rays and ionizing radiation, and (c) biological carcinogens—viruses, i.e. HPV, Epstein-Barr virus, HBV, HTLV-1, bacteria—*Helicobacter pylori, Borrelia burgdorferi, Campylobacter jejuni* and *Chlamydia psitacci,* parasites—*Schistosoma haematobium, Clonorchis sinensis* and *Opisthorchis viverrini*. Latent period is the time period between the exposure of the cell to the initiator carcinogenic agent till the malignant tumor becomes clinically detectable. However, some of these etiological factors can act both initiator and promoter in the process of carcinogenesis. There is

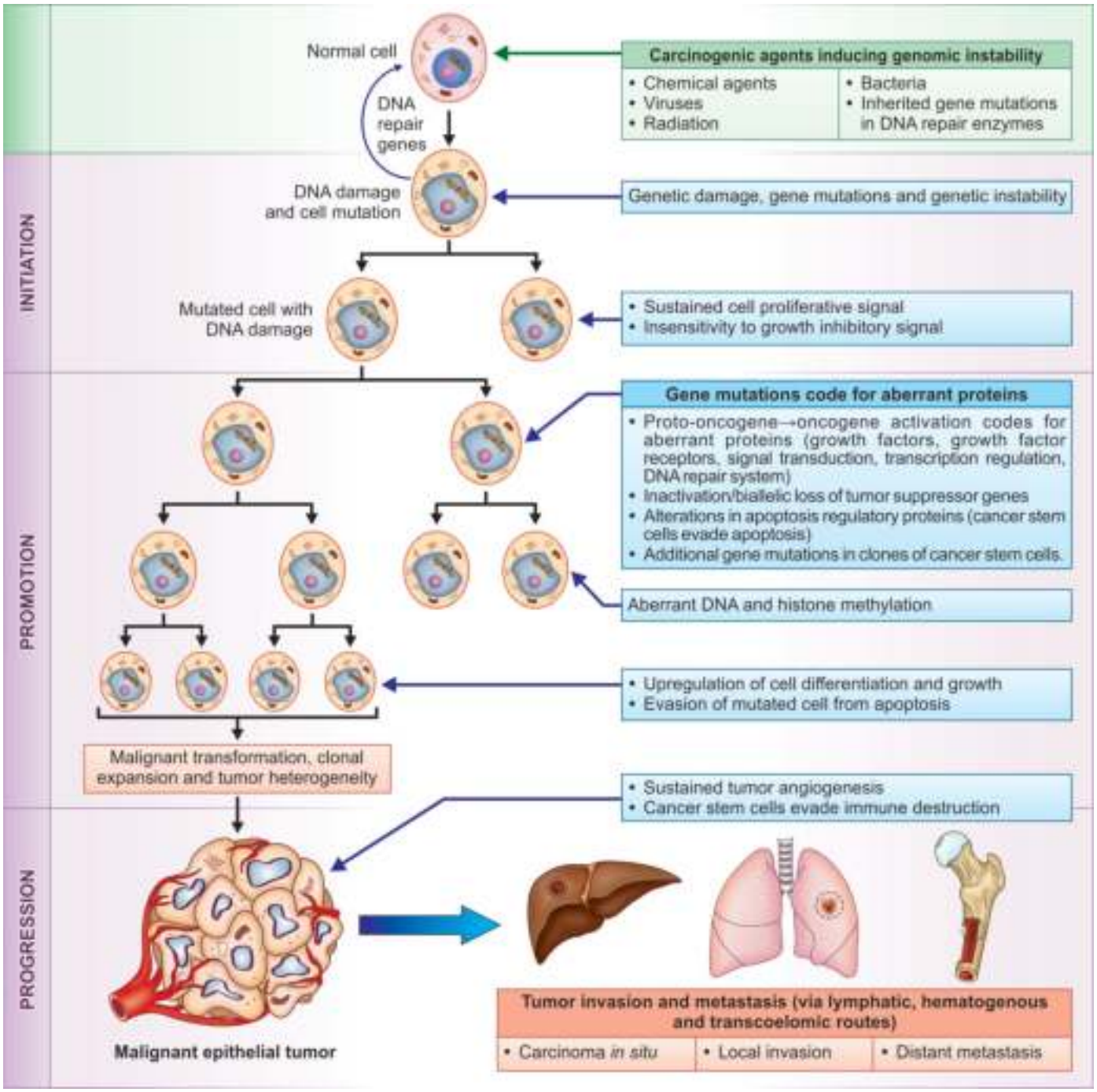


Fig. 6.94: Multistep molecular carcinogenesis. Schematic representation shows inherited/acquired gene mutations transforming normal cell to cancer cell by uncontrolled monoclonal proliferation and decreased apoptosis. Tumor progression occurs by additional gene mutations, angiogenesis, escape from host immune response. Cancer cells invade and metastasize to distant organs.

a normal history from metaplasia to dysplasia to invasive malignant neoplasm. This is best evidence in development of malignant neoplasms in uterine cervix and respiratory tract.

- **Occupational and air pollutants:** Environmental air pollutants such as arsenic, asbestos, polyvinyl chloride, other industrial emissions as well as vehicle exhaust have carcinogenic properties. In certain occupations, workers exposed to asbestos are at high-risk for lung carcinoma and mesothelioma. Workers involved in the production of aniline dyes, rubber, paint and β-naphthylamine are at high-risk for urinary bladder carcinoma.
- **Cigarette smoking:** Cigarette smoke contains carcinogens, which cause gene mutations. Risk of development of lung carcinoma correlates with

the duration of cigarette smoking and number of cigarettes smoked per day. Researches also showed that a person who stops cigarette smoking has lower risk for development of lung carcinoma. Inhalation of 'second hand cigarette smoke' (passive tobacco smoking in nonsmokers) also increases risk for lung carcinoma and oral squamous cell carcinoma.

- **Alcohol consumption:** Excessive alcohol consumption is linked to cirrhosis, a precursor of hepatocellular carcinoma, breast carcinoma and colorectal carcinoma. Excessive alcohol consumption and cigarette smoking synergistically increase the incidence of cancers of the mouth, esophagus and larynx. It is likely that alcohol acts as a solvent for the carcinogenic substances in cigarette smoke, thus enhancing its absorption.
- **Sexual factors:** The early age of first intercourse and women with multiple sexual partners is positively correlated with risk of cervical carcinoma. The suspected mechanism of developing cervical carcinoma involves transmission of human papillomavirus (HPV 16, 18). Steroid hormones, i.e. estrogen, progesterone and testosterone have been implicated as promoters of breast carcinoma, endometrial carcinoma and prostatic carcinoma.
- **Ultraviolet and ionizing radiation:** Exposure to solar ultraviolet radiation induces TP53 tumor suppressor gene mutation, that induces pyrimidine dimers in DNA formation and promotes development of skin cancers (e.g. squamous cell carcinoma, basal cell carcinoma and melanoma). Sunlight also releases tumor necrosis factor-α (TNF-α) in exposed skin, possibly diminishing immune response. Ionizing radiation such as X-rays is linked to developing acute leukemia, multiple myeloma and cancers of thyroid, gastric region, colon, lung, urinary tract. **Ionizing radiation** causes DNA damage via two mechanisms: (a) direct mechanism—direct toxicity and DNA breakage and subsequent cellular death, and (b) indirect mechanism—based on the ionizing radiation-induced free radicals with further fragmentation of the DNA or ionization of water or other molecules within the cells. The damaged tissue and blood vessels are replaced by fibroblasts that are unable to synthesize collagen fibers. Low doses of radiation can cause DNA mutations and chromosomal abnormalities.
- **Dietary factors:** Numerous aspects of diet are linked to various human cancers resulting from obesity, high consumption of smoked foods and salted fish or meat containing traces of polycyclic aromatic hydrocarbons and foods containing nitrates and nitrites, diet low in fibers, and naturally occurring carcinogen such as aflatoxin B1 produced by *Aspergillus flavus* in stored food crops. Aflatoxin B1 produced by *Aspergillus flavus* is linked to hepatocellular carcinoma. High-fat diet-related obesity is linked to cancers of colon, breast, kidney, esophagus, endometrium and gallbladder. Adequate consumption of fruits and vegetables probably lower the risk of developing gastrointestinal tract carcinomas.
- **Infectious agents:** About 20% of human cancers are attributable to biological pathogens (e.g. viruses, bacteria and fungi). Pathogenetic infections result in deregulation of gene expression both by genetic and epigenetic mechanisms and eventually result in transformation of normal cell to CSC and malignant phenotype. Another characteristic of pathogen-induced human cancers is the occurrence of chronic inflammation as a result of activation of innate and adaptive immune system. Human papillomavirus (HPV 16, 18) is implicated in cervical carcinoma. Epstein-Barr virus (EBV) is linked in African Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's disease. Hepatitis B virus (HBV) and hepatitis C virus (HCV) are implicated in development of hepatocellular carcinoma. Human T cell leukemia virus type 1 (HTLV-1) is linked to T cell leukemia/lymphoma. Infection with human immunodeficiency virus (HIV) increases risk of developing Kaposi sarcoma. Microbial carcinogenic agents associated human cancers are given in Table 6.75.

- **Genetic and epigenetic alterations and cancers:** Multiple genetic alterations are required before transformation of a normal cell to CSC. In the early studies on carcinogenesis, it has been observed that a long latent period could elapse from exposure to carcinogen to the development of malignant phenotype.
  - Any change in the DNA sequence can alter the genetic code and therefore may alter synthesis of the protein that it encodes. DNA sequence of a CSC genome generally acquires molecular alterations such as multiple cancer driving mutations (oncogenes and inactivation/biallelic loss of tumor suppressor genes), substitutions, insertions, deletions of small or large fragments of DNA, gene amplification, gene rearrangements and post-translational modifications. These genetic alterations modulate gene expression during cancer progression. DNA methylation and demethylation are also common occurrence in cancer and leads to altered gene expression. Whole genomic sequencing analysis reveals recurrent somatic mutations in various epigenic regulators.

Table 6.75 Microbial carcinogenic agents associated human cancers

Cancer	Associated Pathogens
Burkitt's lymphoma	Epstein-Barr virus
Cervical carcinoma	Human papillomavirus (16, 18)
Cholangiocarcinoma	*Clonorchis sinensis* (liver fluke), *Opisthorchis viverrini, Fasciola hepatica, Salmonella typhi*
Colorectal carcinoma	*Streptococcus bovis*, John Cunningham virus
Cutaneous MALT lymphoma	*Borrelia burgdorferi*
GIT non-Hodgkin's lymphoma	*Borrelia burgdorferi*
Gastric carcinoma (distal half of stomach), gastric MALT lymphoma	*Helicobacter pylori*
Gliomas (astrocytomas)	John Cunningham virus
Hepatocellular carcinoma	HBV, HCV, *Clonorchis sinensis* (liver fluke), *Schistosoma japonicum*, aflatoxin B1 (*Aspergillus flavus*)
Hodgkin's disease	Epstein-Barr virus (EBV)
Kaposi sarcoma	Human herpesvirus 8 (HHV8)
Merkel cell carcinoma	Merkel cell polyomavirus
Malignant mesothelioma	Simian virus 40 (SV40), oncogenic DNA virus
Non-Hodgkin's lymphoma	Simian virus 40 (SV40), oncogenic DNA virus
Orbital adnexal lymphoma	Orbital adnexal lymphoma
Primary brain tumors	Simian virus 40 (SV40), oncogenic DNA virus
Prostatic carcinoma	Xenotropic murine leukemia virus
T cell acute lymphoblastic leukemia	Human T-lymphotropic virus 1
Urinary bladder carcinoma	*Schistosoma haematobium*

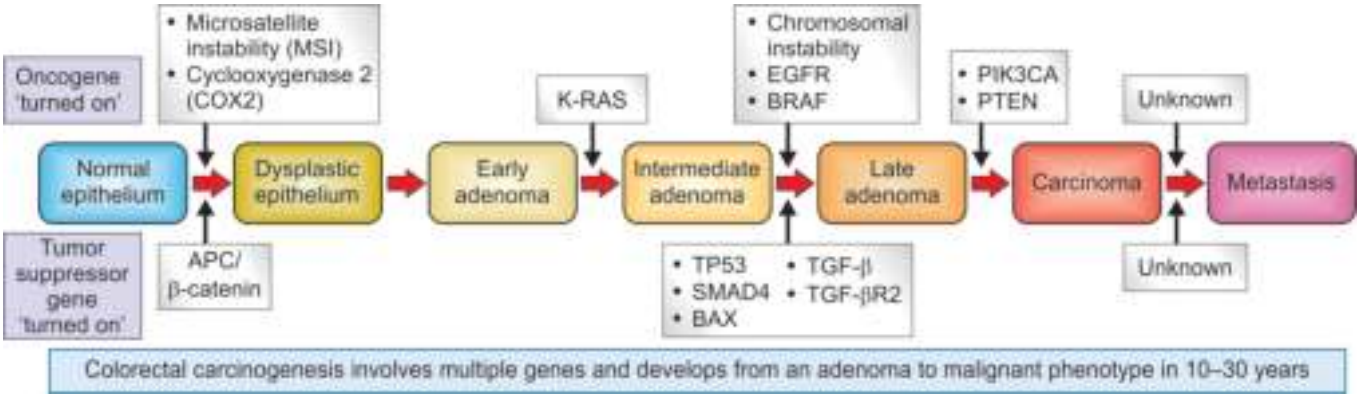


Fig. 6.95: Molecular carcinogenesis revealing adenoma–colorectal carcinoma sequence. It is postulated by Knudson's hypothesis that losses of normal copy of tumor suppressor APC gene occurs early in life. This is the first hit. Loss of intact copy of APC gene follows (second hit). Other gene mutations include K-RAS, losses at 18q21 involving SMAD2 and SMAD4 and inactivation/biallelic loss of tumor suppressor TP53 gene. It leads to the emergence of colorectal carcinoma and metastases, in which additional gene mutations occur.

Multistep carcinogenesis showing morphologic and molecular changes in the adenoma–colorectal carcinoma sequence is shown in Fig. 6.95. Molecular carcinogenesis revealing development of pancreatic carcinoma is shown in Fig. 6.96.

- Epigenetics can be defined as heritable changes in gene expression that are not accompanied by changes in DNA sequence. Epigenetic regulation of gene expression is mediated by three main mechanisms: (a) DNA methylation, (b) histone

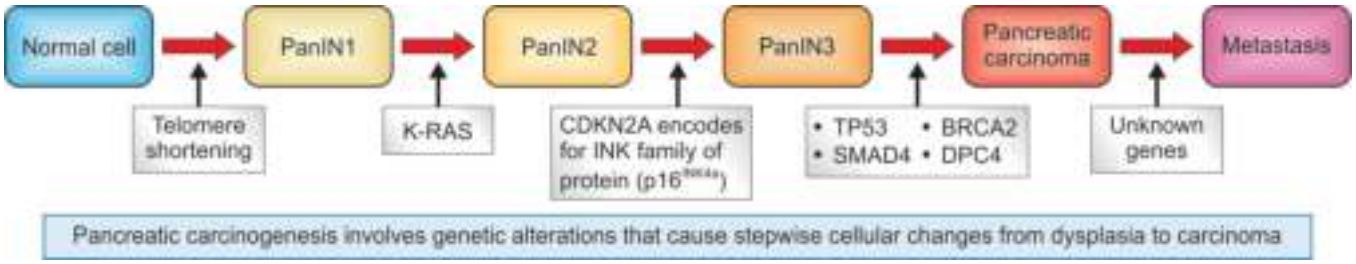


Fig. 6.96: Molecular carcinogenesis revealing development of pancreatic carcinoma. It demonstrates genomic and histopathologic steps from the initiating gene mutation in the founder cell to metastasis formation. It has been convincingly shown that the genomic landscape of solid tumors such as pancreatic carcinoma requires the accumulation of many genetic events, a process that requires decades to complete.

octamer modifications, and (c) microRNAs. Epigenetic alterations result in remodeling of DNA repair genes, which cause DNA repair system failure. Epigenetic alterations lead to increase in permanent DNA damage and a considerable increase in somatic mutations and stable phenotype malignant phenotype.

- Conventional histology is unable to detect genomic changes, and biochemical alterations associated with tumor promotion and progression. Ancillary technologies such as immunohistochemistry and molecular genetics are employed to identify products of activated proto-oncogenes (oncogenes), which offer promise in distinguishing the various stages of progression in the evolution from benign to malignant tumors.

CHEMICAL CARCINOGENESIS

Chemical carcinogenic agents are considered to be the key etiological factor of human malignancies by inducing cellular genetic damage causing DNA abnormalities in somatic cells that alter cell growth, unrestricted proliferation of clonal CSCs and survival. Application of an initiator or a promoter chemical carcinogenic agent alone is not sufficient to induce carcinogenesis. Both initiator and promoter must act sequentially to induce carcinogenesis. The interval between tumor initiation and promotion can be long or short. Tumor initiation occurs due to permanent gene mutations in the target cell. Multiple genetic alterations are required before transformation of a normal cell to CSC. In the early studies on carcinogenesis, it has been observed that a long latent period could elapse from exposure to carcinogen to the development of malignant tumor growth.

- **Chemical carcinogenic agents—classification:** Chemical carcinogenic agents can be classified into several groups—direct and indirect, which include: (a) genotoxic chemical carcinogenic agents cause direct damage to DNA by forming chemical DNA adducts that are prone to damage in DNA replication or resistant to DNA repair mechanisms, (b) mitogenic chemical carcinogenic agents act as mitogen factor that binds to receptors or in cells and stimulate mitosis by activation of receptor tyrosine kinase activity without causing direct DNA damage, and (c) cytotoxic chemical carcinogenic agents cause tissue damage leading to hyperplasia and tissue regeneration. Complete (direct acting) carcinogenic agents can carry out both tumor initiation and promotion functions (e.g. ultraviolet rays, ionizing radiation, tobacco smoke).
  - **Exposure to complete chemical carcinogenic agents:** Exposure to a complete chemical carcinogen alone is sufficient to induce carcinogenesis. Procarcinogens are metabolized to ultimate carcinogen to cause cancer. Hormones can only promote but unable to initiate carcinogenesis. Cocarcinogens cause cancer by acting with another chemical carcinogenic agents. Germline mutation in proto-oncogene (oncogene) or tumor suppressor gene is linked to numerous human cancers.
  - **DNA-adduct formation:** The principal reactive products of the nitrosamines and similar alkylating agents with DNA are N^7 and O^6 guanine derivatives, which bind and react with DNA nucleotide base pairs, RNA and proteins resulting in the formation of DNA adducts with these cellular components. If DNA adducts escape DNA repair mechanisms and persist, they may lead to miscoding, resulting in permanent mutations.
  - **Consequences of DNA-adduct formation:** Polycyclic aromatic hydrocarbon double-helical DNA-adduct formation occurs by frameshift mutation during DNA replication. Alkylated nucleotide base pairs in DNA can mispair with the wrong nucleotide base pairs during DNA replication. There are loss of nucleotide bases due to induction of instability in the glycosidic bond between the purine base and deoxyribose. Some chemical carcinogenic agents cause

conformational transition of DNA from its usual double-helical B form to a Z-DNA form.

- **Chemical carcinogenic agents—metabolism:** Carcinogenic agents may be absorbed via oral, inhalation, cutaneous and injection routes and dispersed among human tissues. The absorption lies on the physiochemical properties of chemical carcinogens through active or passive transport mechanisms, which undergo metabolic conversion through various enzymatic pathways, with differences in saturation levels. Metabolic bioactivation, detoxification and genotoxic property of chemical carcinogens play an important role in carcinogenesis.
  - Metabolic bioactivation of chemical carcinogenic agents and production of reactive electrophilic intermediates leads to the covalent binding to cellular macromolecules such as DNA, RNA and proteins. DNA adducts can be generated from products of lipid peroxidation.
  - Such metabolic conversion by enzymatic pathways may detoxify noxious chemical carcinogenic agents by phase I and phase II enzymes: (a) oxidation, reduction and hydrolytic reactions involve phase I enzymes such as cytochrome P450 dependent monooxygenases and epoxide hydrolases, which enhance the aqueous solubility and causing increased excretion, and (b) conjugation reactions involve phase II enzymes such as glutathione (GSH) transferases, UDP-glucuronosyltransferases and sulfotransferases, which are not only protective but also activate chemical carcinogenic agents.
- **Chemical carcinogenesis:** Chemical carcinogenesis occurs due to biotransformation failure, initiation (covalent binding to DNA), gene mutation, transformation of normal cell to CSC, unrestricted CSC proliferation, tumorigenesis, epithelial–mesenchymal transition (EMT), invasion and metastasis.
  - Numerous cellular and molecular events cause transformation of normal cell to malignant neoplastic cells, i.e. cancer stem cells in the process of carcinogenesis. It has been observed that endogenous molecular signaling pathways could instigate gene mutations in target genes with the support of reactive oxygen species (ROS), which induce to DNA damage.
  - On exposure to a chemical carcinogenic agent, body makes an attempt to eliminate it through a process called biotransformation by making it more water-soluble. When biotransformation fails, most chemical carcinogens require metabolic conversion to become ultimate potent carcinogens.
  - Potent (complete) chemical carcinogenic agents possess the capability of both initiation and promotion of carcinogenesis, which possess electron deficient (electrophilic) molecules, that interact and form covalent or noncovalent bonds with cellular electron-rich DNA, RNA and proteins of human tissues, resulting in alterations in the nucleotide base pairs in DNA and thus impairing DNA replication and transcription.
  - Partial (incomplete) chemical carcinogenic agents can only carry out either tumor initiation or promotion in the process of carcinogenesis. Hormones can only promote but unable to initiate carcinogenesis. Indirect-acting chemical carcinogenic agents do not possess carcinogenic properties in their original state, which require metabolic conversion to become ultimate potent carcinogens by cytochrome P450 dependent monooxygenases.
  - Such cell with DNA damage undergoes apoptosis. Failure of apoptosis, certain genes especially RAS gene and TP53 gene transform normal cell to CSC with DNA damage to malignant phenotype. It is thought that altered cells are primed but require a second change to bring about molecular genetic changes to induce carcinogenesis. Mechanism of chemical carcinogen-induced carcinogenesis is shown in Fig. 6.97.

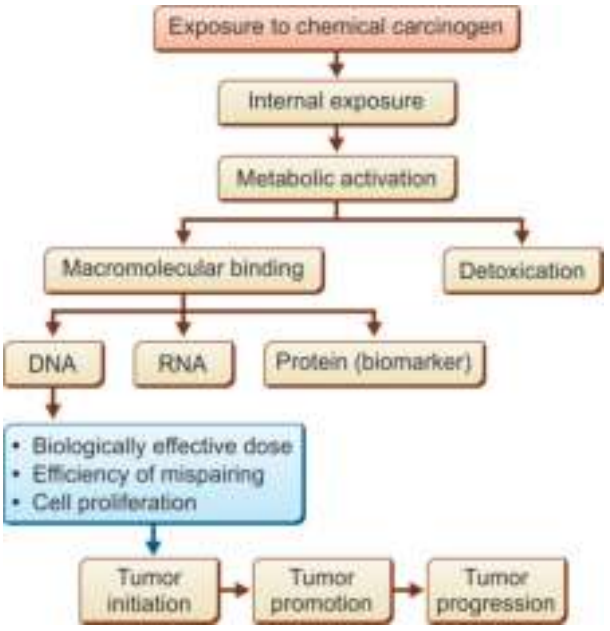


Fig. 6.97: Mechanism of chemical carcinogen-induced carcinogenesis.

MULTISTEP CARCINOGENESIS: MECHANISMS

The multistep carcinogenesis consists of several cellular and molecular events and includes multiple steps—tumor initiation, promotion and progression to bring about transformation of normal cell to CSC, which clearly involve both genetic and epigenetic events.

- Tumor initiation occurs due to exposure of normal cells to an adequate quantity of chemical carcinogenic agent and cause permanent DNA damage. Promoters induce tumorigenesis in an initiated (mutated) cell, but are non-tumorigenic themselves through progression of cell cycle, and propagation of gene mutations.
- Neither an initiator nor a promoter chemical carcinogenic agent acting on its own to cause neoplasia; therefore, both initiator and promoter must act on the cells to induce tumorigenesis.
- Initiation–promotion model of carcinogenesis is shown in Fig. 6.98. Simplified scheme of carcinogenesis is shown in Fig. 6.99. Multistep process of chemical carcinogenesis is given in Table 6.76. Basic classes of chemical carcinogenic agents acting as initiators and promoters are given in Table 6.77.

Tumor Initiation

During initiation, chemical carcinogenic agent can induce rapid non-lethal stable gene mutations in the genome of daughter cell leading to transformation of normal cell to CSC leading to tumorigenesis. Development of malignant tumor depends on the quantity of chemical carcinogenic agent. Increased quantity of carcinogenic agent induces carcinogenesis, whereas decreased quantity inhibits neoplastic process.

- Direct-acting chemical carcinogenic agents do not require metabolic conversion activation or molecular modification in order to induce DNA damage and examples include nitrosamines, ultraviolet rays, alkylating and acylating agents.
- Indirect-acting chemical carcinogenic agents do not possess carcinogenic properties in their original state and require metabolic conversion to become ultimate potent chemical carcinogens by cytochrome P450 oxidases within the body before they are able to bind and induce DNA damage. Examples of carcinogenic agents include polycyclic aromatic amines, nitrosamines, organohalogen compounds, and aflatoxin B1 (synthesized by fungus *Aspergillus flavus* in growing stored grains, nuts and peanut).
- The damaged DNA does not get repaired as the proliferating cells get less time and consequently, the bonds that chemical agents establish with the **DNA (adducts)** are not removed. Thus, this process verifies that cell division stays symmetrical leading to transformation of normal cell to CSC. The process of initiation encompasses a non-lethal gene mutation.
- Genetic alterations can result in aberrant biochemical signaling pathways associated with CSCs proliferation, survival and differentiation, which can be influenced by a number of factors, which include rate and type of metabolism of carcinogenic agents and DNA repair system response. DNA methylation in the genes can transcriptionally kill tumor suppressor genes leading to unrestricted CSC proliferation and survival in individual's life.
- Mitogenic stimulation results in unrestricted CSC proliferation and disruption in apoptosis through extrinsic (death receptor) and/or intrinsic (mitochondrial) pathways, thus leading to clonal expansion of initiated (mutated) cells that survives later. Reactive oxygen species (ROS) are considered to assist in the activation of chemical carcinogenic agents through hydroperoxide-dependent oxidation, which is mediated by peroxyl radicals.

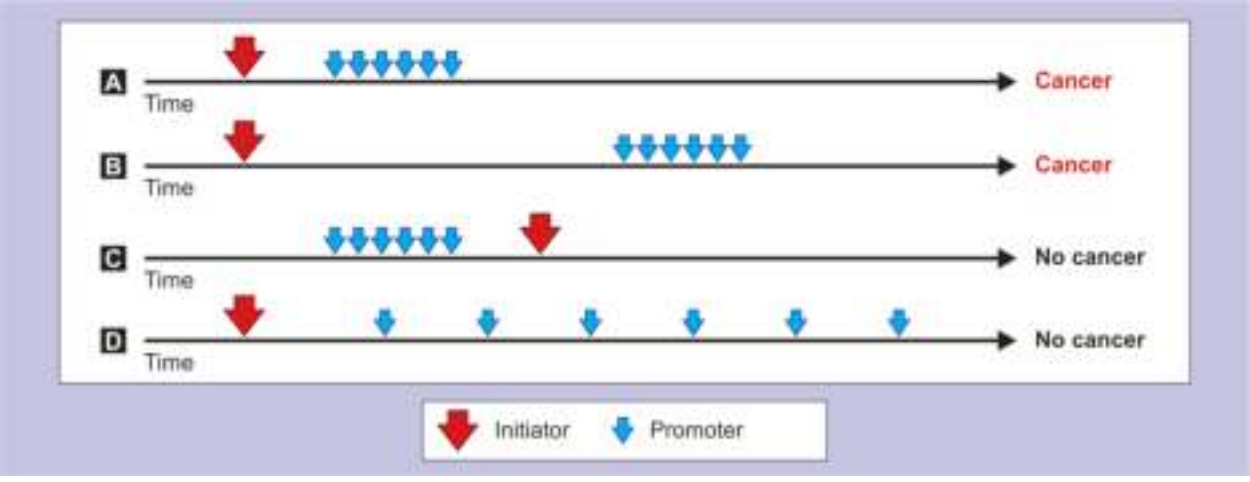


Fig. 6.98: Initiation–promotion model of carcinogenesis developed by Berenblum and P. Shubik by painting polycyclic aromatic hydrocarbons and croton oil on mouse skin. (A) Simultaneously exposure to initiator and then promoter leads to cancer, (B) exposure to initiator and then promoter after long time also causes cancer, (C) exposure first to promoter and then initiator does not cause cancer, (D) exposure to initiator then promoter at wide intervals does not cause cancer.

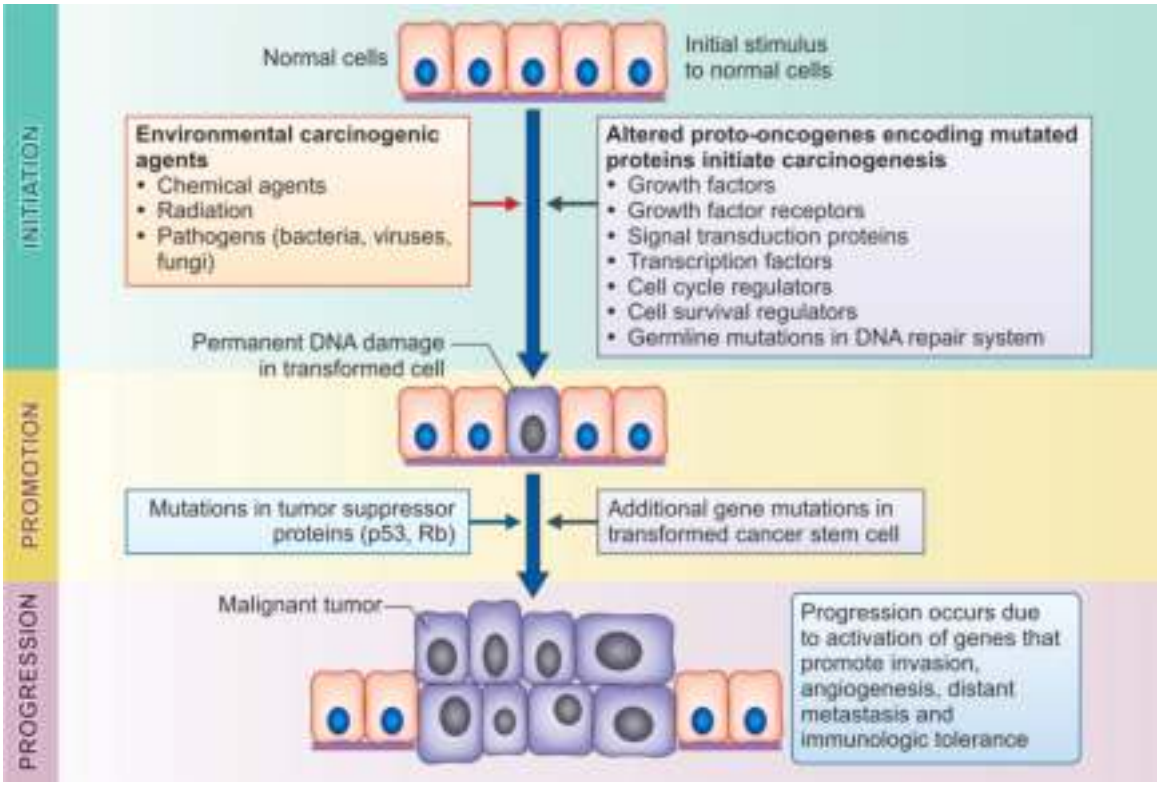


Fig. 6.99. Simplified scheme of carcinogenesis. Exposure to chemical carcinogenic agents, radiation and biological agents and altered genes cause DNA damage. DNA of normal cell resulting in transformed CSCs. Additional mutations and inactivation/biallelic loss of tumor suppressor genes participate in promotion process by proliferation of clonal of cells.

Table 6.76 Multistep process of chemical carcinogenesis

Multistep Process of Carcinogenesis	Normal Cell	Initiation Process	Promotion Process	Progression Process
Chemical carcinogen	Chemical carcinogen binds but does not induce genetic mutations	Chemical carcinogen binds to DNA, forms DNA adducts and induces genetic mutations	Chemical carcinogen induces selective clonal growth of initiated (mutated) cells	Chemical carcinogen induces final conversion of clonally expanded cells into malignant tumor growth
Chemical carcinogen effect	DNA damage undergoes repair	Altered cell (initiated/mutated cell)	Initiated (mutated) cell leads to unrestricted proliferation and formation of focal malignant tumor mass	Growth advantage and genetic instability lead to development of malignant tumor
Apoptosis	Intact	Impaired	Impaired	Impaired

- Reactive oxygen species (ROS) are group of short-lived, highly reactive, oxygen-derived molecules such as superoxide anion, hydrogen peroxide and hydroxyl radical that can induce DNA damage and affect the DNA damage repair response (DDR).
  - Cellular membranes or organelle membrane, due to their high polyunsaturated fatty acids are especially susceptible to ROS damage, which is called 'lipid peroxidation'. ROS activate chemical carcinogens through hydroxyperoxide-dependent oxidation which is mediated by peroxyl radicals. ROS-induced lipid peroxidation directly reacts with DNA to produce DNA adducts.
  - DNA adducts lead to alterations in DNA structure such as DNA nucleotide base pair modifications and frameshifts. Reactive oxygen species (ROS)-induced lipid peroxidation has been implicated in carcinogenesis.

Tumor Promotion

Tumor promotion is defined by propagation of gene mutations by non-mutagenic co-carcinogens (e.g. croton

Table 6.77 Basic classes of chemical carcinogenic agents acting as initiators and promoters

Characteristics	Initiators of Cancer	Promoters of Cancer
Nature of agent	Electrophilic (electron deficient) carcinogenic agents	Non-electrophilic (electron-rich and noncarcinogenic agents
Action	Induces non-lethal mutation(s) in a cell	Induces cell proliferation (clonal expansion)
Reversibility	Irreversible changes in DNA after cell proliferation after one cell cycle	Reversible influence on cell growth without change in DNA
Order	Exposure of the cell to initiator before promoter for carcinogenesis	Exposure of the cell to promoter after the initiator for carcinogenesis
Examples (carcinogen acting as both initiator and promoter)	▪ Polycyclic aromatic amines ▪ Ultraviolet rays/ionizing radiation ▪ Epstein-Barr virus ▪ Hepatitis B and C virus ▪ Pesticides (some)	▪ Polycyclic aromatic amines ▪ Ultraviolet/ionizing radiation ▪ Epstein-Barr virus ▪ Hepatitis B and C virus ▪ Pesticides
Examples (carcinogen acting as initiator only)	▪ Asbestos ▪ Azo dyes ▪ Benzene ▪ Metals (nickel, chromium) ▪ Nitrates/nitrites (N-nitroso compounds)	Not applicable
Examples (carcinogen acting as promoter only)	Not applicable	▪ Alcohol ▪ *Helicobacter pylori* ▪ Hormones ▪ Alcohol ▪ Chronic irritation or wound ▪ Pesticides (some act as initiators) ▪ Polychlorinated biphenols (PCBs)

(a) Initiators induce genomic instability and initiate cancers. Promoters promote cell proliferation and survival.
(b) Carcinogens acting as both initiators and promoters of cancer include ionizing radiation, polycyclic aromatic hydrocarbons and oncogenic viruses (Epstein-Barr virus, hepatitis B virus, hepatitis C virus, and human papillomavirus (HPV).

oil and turpentine oil) induced by initiators leading to unrestricted CSC proliferation (clonal expansion) and accumulation of additional multiple gene mutations. The rate accumulation gene mutation is directly proportional to the rate of cell division.

- Clonal expansion of the initiated (mutated) cells occurs due to promotional non-mutagenic co-carcinogens leading to development of malignant phenotype and CSCs survival. Reversibility is considered to be one of the essential features of the promotion response. This implies that the removal of the promoting agent causes no further proliferation of the initiated (mutated) cell population.
- The promoters such as phorbol esters, phenols, hormones and drugs are themselves non-tumorigenesis, but intensify the carcinogenicity of chemical carcinogenic agents, which induce unrestricted CSC proliferation in vulnerable tissues, fixation of gene mutations, augmentation of genetic changes leading to clonal expansion of initiated (mutated) cells. These promoters could indirectly induce DNA damage by production of reactive oxygen species (ROS), that is directly associated to P450 monooxygenases activity wherein oxidative stress could aid in the clonal expansion of the initiated (mutated) cells.

Tumor Progression

Genetic heterogenicity in the promoted cell population takes place when the initiated (mutated) cell population is extended during promotion and attains further genetic damage, continuous accumulation of new different gene mutations with high-proliferative potential and different behaviors, invasive phenotype with development of new sub-clones of CSCs, angiogenesis and distant metastasis. Metastasis involves the spread of CSCs from the primary tumor site to other parts of the body through lymphovascular and transcoelomic routes. Some of the nonviable cells undergo apoptosis.

- As the heterogenous malignant tumor grows, it becomes complex due to clonal expansion of CSCs, and each clone with different behaviors. CSCs possess several unique capabilities such as self-renewal of cells and metastasis. With time, it becomes increasingly difficult to eliminate all CSCs with chemotherapeutic agents (e.g. tamoxifen for

breast carcinoma) because each group can respond differently to the treatment.

- Malignant tumor heterogenicity is an important characteristic of tumor progression, which allows for antigenic and protein products ability to produce proangiogenic factors, emergence of chromosomal alterations, development of metastatic capability, altered cellular metabolism and decreased sensitivity to chemotherapeutic agents and radiation therapy. Alternatively, genetic heterogenicity observed in malignant tumor progression may be generated by epigenetic regulatory mechanisms operative as a continuation of the process of promotion.
- Associated with progression, there is development of genomic instability and aneuploidy in malignant tumors. Finally, chromosomal rearrangement is associated with malignant tumors, especially chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL).
- Chemopreventive agents are known to inhibit tumor angiogenesis and invasion of primary malignant tumors and thus could be utilized to inhibit the metastasis of cancer. Debulking of malignant tumor done by surgery or radiation is one the strategies in the management of malignant tumors.

CHEMICAL CARCINOGENIC AGENTS: CLASSIFICATION

Chemical carcinogenic agents bring about the process of carcinogenesis by covalently or non-covalently binding to DNA, RNA and proteins of human tissues leading to genetic alterations. Process of carcinogenesis consists of several molecular and cellular events and includes the three major steps—initiation, promotion and progression to bring about transformation of normal cell to CSC.

- Chemical carcinogenic agents that assist to initiate carcinogenesis are classified into two categories: (a) direct-acting chemical carcinogens initiate carcinogenesis without undergoing metabolic conversion, and (b) indirect-acting chemical carcinogens (procarcinogens) promote carcinogenesis involving metabolic conversion.
- Direct-acting chemical carcinogenic agents associated human cancers are given in Table 6.78. Indirect-acting chemical carcinogenic agents associated human cancers are given in Table 6.79.

Direct-acting Genotoxic Chemical Carcinogenic Agents

Direct-acting genotoxic chemical carcinogens do not require metabolic conversion or any molecular modification in order to induce DNA damage. Examples of direct-acting genotoxic chemical carcinogens are alkylating and acylating agents, which have highly reactive electrophilic groups that directly cause DNA damage leading to gene mutations and eventually development of malignant tumor.

Alkylating Agents

Primary mechanism of action for most alkylating drugs after undergoing metabolic activation by cytochrome P450 dependent monooxygenases is via cross-linking and/or transfer of alkyl group to form monoadducts in DNA strands, which results in the misreading of the DNA code and inhibition of DNA replication,

Table 6.78 Direct-acting chemical carcinogenic agents associated human cancers

Direct-acting Chemical Carcinogens	Direct-acting Chemical Carcinogenic Agents: Examples	Associated Cancers
Alkylating agents	Nitrogen mustard (busulfan, chlorambucil, melphalan)	▪ Hodgkin's disease ▪ Non-Hodgkin's lymphoma ▪ Leukemias ▪ Ovarian carcinoma
	Cyclophosphamide	▪ Leukemias ▪ Multiple myeloma
	Hydralazine (temozolomide)	Glioblastoma multiforme
	Platinum-based agents (cisplatin, carboplatin, oxaliplatin cause cross-links between DNA strands → impaired DNA replication and degradation)	▪ Urinary bladder carcinoma ▪ Lung carcinoma ▪ Testicular cancer ▪ Ovarian carcinoma
	Chlorambucil chemotherapeutic agent in treatment of chronic lymphocytic leukemia (CLL)	Secondary malignancy
	Nitrosoureas chemotherapeutic agent in treatment of primary intracerebral tumor and multiple myeloma	Secondary malignancy
	β-Propiolactone, dimethyl sulphate and diepoxibutane	Cancers in experimental animals
Acylating agents	Dimethylcarbamyl chloride, dichloroacetyl chloride (DCAC) and ethyl chloroformate (ECF)	▪ Squamous cell papilloma of skin ▪ Squamous cell carcinoma of skin

Table 6.79 Indirect-acting chemical carcinogenic agents associated human cancers

Indirect-acting Chemical Carcinogens	Indirect-acting Chemical Carcinogenic Agents: Examples	Associated Cancers
Polycyclic aromatic hydrocarbons	Benz(a)anthracene, dibenz(a, h)anthracene, 7-12-dimethylbenz(a) anthracene, benz(a) pyrene and 3-methylcholanthine) in smoked meat and fish consumption and cigarette smoke	▪ Head and neck cancers (oral cavity squamous cell carcinoma and esophageal carcinoma of middle and lower region) ▪ Pancreatic carcinoma ▪ Lung carcinoma ▪ Urothelial carcinoma (renal pelvis/urinary bladder)
Aromatic amines	β-Naphthylamine, benzidine-O, anisidine-O, toluidine used in production of rubber and aniline dyes used to dye fabrics and produce cancer at distant site	Urinary bladder carcinoma (mucosal glucuronidase in the urinary bladder converts β-naphthylamine glucuronide (aromatic amine) to the carcinogenic molecule β-naphthylamine results in urinary bladder cancer)
Azo dyes (derivatives of aromatic amines)	Acid orange 5/7/19, acid red 13/88, alcian yellow R, alizarine yellow R, Allura red AC (used in textile, printing and paper manufacturing, and dimethylaminoazobenzene is used to impart yellow color to butter called 'yellow butter')	Hepatocellular carcinoma
Nitrosamines	Nitrates and nitrites are widely used as fertilizers and as food additives, which get converted to nitrosamines by gut bacteria in hypochlorhydria	Gastric carcinoma
Organohalogen compounds	Polyvinyl chloride, carbon tetrachloride, chloroform, hexachlorobenzene, trichloroethylene	Liver angiosarcoma
Natural occurring carcinogenic products in plants	Aflatoxin B1 synthesized by fungus *Aspergillus flavus* in growing stored grains, betel nuts and peanut, safrole, actinomycin D and mitocin C	Hepatocellular carcinoma
Industrial carcinogens in occupational workers	Nickel (used in nickel plating ceramics, ferrous alloys, batteries and stainless-steel welding)	▪ Nasal cavity squamous cell carcinoma ▪ Lung carcinoma
	Arsenic (used in alloys, medication, preparation of fungicides and herbicides)	▪ Squamous cell carcinoma of skin ▪ Basal cell carcinoma of skin ▪ Squamous cell lung carcinoma ▪ Liver angiosarcoma
	Asbestos (used in building material)	▪ Mesothelioma ▪ Bronchogenic carcinoma ▪ Esophageal carcinoma ▪ Gastric carcinoma
	Beryllium (used in missile fuel, nuclear reactors, aerospace application and light weight alloys)	Lung carcinoma
	Chromium (used in paints, pigments and preservatives)	Lung carcinoma
	Uranium exposure in mine workers	Lung carcinoma
	Cadmium (used in batteries and metal paintings)	Prostatic carcinoma
	Vinyl chloride	Hepatic angiosarcoma
	Benzene in crude oil	Leukemia
	Benzanthracene	Skin cancer
Drugs	Thorotrast (radiocontrast media)	Hepatic angiosarcoma
	Diethylstilbestrol (DES)	Clear-cell adenocarcinoma of vagina (occurs in daughters of patients who received diethylstilbestrol during pregnancy)

Contd...

Table 6.79 Indirect-acting chemical carcinogenic agents associated human cancers (*Contd...*)

Indirect-acting Chemical Carcinogens	Indirect-acting Chemical Carcinogenic Agents: Examples	Associated Cancers
Lifestyle associated cancers	Tobacco smoking	▪ Urinary bladder carcinoma ▪ Renal cell carcinoma
	Betel nuts	Oral cancer
	Diet (low in vegetable, high in nitrates, salts)	▪ Gastric carcinoma ▪ Esophageal carcinoma
	Diet (high fat, low-fiber, broiled/fried foods)	▪ Colon carcinoma ▪ Pancreatic carcinoma ▪ Prostatic carcinoma ▪ Breast carcinoma
	Alcohol plus tobacco smoking	▪ Oral squamous cell carcinoma ▪ Esophageal squamous cell carcinoma
	Alcohol	Cirrhosis-linked hepatocellular carcinoma
	Snuff	Oral squamous cell carcinoma
	Hardwood dust	Nasal adenocarcinoma

transcription of RNA, and protein synthesis, and triggering apoptosis in unrestricted rapidly proliferating CSCs.

- Alkylating agents are used to treat leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, breast cancer and sarcoma including cancers of ovary, and lung. Alkylating agents can cause bone marrow depression (i.e. anemia, leukopenia and thrombocytopenia), nausea and vomiting.
- Therapeutic administration of alkylating agents in cancer patients can cause another cancer, i.e. leukemia. Alkylating agents exert their lethal effects on cells throughout the cell cycle, but tend to be more effective against rapidly dividing cells, which do not have time for DNA repair.

Pathology Pearls: Six Classes of Alkylating Agents

- Nitrogen mustards cyclophosphamide, mechlorethamine, melphalan, chlorambucil, ifosfamide
- Ethylenimine and methylenamine derivatives (altretamine, thiotepa)
- Alkyl sulfonates (busulfan)
- Nitrosoureas (carmustine, lomustine)
- Triazenes (dacarbazine, procarbazine, temozolamide)
- Platinum-containing chemotherapeutic agents (cisplatin, carboplatin, oxaliplatin).

Acylating Agents

In chemistry, acylation is a chemical process in which an acyl group (R-C=O) is added to a chemical molecule or compound. Acylating agents and their derivatives are important class of highly reactive electrophilic agents with mutagenic and/or carcinogenic properties, which can interfere with normal biological processes. Dimethylcarbamyl chloride (DMCC), which is being used in the preparation of pharmaceutical agents, which possesses direct-acting chemical carcinogenic potential linked to squamous cell papilloma of skin, squamous cell carcinoma of skin and sarcomas.

Indirect-acting Chemical Carcinogenic Agents

Most chemical carcinogenic agents require metabolic conversion from procarcinogen to active ultimate carcinogen. The metabolic conversion process occurs by hydroxylation by aryl carbohydrate hydroxylase in microsomal fraction of endoplasmic reticulum with cytochrome P450 dependent monooxygenase. Hydroxylating enzyme (e.g. aryl carbohydrate hydroxylase) is ubiquitous in human tissues and readily induces malignant tumors in susceptible individuals.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are among the most extensively studied carcinogens, which include benzene, naphthalene, phenanthrene, anthracene and benz(a) pyrene. PAHs are organic compounds containing carbon and hydrogen and composed of multiple aromatic rings.

- PAHs occur naturally in coal, crude oil and gasoline, which are also derived from incomplete combustion of organic matter such as coal, wood and garbage, and animal fat by process of broiling of meats (e.g. smoked meat and fish), and tobacco smoking.
- PAHs induce DNA damage by frameshift mutations, mispairing with wrong nucleotide base pairs during

DNA replication, loss of nucleotide base pairs, breakage of glycosidic bonds between purine bases and deoxyribose sugar.

- Some polycyclic aromatic hydrocarbons (PAHs) cause conformational transition of DNA from its usual double-helical B form to a Z-DNA form. PAHs are linked to human cancers such as squamous cell carcinoma (oral cavity, mid and lower esophagus), lung carcinoma, pancreatic carcinoma, and transitional cell carcinoma of renal pelvis and urinary bladder.
- Polycyclic aromatic hydrocarbons (PAHs) in smoked fish and/or meat undergoes hydroxylation in liver and metabolized by cytochrome P450 dependent monooxygenases to form benzopyrene epoxide, which irreversibly bind to cellular DNA, RNA and proteins and alter the genome. PAHs are linked to development of oral squamous cell carcinoma, esophageal squamous cell carcinoma and pancreatic carcinoma.
- Benz(a)pyrene is well studied prototype of (PAHs due to its mutagenic and carcinogenic activity.
  - Benz(a) pyrene is **highly reactive chemical carcinogenic component** in tobacco smoke that reaches liver via circulation and is metabolized in the liver by cytochrome P450 dependent monooxygenases.
  - Benz(a) pyrene gets converted to benz(a) pyrene-epoxide resulting in formation of vicinal-diol-epoxide (unstable compound) and then highly reactive benz(a) pyrene-diol-epoxide (**BPDE**) compound, that can irreversibly bind to DNA at N2 position of guanine to form benz(a) pyrene-diol-epoxide adducts and produce N2 guanine mutagenic lesions resulting in lung carcinoma (local site) and renal cell carcinoma and urinary bladder carcinoma (distant sites).
  - In addition to benz(a) pyrene, other components present in tobacco smoke produce oxidative free radicals, that cause DNA fragmentation. If DNA damage left unrepaired, BPDE-DNA adducts may lead to permanent gene mutations resulting in transformation of normal to malignant cells, i.e. CSCs, tumorigenesis, angiogenesis, invasion and metastasis.

Aromatic Amines

Aromatic amines are derivative of aromatic hydrocarbons in which a hydrogen of benzene ring has been replaced by amino group. All such compounds in which an amino or substituted amino group is bonded directly to an aromatic ring are termed aromatic amines. Examples of aromatic amines include aniline, 4-methylaniline, 4-chloroaniline and 4-nitroaniline. Aromatic amines are used as intermediates as antioxidants, in the manufacture of drugs, pesticides, rubber (tyres and cables), as cutting agents in plastics preparation. In addition, these are widely used as intermediates in the preparation of dyes and pigments extensively employed to color textiles, leathers, rubber, printing inks, paints, lacquers, metal finishing, plastic and paper products.

- **β-Naphthylamine:** β-naphthylamine is used in the manufacture of synthetic aniline dyes. Incidence of transitional cell carcinoma of urinary bladder is increased in aniline dye workers heavily exposed to β-naphthylamine (aromatic amines).
  - The β-naphthylamine undergoes hydroxylation in the liver and converted to 1-hydroxy-2-naphthylamine (water-soluble active carcinogenic metabolite) by cytochrome P450 dependent monooxygenases.
  - The active carcinogenic metabolite 1-hydroxy-2-naphthylamine combines with glucuronic acid in liver, and excreted and deconjugated in urinary bladder by mucosal enzyme glucuronidase, thus exposing the urothelium to the active chemical carcinogenic metabolite results in development of urinary bladder carcinoma. Role of β-naphthylamine (aromatic amines) in the pathogenesis of urinary bladder carcinoma is shown in Fig. 6.100.
- **Benzene:** Benzene is a colorless or light-yellow liquid chemical carcinogenic agent at room temperature. It is used primarily as a solvent in the chemical and petrochemical industries to synthesize numerous chemical agents.
  - Benzene is produced by both natural and man-made processes.
  - Benzene is derived from crude oil, gas emissions from volcanoes and forest fires, glues, adhesives, cleaning products, paint strippers, gasoline fumes, motor vehicle exhaust and industrial emissions.
  - Occupational workers in benzene chemical agent used in petrochemical industries are at risk of development of leukemia and Hodgkin's disease.
- **Dimethylaminoazobenzene:** Dimethylaminoazobenzene is a yellow crystalline solid compound. It was used as an azo-dye for coloring wax products, polishes, and soap and butter. It is reasonably anticipated to be a human chemical carcinogenic agent. Azo dyes have been used to color the butter and linked to hepatocellular carcinoma.

Nitrosamines

Nitrosamines are produced by chemical reactions of nitrates, nitrites and other proteins. Nitrosamines are present in tobacco smoke, drinking water contaminated by fertilizers, many dietary foods such as fruits, vegetables, fish, processed meat, beer and fried foods. In the same time, nitrates and nitrites are often used as

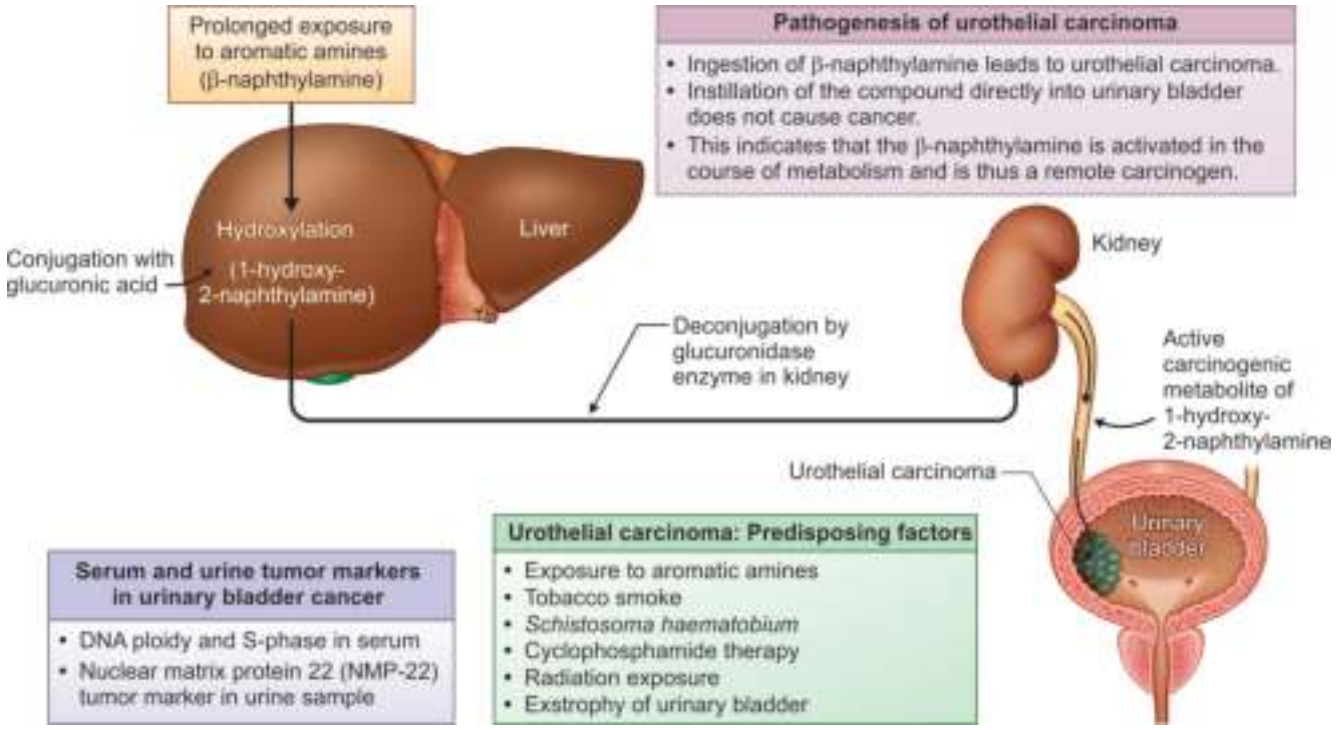


Fig. 6.100: Role of β-naphthylamine (aromatic amines) in the pathogenesis of urinary bladder carcinoma. β-Naphthylamine is metabolized in the course of metabolism in the body to become carcinogen involved in urothelial carcinoma.

food additives in the processed meats to retard microbial spoilage and preserve meat products.

- Dietary foods contain nitrates and nitrites. During gastric hypochlorhydria, overgrowth of commensal bacteria in stomach converts dietary nitrates and nitrites into nitrosamines (potent carcinogen) resulting in development of gastric carcinoma. A high consumption of processed meat products rich in nitrosamines is linked to gastric carcinoma.
- Our diet contains nitrosamine in the form of N-nitrosodimethylamine (NDMA), which is a potent chemical carcinogen, capable of carcinogenesis and development of malignant tumors in stomach, liver and lung.

Organohalogen Compounds

Organohalogen compounds contain at least one halogen, i.e. fluorine (F), chlorine (Cl), bromine (B) or iodine (I) bonded with carbon, which are subdivided into acyl, vinyl, aryl and acyl halides.

- Organohalogen compounds include polyvinyl chloride, carbon tetrachloride (CCL_4), chloroform, vinylidene chloride, hexachlorobenzene and trichloroethylene.
- Polyvinyl chloride (PVC) is used in a variety of applications in the building and construction, electronics, healthcare and automobiles sectors to manufacture products ranging from pipes and dashboards, blood bags and tubing, wire and cable insulation, windshield system components, raincoats, toys and food packaging.
- Persistent exposure to organohalogen compounds chemical carcinogenic agents (i.e. carbon tetrachloride, chloroform, vinyl chloride, hexachlorobenzene and trichloroethylene) is linked to hepatic angiosarcoma.
  - Carbon tetrachloride is used to make refrigerants and propellants for aerosol cans, as a solvent for fats, oils, lacquers, varnishes, rubber waxes and resins as a grain fumigant and a dry-cleaning agent.
  - Chloroform is used as a solvent for floor polishes. Lacquers, resins, adhesives, alkaloids, oils, fats and rubber.
  - Vinylidene chloride is used to manufacture certain plastics such as flexible films like food wrap and in packaging materials and coating steel pipes.
  - Hexachlorobenzene has been used as a pesticide to protect the seeds of onions, sorghum, wheat and other grains against fungus; and to make fireworks. Synthetic rubber and ammunition.
  - Trichloroethylene is used as a solvent in paints, adhesives and cleaners, as a reactant to produce pesticide intermediates and pharmaceutical drugs.

Aflatoxin B1: Natural Carcinogenic Agent

In human beings, *Aspergillus flavus* is predominantly an opportunistic pathogen in immunocompromised patients. Aflatoxin B1 is potent natural carcinogen

synthesized by *Aspergillus flavus* grown on crops stored in hot humid conditions (e.g. peanuts, maize, oil-seeds, cotton-seeds, dried fruits and grains).

- *Aspergillus flavus* synthesizes toxic metabolites (aflatoxin B1, aflatrem, aflavinin, aspergillic acid, neoaspergillic acid, kajoic acid, paspalinine and β-nitropropionic acid).
- After consumption of contaminated crops, aflatoxin B1 reaches the liver and activates cytochrome P450 dependent monooxygenases resulting in formation of aflatoxin-2,3-epoxide, a chemical carcinogenic compound that binds to guanine of DNA and induces specific point mutations in the **TP53 gene** resulting in development of hepatocellular carcinoma in Asian and African population.

Occupational Workers Exposed to Chemical Carcinogenic Agents

Occupational workers are exposed to numerous hazardous chemical carcinogenic agents, which include aniline, chromates, arsenic, nickel, beryllium compounds, dusts (asbestos, and crystalline form of silica), coal tar pitch volatiles, coke oven emissions, diesel exhaust, and tobacco products and certain industrial processes (e.g. nickel, arsenic, beryllium, aluminum, cadmium, chromium, asbestos, underground mining of uranium, iron and steel founding).

- Prolonged exposure to chemical carcinogenic agents has been linked to lung carcinoma, mesothelioma, urinary bladder carcinoma, prostatic carcinoma, sinonasal carcinoma and nasopharyngeal carcinoma, esophageal carcinoma, gastric carcinoma, liver angiosarcoma, and squamous cell carcinoma and basal cell carcinoma of skin. Prolonged exposure to asbestos increases the risk for development of mesothelioma, bronchogenic carcinoma, esophageal carcinoma and gastric carcinoma.
- Exposure to nickel compounds are linked to sinonasal and nasopharyngeal carcinoma. Arsenic compounds are linked to skin cancers (e.g. squamous cell carcinoma, basal cell carcinoma), squamous cell lung carcinoma and liver angiosarcoma.
- Exposure to beryllium, chromium and uranium compounds are linked to lung carcinoma. Persistent exposure to cadmium can cause prostatic carcinoma. Exposure to vinyl chloride is linked to liver angiosarcoma.

Thorotrast Radiocontrast Medium used in Imaging Techniques

Thorotrast radiocontrast medium is drug used to improve the visibility of internal organs and structures in X-ray based imaging technique. Thorotrast is a suspension containing particles of the radioactive compound thorium dioxide, which was used as radiocontrast medium in medical radiography linked to development of hepatic angiosarcoma.

Lifestyle-related Exposure to Chemical Carcinogenic Agents

Lifestyle-related exposure to chemical carcinogenic agents include tobacco smoke, dietary factors (low in vegetable, high in nitrates, salts, high fat, low fiber, broiled/fried foods, saccharin and sodium cyclamates).

- **Tobacco smoking:** Tobacco smoking is linked to carcinomas of lung, kidney, urinary bladder, oral cavity, pharynx, larynx, esophagus, colon, rectum, stomach and liver. Tobacco smoking is also linked to acute myelogenous leukemia (AML).
- **Dietary factors:** Diet low in vegetable, and rich in nitrates may cause gastric carcinoma and esophageal carcinoma. High fat, low fiber, broiled/fried dietary foods increase risk for development of carcinomas such as colon, pancreas, prostate and breast. Tobacco smoke, alcohol and snuff are linked to oral squamous cell carcinoma.
- **Hardwood dust:** Hardwood dust may cause sinonasal adenocarcinoma. Chronic alcoholics may develop oropharyngeal carcinoma, esophageal carcinoma, hepatocellular carcinoma, breast carcinoma and colorectal carcinoma.
- **Alcohol:** Alcohol acts as indirect-acting carcinogen. Normally, alcohol is metabolized to acetaldehyde and then converted to acetate by aldehyde dehydrogenase enzyme.
  - Chronic consumption of alcohol reduces the function of aldehyde dehydrogenase enzyme leading to accumulation of acetaldehyde, that acts as a complete carcinogen responsible for DNA adducts formation, cellular damage and unrestricted CSC proliferation and malignant tumor growth.
  - Alcohol abuse also causes oxidative stress of CYP2E1, allowing reactive oxygen species (ROS) to react with DNA, and then DNA damage. Further, chronic alcoholics develop multiple vitamin (B_{12}, B_6 and A) deficiencies, that may contribute to development of cancer.

RADIATION-INDUCED CARCINOGENESIS

Radiation-induced carcinogenesis is a biological phenomenon, whereby damage to normal cells results in malignant phenotype. Ionizing radiation has enough energy to break chemical bonds and cause DNA damage resulting in the development of human cancers. Some of the elementary forms of radiation are protons, neutrons, X-rays, and γ-rays. X-rays originate outside of the nucleus of an atom while γ-rays originate within

Table 6.80 Radiosensitivity of specialized cells

Degree of Sensitivity	Characteristics
Most radiosensitive cells	Lymphoid cells, hematopoietic cells, germ cells, gastrointestinal mucosal cells, and rapidly dividing cancer stem cells
Intermediate radiosensitive cells	Fibroblasts; cells of endothelium, elastic tissue, salivary glands, and eye
Radioresistant cells	Cells of bone, cartilage, muscle, central nervous system, kidney, liver, and most endocrine glands. These cells cease division shortly after complete fetal development

genomic nucleus, which have variable energies. Radiosensitivity of specialized cells vary in various tissues (Table 6.80).

- Exposure to non-ionizing radiation is usually non-carcinogenic and described as series of low energy waves on the electromagnetic spectrum travelling at the speed of light, covering two main regions: optical radiation (ultraviolet rays, visible rays and infrared rays), and electromagnetic fields (e.g. radiowaves, microwaves, infrared waves).
- Exposure to ionizing radiation (electromagnetic X-rays, γ-rays, and particulate radiation) poses a potential world-wide threat in this nuclear age. Exposure to ionizing radiation increases risk of human cancers (Table 6.81).
  - Ionizing radiation is strong enough to induce ionization of atoms and cause damage to cellular DNA, RNA and proteins, which depends on the magnitude of exposure, penetration and capacity to cause ionization in the tissues.
  - Survivors of atomic bombs dropped in World War II develop AML, CML, but not lymphoid leukemias and other cancers.
  - Radium watch-dial workers have higher risk to develop osteosarcoma. Radiologists may develop AML and CML and other cancers.

ULTRAVIOLET RADIATION-INDUCED CARCINOGENESIS

The sun is the dominant source for visible light waves our eyes can detect wavelengths from 380–780 nm. The ultraviolet (UV) region covers the wavelength range 100–400 nm and is divided into three bands: UV A (90–95%), UV B (5–10%) and UV C. Both UV A (320–400 nm) and UV B (280–320 nm) radiations are able to penetrate to different depths of skin, and hence affect cells in the epidermis and dermis.

- The skin is organized into two regions—the superficial epidermis composed of epithelium and an underlying

Table 6.81 Radiation-induced human cancers

Radiation Source	Radiation Types	Radiation-induced Malignant Tumors
Sunlight radiation	Ultraviolet B (UV B) rays: 290–320 nm	▪ Melanoma of skin ▪ Squamous cell carcinoma of skin ▪ Basal cell carcinoma of skin
Nuclear explosions (e.g. atom bombs)	Ionizing radiation	▪ Acute myelogenous leukemia (AML) ▪ Chronic myelogenous leukemia (CML) ▪ Lung carcinoma ▪ Breast carcinoma ▪ Thyroid carcinoma
Therapeutic radiation	Ionizing radiation	▪ Carcinomas (various sites) ▪ Thyroid carcinoma ▪ Sarcomas ▪ Leukemia
Mining radioactive substances, i.e. uranium induces DNA mutations and chromosomal abnormalities	Ionizing radiation	Lung carcinoma
X-ray workers	Ionizing radiation	▪ Skin carcinoma ▪ Leukemia
Thorium dioxide byproduct of uranium	Ionizing radiation	Liver angiosarcoma

**Ultraviolet radiation causes TP53 gene mutation, tumor necrosis factor-α (TNF-α) released by exposed skin diminishes immune response. It induces dimer formation between neighboring thymine pairs in DNA.*

connective tissue dermis, which are firmly attached to each other. UVA radiation penetrates deeply into dermis reaching well into the dermis and dermal fibroblasts. In contrast, UVB radiation has shorter wavelengths, and is almost completely absorbed by the skin epidermis causing DNA and cellular damage, with comparatively little reaching the dermis.

- Ultraviolet radiation is classified as a 'complete carcinogen' because it acts as a mutagenic agent that causes DNA damage and has properties of both a tumor initiator and a tumor promoter.
- Ultraviolet radiation (UVA and UVB)-induced skin cancers include squamous cell carcinoma, basal cell carcinoma and melanoma. Unprotected exposure to UVA and UVB radiations can also cause ocular damage including cataracts and eyelid cancers.
- Ultraviolet and ionizing radiation associated human cancers are given in Table 6.82.

Ultraviolet A (UVA) Radiation

Ultraviolet A (UVA) radiation has longer wavelength (320–400 nm), that penetrates deeply into the skin dermis, and causes damage to DNA, RNA and proteins via indirect photosensitizing reactions. It also induces photo-aging (e.g. wrinkling) and immune system suppression.

Ultraviolet B (UVB) Radiation

Ultraviolet B (UVB) radiation has shorter wavelengths (280–320 nm), that is mainly absorbed by skin epidermis and causing damage to DNA, RNA and proteins. UVB radiation has been described as the most carcinogenic agent among all types of solar radiation. UVB radiation is linked to development of cutaneous squamous cell carcinoma, basal cell carcinoma and melanoma. Early detection of skin cancers helps in the management of these patients.

- **Squamous cell carcinoma of skin** is a malignancy of epidermal keratinocytes, that displays variable degrees of differentiation, and cytologic features that most often affects sun-exposed regions such as face, ears, lips, nose, eyelids chest, upper back, arms, hands and legs.
  - Exposure to ultraviolet B radiation, patients with Bowen disease, xeroderma pigmentosum, AIDS and immunocompromised persons increase risk for squamous cell carcinoma of skin.
  - Tumor may grow beyond epidermis and appear as flat reddish or brown patches in the skin, often with a rough, scaly, or crushed surface. It may sometimes invade surrounding tissues and metastasize to lymph nodes and bones.
  - Ultraviolet B (UVB) radiation-induced mutations involving genes (e.g. TP53, CDKN2A, NOTCH1, NOTCH2, EGFR and TERT) and RAS/RAF/MEK/ERK (MAPK) and PI3K/AKT/mTOR signaling pathways play key role in the pathogenesis of squamous cell carcinoma of skin.
  - Most patients have a favorable outcome after surgical resection. Only a subset of patients may

Table 6.82 Ultraviolet and ionizing radiation associated human cancers

Types of Radiation	Mechanism	Associated Cancers
Ultraviolet radiation (excessive exposure to sunlight)	▪ UV radiation causes genetic mutation in TP53 tumor suppressor gene ▪ UV radiation releases tumor necrosis factor-α (TNF-α) in exposed skin, possibly diminishing immune response ▪ UV radiation induces dimer formation and causes damage to DNA, RNA and proteins ▪ Xeroderma pigmentosum is an autosomal recessive disorder due to failure of DNA excision repair mechanisms	▪ Squamous cell carcinoma of skin ▪ Basal cell carcinoma of skin ▪ Melanoma of skin
Ionizing radiation	▪ Low doses of ionizing radiation can cause DNA mutations and chromosomal abnormalities, and large doses can inhibit cell division ▪ Ionizing radiation also can enhance the effects of genetic abnormalities	▪ Lung cancer in uranium workers ▪ Osteosarcoma in radium watch-dial workers ▪ Acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (but not lymphoid) in survivors of atomic blasts ▪ Thyroid cancer in patients, who have received head and neck radiation therapy ▪ Skin cancer and acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) may occur in radiologists

develop local recurrence and distant metastasis associated with fatal outcome.

- **Basal cell carcinoma of skin:** Basal cell carcinoma of skin is most common malignant tumor on ultraviolet-exposed skin in the head and neck region. Multiple basal cell carcinomas develop early in life with basal cell nevus syndrome (Gorlin syndrome), or Bazex syndrome or xeroderma pigmentosum.
  - Mutation of PTCH gene located on chromosome 9q22.3 is detected in most syndromic and sporadic basal cell carcinoma. Multiple basal cell carcinomas develop early in life with basal cell nevus syndrome (Gorlin syndrome), or Bazex syndrome or xeroderma pigmentosum.
  - Mutated PTCH gene mapped on chromosome 9q22.3 in the mitogenic Sonic Hedgehog signaling pathway, and inactivated/biallelic loss of TP53 tumor suppressor gene plays key role in development of syndromic and sporadic basal cell carcinoma.
- **Melanoma of skin:** Melanoma is an aggressive skin cancer, that arises from melanin producing melanocytes, that most often disseminates to distant organs. Classic features of melanoma include junctional activity with obscured dermoepidermal junction and pagetoid spread individually and in clusters throughout epidermis.
  - Risk factors for melanoma include prolonged exposure to ultraviolet radiation, fair-skin persons, and a family history of melanoma. In most cases, melanomas have an irregular shape and are more than one color.
  - Melanoma can be superficial spreading melanoma (fair colored persons), nodular melanoma (head and neck, chest and back regions), lentigo maligna melanoma, acral lentiginous melanoma (face region) and amelanotic melanoma (little or no color).
  - An activated mitogenic RAS/RAF/MEK/ERK (MAPK) signaling pathway and inactivated/balladic loss of TP53 and p16 tumor suppressors are linked to melanoma.

Pathology Pearls: Ultraviolet B (UVB) Radiation-induced Carcinogenesis

Ultraviolet B (UVB) radiation has slightly more energy than UVA radiation. During cutaneous carcinogenesis, variable alterations in the oncogenes, tumor suppressor genes, and cell cycle control signaling pathways occur.

- UVB radiation is directly absorbed by DNA, which causes molecular rearrangements forming **photoproducts** such as **cyclobutane pyrimidine** dimers (CPDs), and **pyrimidine** (6–4) photoproducts (6–4 PPs), which may induce DNA breaks.
- Besides formation of photodimers in the genome, UVB radiation induces gene mutations by generating reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radical. Nucleotide base pairs in DNA are highly susceptible to oxidative free radical induced injury.
- Oxidation of nucleotide base pairs promotes mispairing outside of normal Watson-Crick parameters causing mutagenesis. These oxidative free radical avidly attack DNA macromolecules such as RNA, proteins and lipids altering their structure and interfering with their function. These lesions are associated with DNA replication/transcription blockage, that may result in production of DNA double-strand breaks (DSB1) at the sites of collapsed DNA replication forks of cyclobutane pyrimidine dimers (CPDs)-containing DNA.
- Most of the DNA damage is temporary. The human body has inbuilt DNA repair systems (e.g. nucleotide excision repair and base excision repair) to recognize, excise and repair by replacing with correct DNA sequence.

Ultraviolet C (UVC) Radiation

Ultraviolet C (UVC) rays (200–280 nm) are highly mutagenic that do not reach the earth's surface and are completely absorbed by the stratospheric ozone layer in our atmosphere.

IONIZING RADIATION-INDUCED CARCINOGENESIS

Ionizing radiation can penetrate the human body and the radiation energy can be absorbed in tissues. This has the potential to induce harmful effects to persons especially at high levels of exposure.

- Ionizing radiation can fall into two categories: Natural and man-made. Natural sources of ionizing radiation include: (a) radiation from space (cosmic and solar radiation), (b) radiation from earth (terrestrial radiation, i.e. radon gas), and (c) radiation from building materials.
  - Ionizing radiation from natural sources is typically at low levels. The usual low dose of ionizing radiation from natural sources is absorbed by our body.
  - Ionizing radiation from man-made sources includes X-rays, computed tomography, positron emission tomography, fluoroscopy and nuclear medicine procedures. The direct and indirect effects of ionizing radiation on DNA are given in Fig. 6.101.
- In natural or man-made ionizing radiation, energy is released by atoms in the form of electromagnetic waves or radioactive particles.
- Ionizing radiation can travel unseen and pass through these materials. Ionizing radiation has more energy

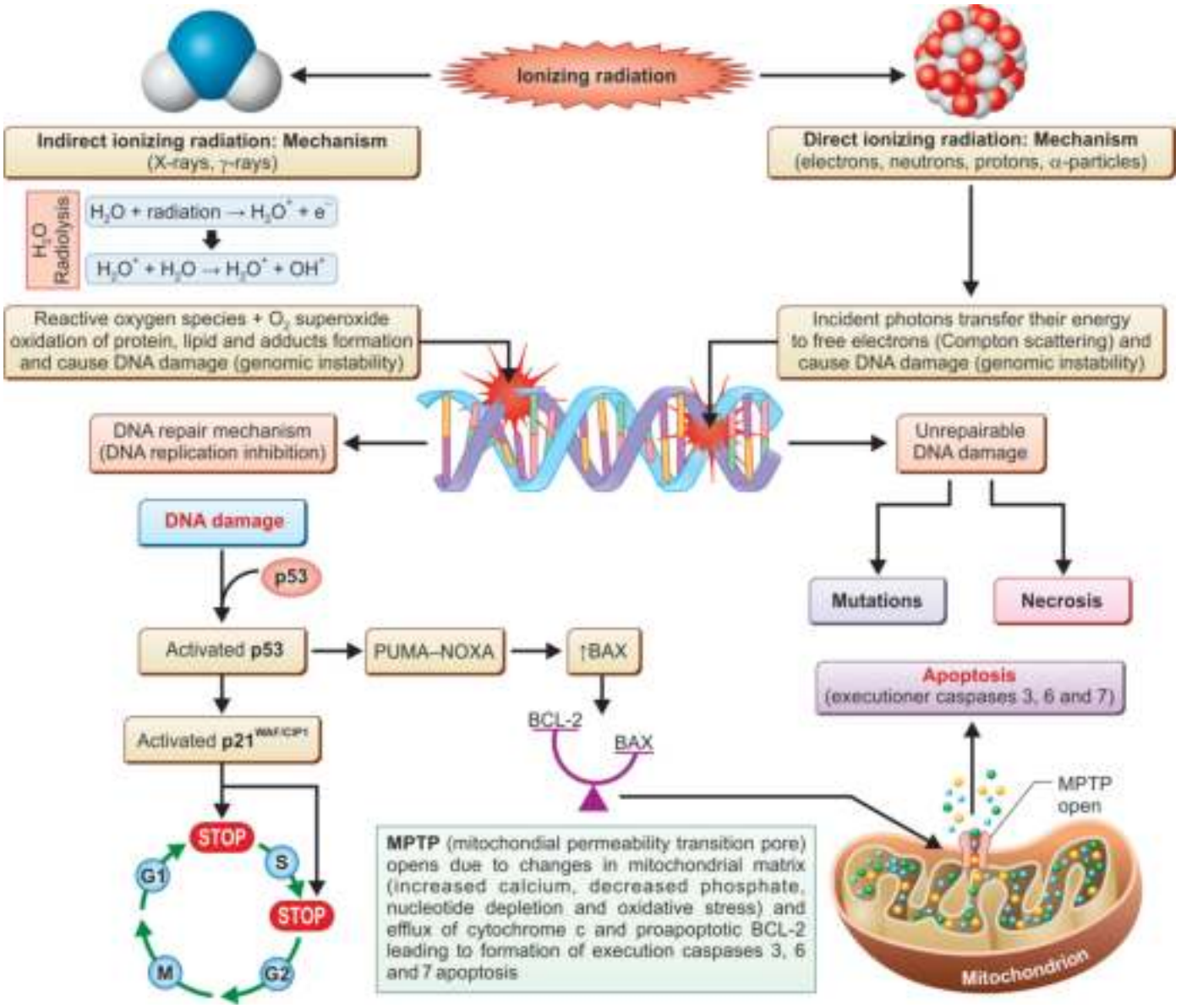


Fig. 6.101: The direct and indirect effects of ionizing radiation on DNA. Incident photons transfer part of their energy to free electrons (Compton scattering). These electrons can directly interact with DNA to induce DNA damage or they can first interact with water to produce hydroxyl radicals that can then induce DNA damage. Indirect ionization occurs when non-charged particles, e.g. interact with cellular water. The energy absorbed by an H_2O molecule results in the formation of ion pairs and reactive oxygen metabolites such as hydroxyl radicals. In turn, these free radicals interact with cellular atoms and molecules damaging cellular proteins and may form additional free radicals. The process is known as indirect effect because of the intermediate step of H_2O-based free radical formation.

than non-ionizing radiation, that is absorbed by material surrounding the ionized atom.

- Ionizing radiation deposits a large amount of energy into a small region. In fact, energy from ionizing radiation is enough to break chemical bonds between the carbon bonds, which can cause damage to the living tissues.
- For clinical oncologists, it is essential to understand the mechanism of ionizing radiation-induced carcinogenesis and development of ionizing metachronous malignancies after radiotherapy for proper patient's follow-up. In medical practice, radiation is used for diagnosis (0.1–10 mSv procedure) and therapeutic purposes (20–60 Gy unit per targeted tissue).

Ionizing Radiation-induced Carcinogenesis—Mechanisms

The mechanisms by which ionizing radiation may induce carcinogenesis by various mechanisms: (a) DNA mutations including alterations in the structure of genes and chromosomes, (b) alterations in gene expression with mutations, and (c) induction of oncogenic viruses. Patient receiving radiotherapy in head and neck may thyroid carcinoma.

Ionizing Radiation-induced Direct DNA Damage

The incident photons of ionizing radiation (>2000 rad) penetrate cell membrane, transfer part of their energy to free electrons (known as Compton scattering) in the cytoplasm, and breaks bonds in DNA and RNA. Ionizing radiation induce breaks in single stand or double-strands and deamination or depurination. In addition, ionizing radiation can degrade the amino acid in the side chains of proteins to cause reduced function, and can cause alterations in the tertiary protein structure. DNA-DNA and DNA-protein cross-links and subsequent cellular necrosis with formation of oxidative free radicals and fragmentation of DNA.

Ionizing Radiation-induced Indirect DNA Damage

Incident photons of ionizing radiation (300–1000 rad) first interact with water to produce reactive oxygen species (ROS) such as hydroxyl (OH^-) radicals, that interact with DNA nucleotide base pairs of proliferating cells and induce DNA damage by breaking of hydrogen bonds in macromolecules and formation of protein and lipid adducts resulting in apoptosis.

Ionizing Radiation-induced Bystander Effects

Besides causing gene mutations and macromolecules destruction, ionizing radiation can also cause alterations in signaling pathways leading to release of cytokines and growth factors. The bystander effects may be responsible for inducing cell proliferation in irradiated cells, allowing promotion to take place. Cells can adapt to prolonged radiation exposure; the cells have been primed by low-dose of radiation. DNA repair and cell cycle regulation pathways are upregulated to prepare for impending ionizing radiation damage. The bystander effect lasts for weeks to months.

Therapeutic Ionizing Radiation in Human Cancers

Therapeutic ionizing radiation used for malignant tumors includes: (a) photon ionizing radiation (X-rays or γ-rays: pure energy photons), and (b) particle ionizing radiation (e.g. electrons, protons, neutrons, carbon ions, α, and β particles). The α, and β particles are not part of electromagnetic spectrum, but are energetic particles as opposed to pure energy photons. Photon ionizing radiation is used to treat deep-seated localized malignancies. Carbon ion radiation is used for radiation-resistant malignant tumors.

Ionizing Radiation using α and β Particles

Ionizing radiation using α and β particles or high-energy photons (γ-rays and X-rays) are contained in radioactive particles, that can be swallowed or inserted into the body.

- Based on the source of radiation, therapeutic radiation is classified into two main groups: (a) **external beam radiotherapy** by placing X-ray tube outside the patient's body, and (b) **brachytherapy** by using an isotope (e.g. cadmium-226, cesium-137, iridium-192, iodine-125) is inserted within the malignant growth or cavity.
- Ionizing radiation using α and β particles scan, travel different distances, and interact with the atoms of absorbing materials in their paths, causing ionization of the atoms. In ionizing radioactive α and β particles, electron beams produced by a linear accelerator are used to treat cutaneous malignancies close to the body surface.
- Proton beam radiation needs highly advanced equipment and hence is not routinely performed in oncology departments.
- Neutron beam radiation is used for head and neck cancers especially inoperable malignancies, but its use adversely affects surrounding normal tissues.

X-Rays and γ-Rays—Ionizing Radiation

X-rays and γ-rays are examples of ionizing radiation at upper end of electromagnetic spectrum. Ionizing radiation in this electromagnetic spectrum has high energy, that can remove electrons from an atom. Very high-energy ionizing radiation removes electrons and breaks up the nucleus of the atom. A familiar example of ionizing radiation is the X-rays frequently used in clinical practice. X-rays have unique capability to remove electrons from atoms and molecules in the matter through which they pass. Ionizing radiation activity can alter the molecules within the cells, that may produce damage to skin and tissue and increased risk for malignant tumors.

Basis of Ionizing Radiation Therapy for Malignancies

Ionizing radiation can be used to treat malignancies. The principle of ionizing radiation therapy is to irradiate the malignant tumor at specific time intervals. Each ionizing radiation exposure induces DNA damage in both CSCs and surrounding normal cells by direct and indirect mechanisms. Following ionizing radiation, normal cells can stop the cell cycle to repair DNA damage, while CSCs continue to undergo unrestricted proliferation despite lethal DNA damage inducing apoptosis causing cell death. The fractional killing term refers to the proportion of CSCs killed by each dose of ionizing radiation.

Pathology Pearls: Effects of Ionizing Radiation Depend on Several Factors

- **Size of irradiation field, dose fractionation and total dose:** Whole body ionizing irradiation of 100–300 rad can induce acute radiation sickness. While higher doses (500 or >1000 rad) over couple of days can cause bone marrow depression and multi-organs failure leading to mortality.

- **Exposure time and time interval between fractions:** Patient develops bone marrow suppression and mucosal gastrointestinal ulcerations. Radiation-induced second malignancy usually occurs years after therapeutic ionizing radiation.
- **Type of technique of irradiation:** Most aggressive forms of ionizing radiation are α-particle radiation followed by β-particle radiation. X-rays possess highly penetrative effect with minimal tissue damage, but can break weak bonds between nucleic acids and induce chromosomal alterations.
- **Radiation sensitivity of tissue:** Bone marrow, ovaries, testes, lymphatic tissue and gastrointestinal mucosa are highly sensitive to ionizing radiation. Connective tissue, blood vessels and urothelium are medium sensitive to radiation. Relatively radiation resistant tissues include skeletal muscle, cardiac muscle, smooth muscle, cartilaginous tissue, ovarian stroma, corpus luteum, liver, kidneys, pancreas and brain.
- **Individual sensitivity:** Consequences of ionizing radiation therapy are more severe in patients, who have previously received radiotherapy and also depend on the patient's gender and genetic factors.

Therapeutic Radiation-induced Second Malignancies

Radiation therapy is one of the modalities of treatment of malignancies in more than 50% of cases. Therapeutic radiation-induced malignancies (RIMs) are late complications of radiotherapy, seen among the survivors of both adult and pediatric cancers in 15–20% of cases. Second malignancy occurs primarily in the region or near the first irradiated site. Many factors contribute to radiation-induced second malignancies such as age of patient at radiation, volume of irradiated region, type of irradiated organ and tissue, radiation technique and personal history and family history of cancer.

- **External beam radiation therapy** is used to treat various human cancers. Brachytherapy is most often used to treat breast cancers of the head and neck, breast, cervix, prostate and eye. Systemic therapeutic radiation is called radioactive iodine (^{131}I) and is most often used to treat thyroid carcinoma. Another type of systemic therapeutic radiation is called targeted radionuclide therapy or molecular therapeutic radiation is used to treat some patients suffer from advanced prostatic carcinoma and gastrointestinal tract carcinoma, and pancreatic neuroendocrine tumors.
- Genetic susceptibility is a well-known fact for development of cancer. Ionizing radiation or chemotherapeutic agents induce clonal unbalanced cytogenetic abnormalities (i.e. chromosome 5 or 7 and TP53 gene mutation), DNA repair genes, and genes regulating hematopoietic microenvironment leading to increased susceptibility to treatment-related leukemia. This supports the thought of genetic susceptibility to therapeutic radiation-related second malignancy.
- Carcinogenic potential of ionizing radiation is well-known effect. Exposure to ionizing radiation induce single strand and double DNA breaks. Double-strand break can result in gene mutation and subsequent transformation of irradiated cell to cancer stem cell (CSC). Alterations in the DNA repair proteins may also lead to increased risk for development of second malignancies. For example, ataxia-telangiectasia mutated (ATM) protein senses the DNA damage and initiates DNA repair. Mutation in ATM gene can lead to increased cancer susceptibility to develop second malignancy. Recent study revealed that patient with tongue carcinoma undergoing radiation therapy developed second malignancies such as osteosarcoma of mandible, and papillary thyroid carcinoma after many years.

Therapeutic Ionizing Radiation-induced Second Malignancies: Examples

Most common second malignancies develop in patients following therapeutic radiation to treat primary cancers such as cutaneous carcinomas, gastrointestinal carcinomas, head and neck malignancies, lymphomas, breast carcinoma, sarcoma and lung carcinoma in decreased frequency. Mutagenesis of normal tissues is the basis for therapeutic radiation-induced malignancies. Therapeutic ionizing radiation-induced second malignancies are given in Table 6.83.

VIRAL CARCINOGENESIS

Carcinogenesis is a multistep process that results in the transformation of normal cell to CSC, which requires a progression of changes at the cellular, genetic and epigenetic levels that ultimately results in cellular changes for unrestricted CSC division, and development of malignant tumor growth. The essential feature of mode of action of **oncogenic DNA viruses** is addition of new viral DNA into the nucleus of host cells resulting in induction of mutant cells. Mode of action differs in oncogenic DNA and RNA viruses.

- Some oncogenic viruses store their genetic information using DNA and others use single-stranded RNA. Oncogenic viruses are classified as either DNA and RNA viruses.
  - DNA viruses contain two complementary and intertwined strands of nucleic acid (double-helix).
  - Oncogenic RNA viruses include hepatitis C virus (HCV) and HTLV-I and HTLV-II.
  - Examples of oncogenic DNA viruses include HPV (16, 18), Epstein-Barr virus (EBV), HBV, and

Table 6.83 Therapeutic ionizing radiation-induced second malignancies

Ionizing Radiation given for Primary Tumors	Post-radiation-induced Second Malignances in the Vicinity of Organs
Head and neck and mediastinal malignancies	Papillary thyroid carcinoma (8–20 years after radiotherapy), laryngeal carcinoma (20–40 years after radiotherapy), lung carcinoma and prostatic carcinoma
Breast carcinoma	Lung carcinoma, esophageal carcinoma, breast angiosarcoma, and contralateral breast carcinoma after many years
Prostatic carcinoma	Urinary bladder carcinoma and rectal carcinoma
Cervical carcinoma	Colorectal carcinoma, carcinoma of anal canal, and malignancies of uterus and other pelvic structures even after long follow-up
Pelvic malignancies	Colorectal rectal carcinoma (50 Gy-median, dose) and carcinosarcoma of uterine body (months to five years)
Non-Hodgkin's lymphoma (NHL)	Solid malignancies and leukemia
Pediatric malignancies	Glioma, meningioma and gastrointestinal malignancies
Chondrosarcoma of bone	Fibrosarcoma, osteosarcoma with chondroid differentiation, and chondrosarcoma of urinary bladder
Basal cell carcinoma of skin	Brain sarcoma
Desmoid tumor	Fibrosarcoma

HHV-8, which integrate their genomes into host cells and enter a period of latency.

- Both oncogenic RNA and DNA viruses play an important role in carcinogenesis by disrupting cellular genes in the host. Oncogenic viruses associated human cancers are given in Table 6.84.

- The mode of virally induced tumors can be divided into acute transforming viruses, and slowly transforming viruses.
  - Acute transforming oncogenic DNA and RNA viruses induce tumorigenesis as they carry an overactive viral-oncogene. The infected cell is transformed to CSCs as soon as v-onc is over-expressed. An example of an acute transforming virus is the Rous sarcoma virus (RSV), that carries the v-src oncogene.
  - Slowly transforming viruses induce slow tumor growth, since they do not carry any viral oncogenes,

Table 6.84 Oncogenic viruses associated human cancers

Virus	Type of Virus	Virus Associated Malignant Tumors
Epstein-Barr virus (EBV)	DNA virus	Burkitt's lymphoma, Hodgkin's disease (mixed cellularity variant), nasopharyngeal carcinoma, extranodal marginal zone B cell/T cell NHL, extranodal NK/T cell lymphoma (nasal type), CNS lymphoma in AIDS, primary CNS diffuse large B cell lymphoma (DLBCL), gastric MALT lymphoma, follicular dendritic cell sarcoma, primary effusion lymphoma, lymphomatoid granulomatosis, post-transplant lymphoproliferative disease and gastric and smooth muscle tumors
Human herpesvirus 8 (HHV-8)	DNA virus	Kaposi sarcoma in AIDS, primary effusion lymphoma, Castle disease
Human papillomavirus (e.g. HPV 16, 18)	DNA virus	Cervical carcinoma (70%), carcinoma of vulva and vagina, anorectal carcinoma, oropharyngeal carcinoma, penile carcinoma, head and neck cancers
Hepatitis B (HBV)	DNA virus	Hepatocellular carcinoma
Merkel cell polyomavirus	DNA virus	Merkel cell carcinoma
Hepatitis C persists in hepatocytes	RNA virus	Hepatocellular carcinoma, lymphoplasmacytic lymphoma
Human T cell lymphotropic virus 1 (HTLV-I retrovirus) persists in T cells and B cells	RNA virus	Adult T cell leukemia/lymphoma, primary effusion lymphoma
Human T cell lymphotropic virus 1 (HTLV-II retrovirus) persists in T cells	RNA virus	Hairy cell leukemia

which induce tumor growth by transcriptionally acting proto-oncogenes particularly the long terminal repeat (LTR) in the proto-oncogenes.

- Oncogenic viruses utilize a variety of carcinogenic mechanisms to transform normal cell to CSC: (a) oncogenic viruses express viral oncogenes, that directly transform an infected normal cell to CSC; and (b) several oncogenic viruses such as human papillomavirus (HPV), Epstein-Barr virus (EB virus), HCV, HTLV-1, KSHV and SV40 encode oncoproteins that employ several mechanisms to inactivate two of the major regulators of genome stability, cell viability and cell cycle; namely the TP53 tumor suppressor gene product (p53 protein) and retinoblastoma (RB) gene product (pRB).

Pathology Pearls: Viral Carcinogenesis

- Virus integrates into the cellular genome and disrupts cellular genes.
- Virus carries mutated cellular proto-oncogene (oncogenes).
- Viral gene products act as transcription factors.
- Viral gene products inactivate cell cycle regulatory proteins (p53, pRB).
- Viral infection results in immunodeficiency state.

ONCOGENIC RNA VIRUSES ASSOCIATED CANCERS

RNA viruses are also known as 'retroviruses' and most common cause of emerging diseases in human beings, attributable to the high mutation rate in RNA viruses compared to DNA viruses. Oncogenic RNA viruses include hepatitis C (HCV), and human T lymphocyte virus type I and II (HTLV-I and HTLV-II). The range of following infection with oncogenic RNA is shown in Fig. 6.102.

- Oncogenic RNA viruses generally undergo replication by binding to a cellular receptor and causing transcription of genomic RNA into proviral DNA and integration of proviral DNA into chromosomal DNA. Latency may be established at this point. Transcription may occur to generate new genomes and messenger RNA. RNA virus is released by budding usually without cytopathology. Carriers of RNA viruses are symptomatic. There is usually a latency period of 20–30 years following primary infection with HTLV-I.
- Francis Peyton Rous in 1910 isolated retrovirus from a chicken tumor later named the Rous sarcoma virus. Non-oncogenic retroviruses possess no oncogenes and hence direct their own life cycle. For example, HIV infects CD4+ helper T cells and destroys them.
  - **Transduction:** Retroviral oncogenes are formed from proto-oncogenes (cellular genes), which cause cancer. The process is called transduction. Genomic DNA sequences in retroviruses are known as viral oncogenes (v-onc), genomic DNA sequences in human beings are known as cellular oncogenes (c-onc).
  - **Genes in RNA viruses:** Most retroviruses contain three types of genes: (a) gag (group antigen) codes for protein core, (b) pol (polymerase) codes for reverse transcriptase enzyme for proviral integration, and (c) env (envelope) codes for envelope glycoproteins.
  - **Entry of retrovirus:** Oncogenic retroviruses (v-onc) enter the host cell by reverse transcriptase and mutate genome. Proviral DNA and host chromosome DNA crossover and are joined by recombination.
  - **Transcription:** Host RNA polymerase transcribes proviral DNA and produces viral mRNAs required for the virus life cycle. Proviral promoters can activate transcription of nearby genes.
  - **Oncogenic RNA viruses induced carcinogenesis:** Oncogenic RNA viruses are also called transducing viruses. Most transducing viral oncogenes are defective and cannot replicate independently. Viral encoded TAX protein induces carcinogenesis by different mechanisms: (a) TAX protein inactivates $p6^{INK4a}$ and enhances cyclin deactivation resulting in increased DNA replication, (b) TAX protein interferes with DNA repair mechanism resulting

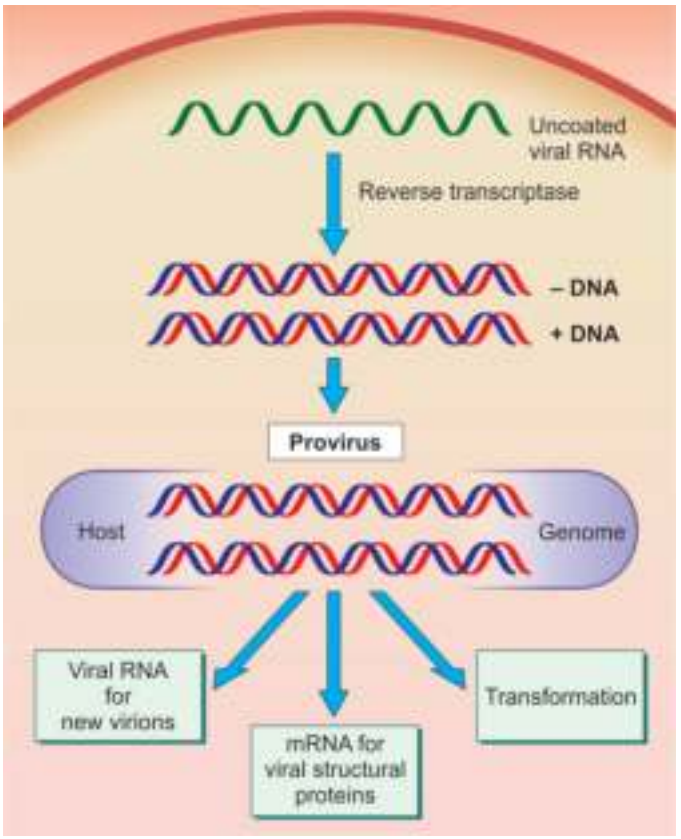


Fig. 6.102: The range of following infection with oncogenic RNA virus.

in genomic instability, and (c) TAX protein also activates NF-κB transcriptional factor to form monoclonal CSCs and malignant phenotype.

Human T Cell Leukemia Virus I and II (HTLV-I and II)

Human T cell leukemia virus I (**HTLV-I**) is linked to development of human **T cell leukemia/lymphoma** endemic in Southern islands of Japan and Caribbean basin, and primary effusion lymphoma. On the contrary, **HTLV-II** is linked to **hairy cell leukemia**. HTLV-I differs from other oncogenic retroviruses, which lacks a viral oncogene, and does not integrate into specific sites of the human genome to disrupt proto-oncogenes.

HTLV-I and II Infection-linked T Cell Leukemia/Lymphoma

Human T cell leukemia virus I (HTLV-I) infects CD4+ helper T cells via sexual intercourse, blood transfusion or breastfeeding. Only 1% of infected persons will develop T cell leukemia/lymphoma and only after a period of 20–30 years.

- During HTLV replication, **TAX gene** encodes TAX protein, which acts through binding host cAMP response protein (CREB) to recruit histone acetyl transferases to the TAX-response element 1 (**TRE1**) to promote transcription of host genes (c-Fos).
- TAX protein alters function of cellular proteins resulting in activation, proliferation and survival of infected CD4+ helper T cells. TAX protein can also inactivate TP53 gene product (p53 protein), which results in DNA repair failure induces cell cycle progression.
- TAX protein also alters the expression of many host cellular proteins associated with cell cycle progression through G1 phase by disabling checkpoints of cell cycle. TAX protein also inhibits apoptosis and stimulates telomerase expression resulting in unrestricted cell proliferation, and development of human T cell leukemia/lymphoma.
- The host cellular alterations confer a fitness advantage for viral replication, which cause chromosomal instability, and accumulation of host genomic mutations.

Clinical Features of T Cell Leukemia/Lymphoma

Patient presents with hypercalcemia, lymphadenopathy, skin lesions caused by infiltration of leukemic cells, spleen and liver involvement and immune suppression.

Hepatitis C Virus (HCV)-induced Cirrhosis and Hepatocellular Carcinoma

Hepatitis C virus (HCV) affects persons across world. It is mainly acquired by blood transfusions, and intravenous drug abuse. It may be transmitted by transplacental infection of the fetus, breastfeeding and co-infection with HBV, but is not efficiently sexually transmitted. In contrast to HBV, HCV cannot integrate into the human genome. Majority of infected patients are unable to clear the HCV infection and develop chronic hepatitis, that induce hepatic lesions frequently associated with steatohepatitis, progressive fibrosis, and cirrhosis over 20–40 years involving a variety of signaling pathways.

- Hepatitis C virus (**HCV**) is major cause of cirrhosis that may progress to hepatocellular carcinoma frequently together with HBV. Hepatocellular carcinoma risk appears to be dependent on viral genotype. HCV genotypes 3, 25, 26 and 27 are more carcinogenic.
- Hepatocarcinogenesis is a multistep process that may last for years, which involves progressive accumulation of different genetic mutations leading to malignant transformation of hepatocytes.
  - HCV core proteins can directly upregulate mitogenic signaling pathways, inhibit apoptosis and induce reactive oxygen species (ROS) production. Reactive oxygen species can cause genomic instability by inducing several gene mutations (e.g. TP53, CTNNB1 and TERT).
  - HCV also triggers persistent chronic inflammation in liver and production of various lymphotoxins (LT-α and LT-β), which are linked to hepatocellular carcinoma.
  - Chronic inflammation in liver exacerbates reactive oxygen species (ROS), which are considered a main source of several genetic mutations. Reactive oxygen species induce TGF-β signaling pathway activates hepatic stellate cells leading to fibrogenesis. TGF-β together with TLR4 plays a pivotal role in epithelial–mesenchymal transition (EMT).
  - HCV dysregulates host lipid metabolism resulting in accumulation of fat in the hepatocytes in many patients. HCV can also induce angiogenic and metastatic signaling pathways.
  - Epigenetic regulatory genes are dysregulated in hepatocellular carcinoma, which can result in permanent modification of gene expression. Recent studies revealed polymorphisms in the DEPDC5 and MICA genes increase the risk for hepatocellular carcinoma.

Clinical Features

Majority of patients infected with HCV remain asymptomatic. Symptomatic patients may experience jaundice, malaise, nausea, dark urine and right upper quadrant pain 2–12 weeks after exposure to HCV. Patients with acute HCV infection have moderate to high serum aminotransferase levels. Chronic HCV infection can progress to cirrhosis and hepatocellular carcinoma.

ONCOGENIC DNA VIRUSES ASSOCIATED CANCERS

DNA virus has a genome-made of deoxyribonucleic acid (DNA), that replicates using DNA-dependent DNA polymerase. DNA viruses can be grouped into two classes: **double-stranded (ds) viruses** (e.g. polyomaviruses, adenoviruses and herpesviruses) and **single-stranded (ss) DNA viruses.**

- Most double-stranded DNA viruses replicate within the host cell nucleus. DNA viruses linked to human cancers include Epstein-Barr virus (EBV), human papillomavirus (HPV 16, 18), hepatitis B virus (HBV) and human herpesvirus 8 (HHV-8).
- DNA viruses integrate viral DNA into host genome resulting in expression of viral mRNA coding for specific proteins in host cells, which target key cellular regulatory proteins (pRB and p53 proteins encoded by RB and TP53 tumor suppressor genes).

Epstein-Barr Virus (EBV)-induced Human Cancers

Epstein-Barr virus (EBV) has been linked to various human cancers. EB virus enters through binding with CD21 and infects B cells, one of the principal infection-fighting white blood cells of the immune system.

- EBV viral genes activate the transcription of latent membrane protein 1 (**LMP-1**), which induces activation of NF-κB and JAK/STAT (Janus/kinase) signaling pathway that promotes B cell survival.
- EB virus does not replicate within B cells; instead, it alters genetic code of B cells and transforms these cells into lymphoblasts, which have an indefinite life span. In other words, EB virus renders lymphoblasts immortal. Epstein-Barr virus-linked human cancers are given in Table 6.85.

Table 6.85 Epstein-Barr virus-linked human cancers

Burkitt's lymphoma
Hodgkin's disease (mixed cellularity variant)
Nasopharyngeal carcinoma
Extranodal marginal zone B cell/T cell non-Hodgkin's lymphoma
Extranodal NK/T cell lymphoma (nasal type)
Central nervous system lymphoma in AIDS
Primary central nervous system diffuse large B cell lymphoma (DLBCL)
Gastric MALT lymphoma
Follicular dendritic cell sarcoma
Primary effusion lymphoma
Lymphomatoid granulomatosis
Post-transplant lymphoproliferative disease
Gastric carcinoma, and smooth muscle tumors

EBV Infection-associated Burkitt's Lymphoma

The hallmark of EBV infection-linked Burkitt's lymphoma is the translocation between the Myc gene and one of the immunoglobulins (Ig) heavy or light chain loci, that results in overexpression of the **Myc gene**, which codes for a transcription factor, that modulates genes related to cell cycle progression, apoptosis, and cellular transformation.

- EBV infection may lead to Myc gene mutation that allows human B cells to proliferate indefinitely.
- Depending on the stage of B cell development, EBV infection can cause different forms of Burkitt's lymphoma: **endemic Burkitt's lymphoma** (4–7 years of age group), **sporadic Burkitt's lymphoma** (worldwide), and immunodeficiency (HIV/AIDS)-associated Burkitt's lymphoma. Molecular genetic alterations t(8;14), t(2;8) and t(8;22) are demonstrated in patients with Burkitt's lymphoma.

EBV Infection-associated Hodgkin's Disease

Hodgkin's disease is a malignant neoplasm of single/multiple lymph nodes. Diagnostic criteria include presence of neoplastic component of Hodgkin's disease and presence of Reed-Sternberg cells constituting 1–10% of cell population in Hodgkin's disease. Up to 40% of Hodgkin's disease cases are associated with the Epstein-Barr virus (EBV) infection.

- EBV-associated Hodgkin's disease is more frequent in cases with mixed cellularity histology affecting children and older adults in developing countries. EB virus may directly trigger tumorigenesis through constitutive expression of **Myc oncogene**.
- Clonal viral genomes can be demonstrated in Reed-Sternberg cells, the tumor component of Hodgkin's disease (mixed cellularity) in 50% of cases. EBV may actually protect the Reed-Sternberg cell from apoptosis, and immune response of natural killer T cells and mutation in the TP53 gene.
- Circulating EBV-DNA may serve as a biomarker to monitor response to therapy, and eventually, EBV will become a target for therapeutic intervention in Hodgkin's disease.

EBV Infection-associated Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma is an Epstein-Barr virus (EBV)-associated malignancy that is common in Thailand, which might be a result of virus latency in nasopharyngeal cells.

- EBV is transmitted from the carrier through the saliva and enters the host via oropharyngeal region where it can infect '**naïve tonsillar B cells**' via interaction of the viral envelope glycoprotein gp350 and CD21 expression on B cells.

- Epstein-Barr virus nuclear antigen 2 (**EBVN2**) transactivates latent membrane protein 1 (**LMP1**) leading to phenotypic changes in B cells. The genetic mutations induced by EBV infection depend on the viral type and strain. Tobacco smoking has been associated with nasopharyngeal carcinoma possibly due to reactivation of latent EBV.

EBV Infection-associated T Cell/NK Cell Lymphoproliferative Diseases

EBV-associated T cell/NK cell lymphoproliferative diseases result from ectopic infection of T cell or natural killer (NK) cells with Epstein-Barr virus.

Human Papillomavirus (HPV)

Human papillomavirus (HPV 16, 18) is linked to cervical carcinoma. HPV is the most common sexually transmitted virus in the world. The HPV genome is divided into three regions: (a) noncoding upstream regulatory region, (b) 'early region', (c) and 'late region'. The early region of HPV is involved in viral replication and oncogenesis. The late region of HPV encodes structural proteins for the viral capsid. Oncogenic risk of anogenital human papillomavirus is given in Table 6.86.

HPV 16 and 18 Infection-associated Cancers

HPV 16 and 18 are high-risk types known to significantly increase the risk for cervical, vaginal and vulvar cancers, as well as penile cancer in men. HPV 16 and 18 can infect only the immature squamous epithelial basal cells at the squamocolumnar junction of cervix resulting in a cytopathic effect, 'koilocytic atypia', consisting of nuclear atypia and a cytoplasmic perinuclear halo. HPV does not infect the mature superficial squamous cells that cover the ectocervix, vagina, and vulva.

HPV 16 and 18 Infection-associated Cervical Carcinoma

HPV integrates its DNA into the host genome and induces disruption of E2 protein resulting in significantly, increased expression of the E6 and E7 oncoproteins encoded by E6 and E7 oncogenes, which interfere with the function of TP53 and RB respectively knocking those proteins out of action, and allowing the cell to grow and divide. These cellular alterations cause deregulated cell cycle by interrupting checkpoint proteins (p53 and pRB), inhibition of apoptosis, increased genomic instability, cell proliferation, immortalization and transformation of infected cells to CSCs. E6 and E7 oncoproteins of HPV-16 and HPV-18 bind to the pRB and p53 proteins very tightly; in contrast, the E6 and E7 proteins of HPV-6 and HPV-11 (low-risk types) bind pRB and p53 proteins with low affinity. Principles of HPV E6/E7 oncogene activity in cervical carcinoma are shown in Fig. 6.103.

- **E6 protein of high-risk HPV:** E6 protein binds to the TP53 gene product (p53 protein known as guardian of the genome) tumor suppressor protein and targets it for ubiquitin-mediated degradation of p53. The E6 protein increases telomerase activity in keratinocytes through increased transcription of the telomerase catalytic subunit gene (**TERT**) resulting in increased receptor tyrosine kinase activity and eventually genomic instability and malignant tumor.
- **E7 protein of high-risk HPV:** E7 protein binds to retinoblastoma protein (pRB) and displaces E2F transcription factor. Now displaced free E2F transcription factor binds to the promoters of genes (like c-Myc) that cause the cell to enter the cell cycle. The E7 oncoprotein increases the activity of cyclin D/CDK4 as a result of inactivation of CDK inhibitors (e.g. p21 and p27) resulting in genome instability, and

Table 6.86 Oncogenic risk of anogenital human papillomavirus

Oncogenic Risk for Cervical Cancer	Human Papillomavirus	Histologic Features
High-risk anogenital human papillomavirus	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 59, 66, 68	▪ High-grade squamous intraepithelial lesion (HSIL) ▪ Koilocytosis occasional present ▪ Aneuploidy usually ▪ Abnormal mitoses frequent ▪ Undifferentiated cells and abnormal mitoses in upper two-thirds of cervical epithelium
Low-risk anogenital human papillomavirus	6, 11, 42, 43, 44, 53	▪ Low-grade squamous intraepithelial lesion (LSIL) ▪ Koilocytosis frequently present ▪ Diploid or polyploid most often present ▪ Abnormal mitoses absent ▪ Undifferentiated cells, and bipolar mitoses in lower third of cervical epithelium

Medium risk human papillomavirus includes 33, 35, 39, 51, 52, 56, 57, 58, 59, 68

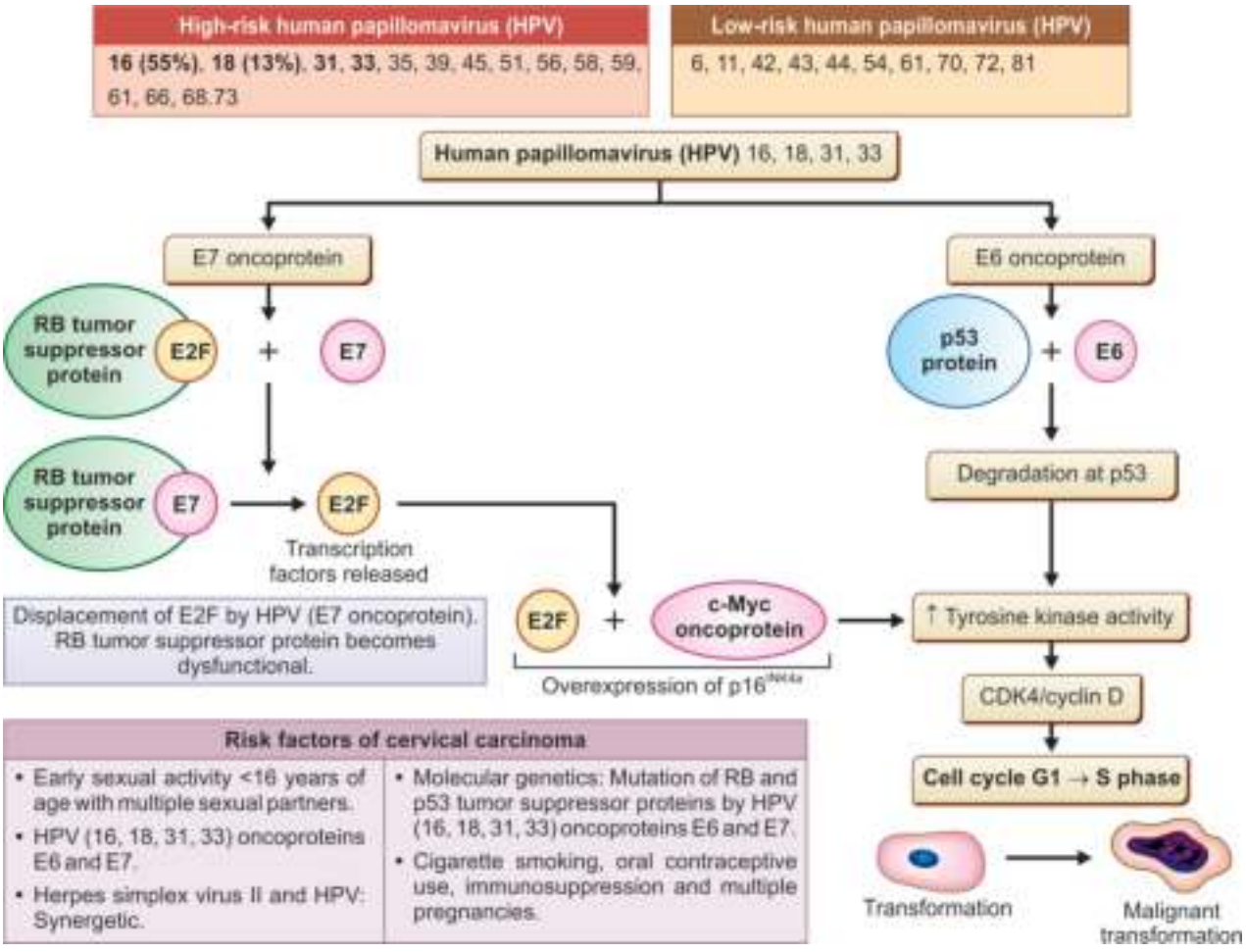


Fig. 6.103: Principles of HPV E6/E7 oncogene activity in cervical carcinoma. E6 protein of HPV binds to the TP53 gene tumor suppressor product (p53 protein known as guardian of the genome) tumor suppressor protein and targets it for ubiquitin-mediated degradation. E7 protein of HPV inhibits retinoblastoma protein (pRB) tumor suppressor.

eventually malignant tumor. The mutagenic potential along with dysregulated cell cycle allows HPV to induce cervical carcinogenesis in host cells.

Cervical Carcinoma Screening, Vaccination and Molecular Techniques

In countries with accessible preventive services, there has been a steep decline in mortality caused by cervical carcinoma due to the development and widespread acceptance of cervical carcinoma screening (Papanicolaou smear).

- Further, the HPV vaccination is now available for women up to 45 years of age, which prevents cervical carcinoma in 70–80% of cases.
- Therapeutic vaccines and gene therapy are primarily directed against E6 and E7 oncoproteins. The link between HPV and cervical carcinoma has led to the development of molecular methods often based on the detection of E6 and E7 oncoproteins, for screening and diagnosis.

Human Herpesvirus 8-induced Kaposi Sarcoma

Kaposi sarcoma is the most common locally aggressive vascular cancer caused by human herpesvirus 8 (HHV-8) in immunocompromised persons with HIV-positive status.

Human Herpesvirus 8 Infection: Pathogenesis

Human herpesvirus 8 infects vascular endothelial cells and modulates signaling pathways, that regulate cell proliferation, gene expression, and metabolism. In HIV/AIDS-related Kaposi sarcoma, most common sites are mucosal surfaces, skin, face, lower extremities, visceral and cutaneous lesions in HIV infected homosexual or bisexual persons.

Clinical Features

Patient presents with cutaneous violaceous patch, plaque, macule or tumor mass usually on lower extremities. Lymphedema is demonstrated in occasional patient. Involvement of viscera and lymph nodes

presents with hemorrhagic mass. In **endemic (African) Kaposi sarcoma**, young male patient presents with bulky lymphadenopathy and soft tissue tumor. Disease has indolent clinical course in adults; and aggressive clinical course in African children with bulky lymphadenopathy.

Hepatitis B Virus-induced Hepatocellular Carcinoma

Hepatitis B virus (HBV) infection is a major risk factor for development of hepatocellular carcinoma (**HCC**), which is endemic in South-East Asia and sub-Saharan Africa and leading cause of cancer deaths. The precise role of hepatitis B virus in causing hepatocellular carcinoma is unknown, but evidence suggests that viral proteins disrupt signal transduction and thereby deregulate cell growth. HBV-related chronic inflammation **cytokines** and reactive oxygen species (**ROS**) play important roles in the hepatocyte cell proliferation and fibrosis leading to development of hepatocellular carcinoma.

- HBV-DNA integrates into the host genome and replicates within endoplasmic reticulum in the hepatocytes and alters expression of cellular genes that are important in cell growth and differentiation. Some of the gene products favor malignant transformation.
- HBV genes have been observed in infected tissues including truncated pre-S2/S, HBV-X gene and a novel spliced transcript of HBV (hepatitis B spliced protein). The proteins expressed from these integrated genes have been shown to induce genomic instability and cell cycle deregulation contributing to hepatic carcinogenesis.
- HBV-X protein binds TP53 gene and interferes with its function. Several cell mediators (e.g. p53, FAS, TNF-α and TGF-β) decrease apoptosis of HBV-infected hepatocytes. **HBsAg** is a popular marker to establish diagnosis of active hepatitis B.

Pathology Pearls: Hepatitis B (HB) and Hepatitis C (HC) Linked to Hepatocellular Carcinoma

Immune-mediated Mechanism

- Immune response
- Chronic hepatocellular damage and regeneration
- Enhanced mutagenesis and cell proliferation
- Development of hepatocellular carcinoma

Molecular Mechanism

- Alteration of DNA repair system
- Alteration of centrosome duplication mechanism
- Viral encoded oncoprotein
- Altered cytokine expression and modulation of microenvironment
- Development of hepatocellular carcinoma

BACTERIAL CARCINOGENESIS

Helicobacter pylori is the first bacterium to be termed a definite cause of human cancers by World Health Organization. Other bacteria such as *Borrelia burgdorferi* and *Campylobacter jejuni* are also linked to human cancers.

HELICOBACTER PYLORI-INDUCED GASTRIC CARCINOGENESIS

World Health Organization has designated *Helicobacter pylori*, a potential human carcinogen, that can induce gastric adenocarcinoma in distal region, and mucosa-associated lymphoid tissue (**MALT**) low-grade B cell NHL by two mechanisms: induction of chronic inflammation and production of reactive oxygen species (ROS), and N-nitroso compounds, that can react with mutated DNA.

- *Helicobacter pylori* induces sequential changes in the stomach, i.e. chronic gastritis, gastric atrophy, intestinal metaplasia, dysplasia, early gastric carcinoma and advanced gastric carcinoma.
- *Helicobacter pylori* itself is not mutagenic but acts solely as promoter by creating an environment where chronic inflammation induces DNA damage, DNA repair failure, DNA methylation and genomic instability, which are emerging hallmarks of cancers.
- *Helicobacter pylori*—Cag PAI gene codes for oncogenic CagA pore-forming cytotoxin that binds to epithelium via interaction with protein–tyrosine phosphatases resulting in morphologic changes of the cell, and influences various intracellular signal transduction pathways such as the RAS/mitogen-activated protein kinase/extracellular signal-regulated tyrosine kinase pathway, nuclear factor kappa B (NF-κB) pathway and β-catenin pathway and proteins that leads to aberrant DNA methylation, decreased expression of DNA repair genes, increased expression of activation-induced cytidine deaminase, and induction of double-strand DNA breaks (DSBs), unrestricted CSC division, epithelial–mesenchymal transition (**EMT**), angiogenesis, invasion and metastasis. Because bacterial infections can be cured by antibiotics, identification of bacterial etiology of human cancers could have important amplifications for cancer prevention.
- The nature of the inflammatory response to *Helicobacter pylori* is also determined by proinflammatory **IL-1** gene cluster polymorphisms in several host genes encoding cytokines, and cytokine receptors, which induce intense inflammatory response leading to

hypochlorhydria/achlorhydria and high-risk for gastric adenocarcinoma.

BORRELIA BURGDORFERI-INDUCED BREAST CARCINOGENESIS

One of the known human pathogens *Borrelia burgdorferi* is the causative agent for Lyme disease and is migrate through mammalian tissue and persists in joints and skin. *Borrelia burgdorferi* has been shown to be present in breast cancer and is associated with poor prognosis. *Borrelia burgdorferi* can invade breast tissue and increase risk for breast carcinoma.

CAMPYLOBACTER JEJUNI-INDUCED COLORECTAL CARCINOGENESIS

All *Campylobacter jejuni* species produce a genotoxin, which induce breaks in DNA double-strands and increase risk of cancer especially in the gastrointestinal tract. *Campylobacter jejuni* promotes colorectal tumorigenesis through the action of cytolethal distending toxin.

PARASITE-INDUCED CARCINOGENESIS

Only three helminths (e.g. *Schistosoma haematobium*, *Opisthorchis viverrini* and *Clonorchis sinensis*) are directly linked to carcinogenesis.

- *Schistosoma haematobium* resides in the venous network around urinary bladder, where it causes foci of squamous metaplasia of urothelium leading to urinary bladder squamous cell carcinoma in Egypt population. Patient initially presents with sudden painless hematuria and in later stage urinary bladder may become irritable, painful urination. Cystoscopy reveals exophytic (cauliflower-like) or ulcerated cancerous lesion.
- *Opisthorchis viverrini*, also known as South-East liver fluke, is a major cause of cholangiosarcoma especially in North-Eastern Thailand. The definitive host (mammals including human beings) becomes infected by ingestion of undercooked fish containing metacercariae. After ingestion, the metacercariae excyst in the duodenum and ascend through the ampulla of Vater into the biliary ducts, which lay eggs after 3–4 weeks. The adult fluke resides in the biliary and pancreatic ducts of the host, where it attaches to the mucosa, and induces cholangiocarcinoma.
- *Clonorchis sinensis* (liver fluke) is transmitted through consumption of raw or undercooked vegetables. It resides in the biliary tree and may result in cholangiocarcinoma in far east population. It also causes hepatocellular carcinoma and pancreatic carcinoma.

HORMONAL CARCINOGENESIS

Hormones are chemical messengers that travel in bloodstream to tissues/organs. Hormones (natural or synthetic) act by binding to receptors on the cell surface and influence biological processes through signaling pathways.

- There are major classes of hormones based on chemical structures, which include: (a) peptide/protein hormones, (b) amino acid or fatty acid-derived hormones and (c) steroid hormones (e.g. estrogen, progesterone and testosterone).
- Excessive hormonal stimulation of cells increases the risk for gene mutations, and subsequent unrestricted cell proliferation of clones of mutated cells.
- Hormones are powerful carcinogens and considered a 'complete carcinogen' because of their ability to both initiate and promote the development of cancers of breast, endometrium, ovary, testis, prostate, thyroid, and bone (osteosarcoma), share a unique mechanism of carcinogenesis.
- Excess of endogenous and exogenous hormones drive unrestricted cell proliferation, and thus the opportunity for the accumulation of random genetic mutations. The emergence of a malignant phenotype depends on a series of somatic gene mutations, that occur during the cell division.
- Endogenous hormones include estrogen, and progesterone and testosterone. Estrogen regulates development of female genital system and secondary sex characters. Progesterone hormone synthesized by corpus luteum in the ovary regulate menstrual cycle and maintenance of early stages of pregnancy.
  - Estrogen hormone is linked to many women cancers (e.g. endometrial carcinoma, breast carcinoma, ovarian carcinoma).
  - Testosterone hormone is mostly synthesized in the testis, and small quantity by adrenal glands. Testosterone regulates changes in boys to go through puberty to become men such as sex drive, spermatogenesis (fertility), bone mass, fat distribution, muscle mass, strength, and erythropoiesis. Testosterone has been implicated in the genesis of prostatic adenocarcinoma.
- Exogenous steroid hormones in the form of oral contraceptives and hormonal replacement therapy (**HRT**) are frequently given to women, that can influence unrestricted cell proliferation, and increase risk of breast carcinoma, ovarian carcinoma, endometrial carcinoma and cervical carcinoma.

ROLE OF HORMONES IN BREAST CARCINOMA

Increased exposure to steroid hormone estrogen to breast tissue in females increases risk for development

of breast carcinoma. The early onset of menstruation, late first pregnancy, obesity, late menopause and the use of oral contraceptives increase risk exposure of breast tissue to estrogen, stimulate unrestricted CSC proliferation, and development of breast carcinoma.

- The primary source of estrogen in postmenopausal women is from the conversion of androstenedione to estrone in the adipose tissue; thus, increased synthesis of estrone in adipose tissue is linked to breast carcinoma.
- Obesity is also associated with decreased serum sex hormone binding globulin (**SHBG**) and increased proportions of free and albumin-bound estrogens.
- Oral contraceptives (birth control pills) are medications that contain synthetic versions of the natural female hormone's estrogen and progesterone, and prevent pregnancy by inhibiting ovulation, and also by preventing spermatozoa to penetrate through cervix. Various studies have provided consistent evidence that increases the risk for breast carcinoma, who use oral contraceptives.
- Combined hormonal replacement therapy (HRT) in postmenopausal women (estrogen plus progestin—synthetic version of the female progesterone hormone) can increase a woman's risk for breast carcinoma.
- Thus, interruption of estrogen hormone induced stimulus is done through anti-hormonal therapy in breast cancer patients. Estrogen receptor-positive breast carcinoma is treated by '**tamoxifen**' to interfere with attachment of steroid hormones to receptors on mammary glands and antagonize the action of estrogen hormone, and thus interrupting unrestricted CSC proliferation. Anti-hormonal therapies have been effective in inhibiting progression of breast cancer disease and thereby increasing the time to recurrence and mortality.
- Polymorphisms in genes such as 17β-hydroxysteroid dehydrogenase 1 (**HSD17B1**), cytochrome P459c17α (**CYP17**), aromatase (**CYP19**) and estrogen receptor (ER)-related to estrogenic action, metabolism and transport can increase risk for breast carcinoma.

HORMONAL REPLACEMENT THERAPY (HRT) IN PREGNANT WOMEN LINKED TO CLEAR CELL CARCINOMA OF VAGINA IN THEIR DAUGHTERS

Diethylstilbestrol (DES) is a form of estrogen that is administered to some pregnant women to prevent miscarriages, premature labor and other pregnancy-related problems. Their daughters have an increased risk for cancers of the vagina and cervix. Patient initially develops **vaginal adenosis** characterized by mucosal columnar epithelium-lined crypts in areas normally lined by stratified squamous epithelium. Vaginal adenosis is thought to be a precursor of clear cell adenocarcinoma of vagina in their daughters.

ROLE OF HORMONES, OBESITY AND LEPTIN IN ENDOMETRIAL CARCINOMA

Endometrial carcinoma is most common malignancy across world. Tumor is usually low-grade associated with good prognosis. Tumor may show microsatellite instability and mutations in PTEN, PIL3CA, K-RAS and CTNNB1. Majority of patients with endometrial carcinoma present with vaginal discharge, and abnormal uterine bleeding in premenopausal or postmenopausal women. A routine Papanicolaou (Pap) smear examination shows endometrial cells in postmenopausal women. Endometrial biopsy is performed to establish diagnosis.

Role of Steroid Hormones in Endometrial Carcinoma

Estrogen and progesterone are steroid hormones that activate receptors in target endometrial tissue to influence endometrial growth. According to the unopposed estrogen hypothesis, increased levels of estrogen and decreased levels of progesterone cooperate to increase the mitotic activity in endometrial cells.

- Sustained endometrial cell proliferation and greater rates of DNA mutation ultimately result in transformation to CSCs. Similarly, women who undergo estrogen replacement without progesterone have an increased risk for endometrial carcinoma.
- Unopposed estrogen partially explains the risk factors for endometrial carcinoma, which include obesity, diabetes mellitus, polycystic ovarian syndrome, nulliparity, and hormonal replacement therapy. In pre-menopausal women, obesity is thought to operate through increased anovulatory menstrual cycles and associated progesterone insufficiency.

Role of Obesity in Endometrial Carcinoma

Obesity has been associated with 2–5-fold increased risk of endometrial carcinoma in post-menopausal women. The mechanism involves conversion of androstenedione to estrone in adipose tissue by the enzyme aromatase present in adipose tissue and fibrovascular stroma.

- Aromatase activity is enhanced by increased adrenal gland synthesis of cortisol, and androgens in obese women.
- Estrone can also be decreased by the enzyme 17β-hydroxysteroid dehydrogenase to form the most potent estrogen and estradiol.

- In obese women, plasma estradiol levels are also elevated due to decrease in sex hormone binding globulin (SHBG) level.

Role of Leptin in Endometrial Carcinoma

Leptin is a protein encoded by the obese gene (Ob) that binds to its receptor (ObR) belonging to cytokine family and induces unrestricted endometrial CSC proliferation by activation of cyclooxygenase-2 through the JAK2/STAT, RAF/MEK/ERK mitogen-activated protein kinase (MAPK) signaling pathway and phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR (mammalian target of rapamycin) signaling pathway.

- Angiogenesis in endometrial carcinoma occurs through activation of vascular endothelial growth factor (VEGF), and VEGF receptor (VEGFR) resulting in increased malignant tumor growth.
- Overexpression of leptin and ObR in endometrial carcinoma is associated with invasion and metastasis. Mechanistically, leptin enhances aromatase expression and increases estradiol synthesis in endometrial CSCs.

ROLE OF HORMONES IN PROSTATIC ADENOCARCINOMA

The causes of prostatic adenocarcinoma are not entirely certain, but it is postulated that steroid androgenic hormones such as testosterone and more potent dihydrotestosterone exert their effects by binding to the androgen receptor (AR) in the cytoplasm of cells causing oncogenic genetic alterations that play a major role in its development. Polymorphisms in genes such as androgen receptor (AR), steroid 5β-reductase type II (SRD5A2), P459c17α (CYP17), and 3β-hydroxysteroid dehydrogenase (HSD3B2)-related to action, metabolism and transport can increase risk for prostatic adenocarcinoma.

ROLE OF ONCOGENES AND TUMOR SUPPRESSOR GENES IN CARCINOGENESIS

Tissue homeostasis depends on the regulated cell division and apoptosis (programmed cell death) of each of its constituents except stem cells. A malignant tumor arises as a result of unrestricted cancer stem cell (CSC) proliferation and failure of apoptosis. DNA stability genes monitor and maintain genomic integrity, and their loss of function results in accumulation of gene mutations. Cellular proto-oncogenes are normal growth-promoting genes which stimulate cell division. Tumor suppressor genes encode proteins that prevent development of malignant tumor by reliably controlling cell division in check, promoting apoptosis and suppressing metastasis. Alterations in proto-oncogenes and tumor suppressor genes induce malignant tumors. Gain-of-function mutations that convert proto-oncogene to oncogene act dominantly; that is, mutation in only one of the two alleles is sufficient to induce malignant tumor. On contrary, recessive loss-of-function mutations in tumor suppressor genes possess oncogenic potential. Role of proto-oncogenes in normal cell growth and differentiation is shown in Fig. 6.104. Tumor suppressor gene proteins and normal cell growth regulatory pathway is shown in Fig. 6.105.

- **Cellular proto-oncogenes normal gene products:** Cellular proto-oncogenes encode normal gene proteins involved in cell proliferation. Binding of growth factor to cell surface receptor sends a signal transduction through the cytoplasm to the nucleus,

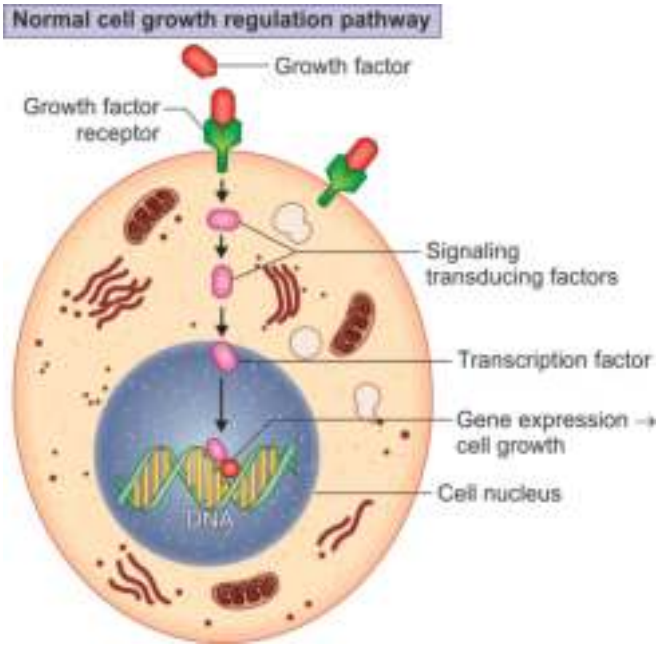


Fig. 6.104: Role of proto-oncogenes in normal cell growth and differentiation. Proto-oncogenes are normal cellular genes that encode intracellular regulatory proteins involved in signal transduction responsible for cell growth and differentiation during embryogenesis and postnatal life. **Oncogenes** are mutated forms of normal proto-oncogenes generally involved in promoting unrestricted cell proliferation. Oncogenes result in dominant gene function. Perturbations in the regulatory pathways lead to alterations of cell proliferation, growth and differentiation leading to development of cancer.

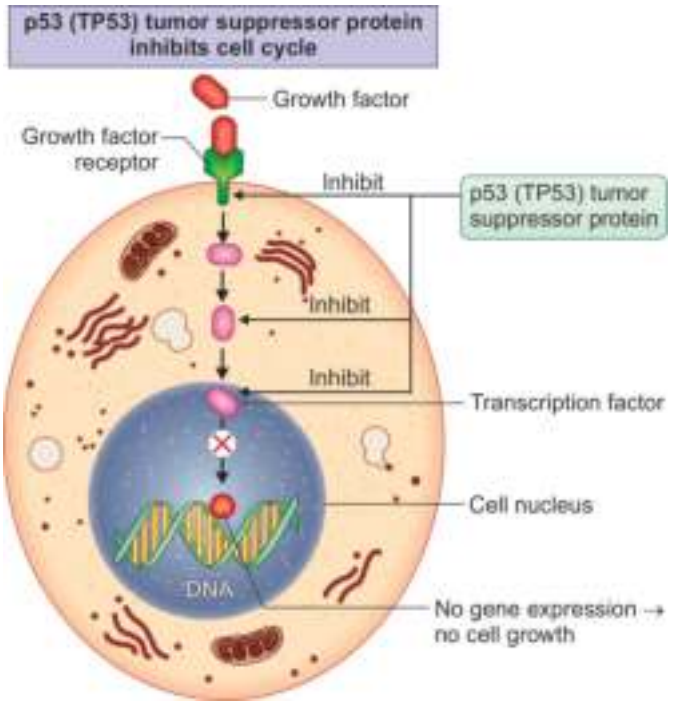


Fig. 6.105: Tumor suppressor gene proteins and normal cell growth regulatory pathway. Tumor suppressor gene encode proteins, which regulate orderly cell growth and cell differentiation, and play a critical role of the normal processes of the cell cycle by sensing the surrounding environment, transmitting signals to the nucleus and directly affecting transcription, translation, survival, or cell division. Inactivation/biallelic loss of tumor suppressor gene is linked to development of malignant tumor. Mutations in tumor suppressor gene result in recessive loss of function.

that activates nuclear regulating proteins, i.e. transcription factors, which promotes cell growth and cell division. When normal cellular proto-oncogenes are permanently altered to become oncogenes, which induce unrestricted CSC proliferation.

- **Cellular proto-oncogenes/oncogenes classification according to function of gene products:** Proto-oncogenes can be classified according to the function of their gene products, which include: (a) growth factors, (b) cell surface growth factor receptors, (c) signal transducing proteins (GTP-binding proteins, and nonreceptor protein kinase), (d) transcription factors (i.e. nuclear regulatory proteins), (e) chromatin modeler proteins, (f) cell cycle regulatory proteins, (g) apoptosis regulatory proteins, and (h) DNA repair proteins.
- **Cellular proto-oncogenes conversion into oncogenes—mechanisms:** Conversion of a proto-oncogene into an oncogene generally involves a gain-of-function mutation. Oncogenes (mutated proto-oncogenes) produce oncoproteins that induce malignant tumor. At least three mechanisms can produce oncogenes from the corresponding proto-oncogenes, i.e. point mutation (deletion/insertion), gene amplification (localized reduplication), and chromosomal rearrangements. Oncogene formed by point mutation encodes an oncoprotein that differs slightly from the normal protein encoded by corresponding proto-oncogene. In contrast, gene amplification and chromosomal rearrangement mechanisms generate oncogenes whose gene products are identical with the normal proteins. **Gain-of-function mutations** that convert proto-oncogene into oncogenes act dominantly; that is, mutation in only one of the gene alleles is sufficient to induce malignant tumor. Activated oncogenes can produce either excess normal protein or functionally abnormal protein. Excess production of nonfunctional protein can occur by two mechanisms: gene amplification (e.g. Myc gene linked to neuroblastoma) and increased mRNA transcription (Burkitt's lymphoma).
  - **Point mutation:** Point mutation (deletion or insertion) in the promoter region of proto-oncogene increases transcription, and production of hyperactive functionally gene product that induces unrestricted CSC proliferation. Point mutation in RAS proto-oncogene in certain codons that renders it constitutively active, and is linked to lung carcinoma, colon carcinoma and pancreatic carcinoma.
  - **Gene amplification:** Gene amplification is frequent genetic abnormality in human CSCs and consists of multiple extra (additional) copies of a proto-oncogene in subchromosomal region of DNA. Sometimes, proto-oncogenes only need to be overexpressed (not mutated) to cause solid malignant tumors associated gene amplification of ribosomal genes, and histone octamer genes can be found clustered in tandem arrays in the genome. HER2/neu gene amplification results in the production of excess HER2 protein on the surface of CSCs in breast carcinoma. Amplification of N-Myc oncogene occurs in subset of **neuroblastomas**. Gene amplification is most often observed in the family members of Myc (transcription factor), RAS (signal transduction protein), FGF (fibroblast growth factor), EGFR (epidermal growth factor receptor), and cell cycle regulatory genes (CCND1, CCNE, MDM2, SDK4). Gene amplification can be detected by several methods: (a) conventional cytogenetics, (b) Southern blotting, (c) polymerase chain reaction (PCR) is a technique, (d) fluorescence *in situ* hybridization (FISH), (e) comparative genomic hybridization (CGH); and (f) microarray technology.

- **Chromosomal translocation:** A chromosomal translocation is defined as genome abnormality in which a chromosome breaks and either the whole or a portion of it reattaches to a different chromosome. Depending on the location of breaks, chromosomal translocation events that relocate a proto-oncogene to a new chromosomal site, that leads to higher expression. Chromosomal translocation that leads to a fusion between a proto-oncogene and second proto-oncogene, which produces chimeric fusion protein with oncogenic activity. Genetic mutation that can occur due to shattering of chromosome known as '**chromothripsis**'. Chromosomal translocation t(8;14)(q24;q32), involving c-Myc oncogene on chromosome 8 and immunoglobulin heavy (IgH) gene locus on chromosome 14 is characteristic of Burkitt's lymphoma (90%). High levels of c-Myc may boost the global gene expression and influence a wide spectrum of cellular signaling pathways by acting on multiple genes leading to increased mRNA transcription and excess Myc protein. Philadelphia chromosome is demonstrated in chronic myelogenous leukemia (**CML**), in which chromosomal translocation t(9;22) that juxtaposes the BCR gene on chromosome 22 with the c-ABL proto-oncogene on chromosome 9. The BCR-ABL-1 fusion creates a constitutively active nonreceptor tyrosine kinase product that promotes cell proliferation independent of extrinsic regulation.

- **Loss of function mutations in tumor suppressor genes with oncogenic activity:** Tumor suppressor genes encode proteins that can inhibit cell proliferation. Since only copy of a tumor suppressor gene is sufficient to inhibit cell proliferation, both alleles of a tumor suppressor gene must be inactivated or lost in order to induce malignant tumor. Thus, oncogenic loss-of-function mutations in tumor suppressor genes act recessively.
- **Carcinogenesis:** Carcinogenesis is multistep process of transformation of normal cell to CSC, and subsequent development of malignant tumor. The process involves genetic and epigenetic alterations, and accumulation of mutated multiple genes coding for abnormal growth factors, cell surface growth factor receptors, signal transducing proteins (G proteins and nonreceptor kinase protein), transcription factors, chromatin remodeler proteins, cell cycle regulatory proteins, apoptosis regulatory proteins and DNA repair proteins, leading to unrestricted CSC proliferation that prevent normal cellular functions.
  - Conversion of one of the two alleles of proto-oncogene to an oncogene is sufficient for initiation, promotion and progression of malignant tumor. However, it requires inactivation/biallelic loss of tumor suppressor genes to promote malignant tumor.
  - Carcinogenesis by oncogenes and mutated tumor suppressor genes is shown in Figs 6.106 and 6.107. Cell proliferation in normal cell and malignant neoplastic cell is shown in Fig. 6.108.
- **Diagnostic molecular techniques to detect oncogenes:** Diagnostic molecular techniques to detect oncogenes include: (a) microarray analysis compares expression of proteins in CSCs versus normal cells, (b) polymerase chain reaction (PCR) assay detects gene arrangement, (c) RNA interface assay is used to detect gene function such as complete or incomplete knockdown, (d) transfection assay is used to study the gene overexpression by introduction of genes under control of an inducible promoter into the cell culture, (e) laser microdissection technique detects expression of genes by cutting out an area of interest on a slide with a laser in order to use other assay (PCR), and (f) cytogenetics refers to study of chromosome translocations in CSCs and identification of genes involved.

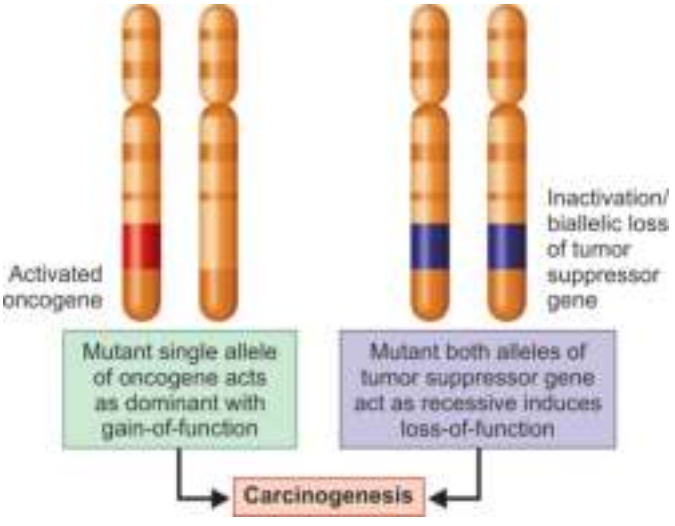


Fig. 6.106: Carcinogenesis by oncogenes and mutated tumor suppressor genes. Proteins produced by proto-oncogenes have normal regulatory functions, whereas proteins produced by oncogenes lack normal regulatory functions. Oncogene (mutated gene) induces aberrant cell division through activation and a gain-of-function; and loss of both copies of tumor suppressor gene (turning off) contribute to oncogenesis. Both types of mutations work together to induce cancer. For example, breast cancer progression involves multiple genetic alterations, which activate dominant-acting oncogenes and disrupt function of specific BRCA1, BRCA2 and p53 tumor suppressors.

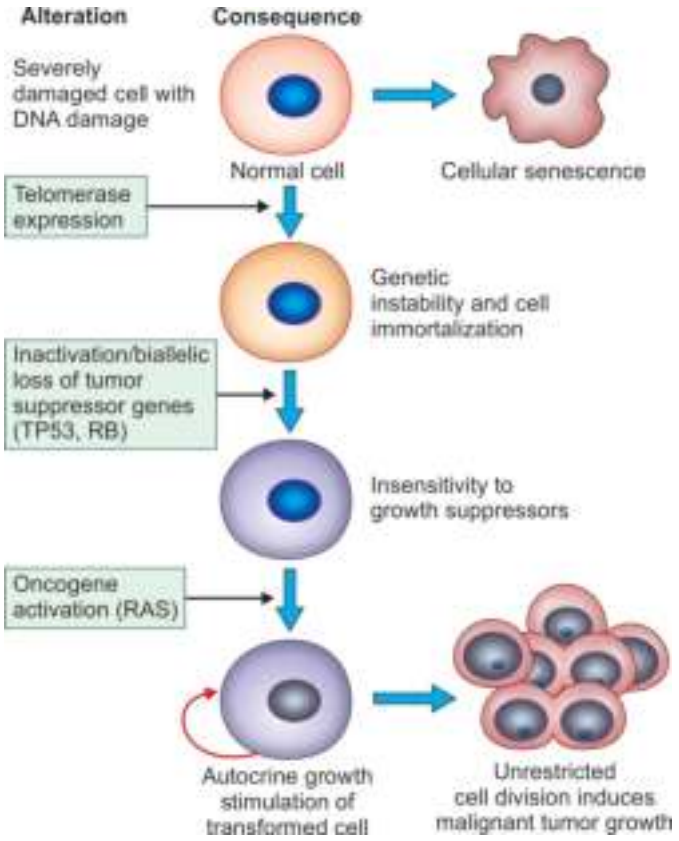


Fig. 6.107: Carcinogenesis by oncogenes and mutated tumor suppressor genes. Proteins produced by proto-oncogenes have normal regulatory functions, whereas proteins produced by oncogenes lack normal regulatory functions. The process by which normal healthy cells transform into malignant neoplastic cells is termed carcinogenesis. Oncogenes are mutated proto-oncogenes, whose alterations cause gain-of-function, while inactivation/biallelic loss of tumor suppressor genes cause loss-of-function that induce transformation of normal cell into malignant neoplastic cell leading to development of malignant tumor.

Pathology Pearls: Oncogenes (Mutated Proto-oncogenes) and Cancer

- Cellular proto-oncogenes encode proteins that promote cell proliferation. When normal cellular proto-oncogenes are permanently altered to become oncogenes, which induce unrestricted cancer stem cell proliferation.
- Gain-of-function (dominant) mutations that convert proto-oncogene to oncogene act dominantly; that is, mutation in only one of the two alleles is sufficient to induce malignant tumor. On contrary, recessive loss-of-function mutations in tumor suppressor genes possess oncogenic potential.
- Activating mutation of one of the alleles of a proto-oncogene to become an oncogene can occur by three mechanisms: point mutation (deletion/insertion), gene amplification (localized reduplication) and chromosomal rearrangements.
- Activated oncogenes can produce either excess of protein or functionally abnormal protein.
- Oncogenes (mutated proto-oncogenes) encode oncoproteins such as growth factors, cell surface growth factor receptors, signal transducing proteins (GTP-binding proteins, and nonreceptor protein kinase), transcription factors (i.e. nuclear regulatory proteins), chromatin modeler proteins, cell cycle regulatory proteins, apoptosis regulatory proteins, and DNA repair proteins.
- RAS oncogene encoding RAS oncoprotein has been isolated from human urinary bladder carcinoma.
- The first recognized oncogene, v-src, was detected in Rouse sarcoma virus—cancer causing virus. Retroviral oncogenes arose by transduction of cellular proto-oncogenes into the viral genome and subsequent mutation.
- Slow-acting retroviruses can induce malignant tumor near a proto-oncogene in such as way that gene transcription is activated continuously and inapparently.

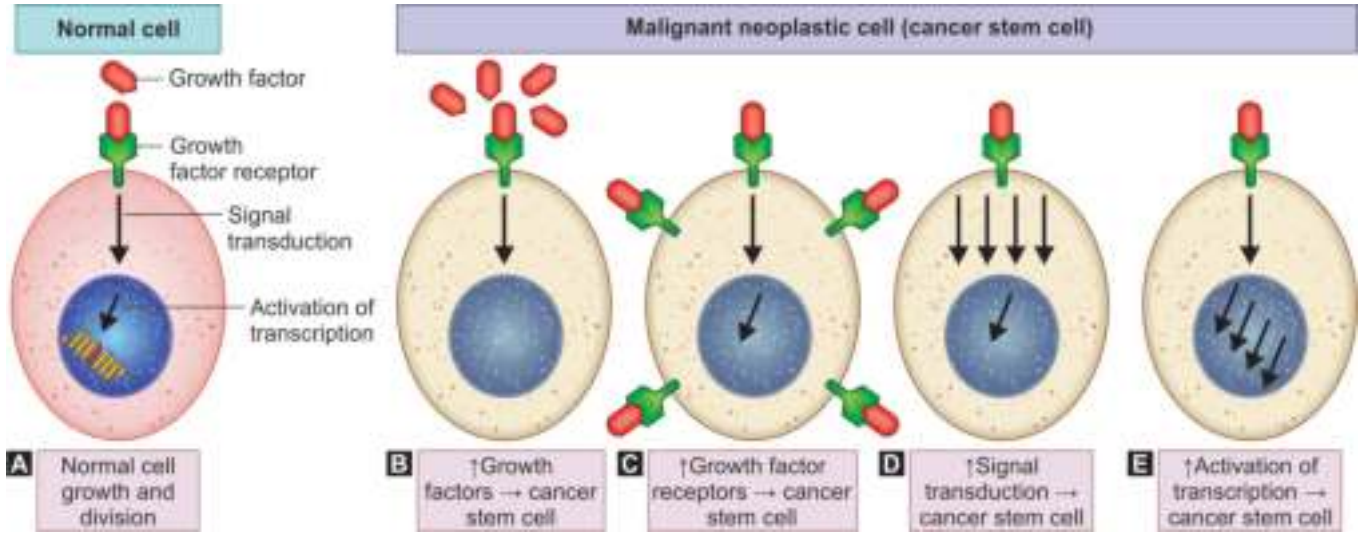


Fig. 6.108: Cell proliferation in normal cell and malignant neoplastic cell: (A) Proliferation of normal cell population result from a sequence of operations involving the binding of growth factors to cell surface growth factor receptors, transcription of the growth promoting signal and activation of DNA transcription. The abnormal degree of proliferation in neoplastic cell populations results from the upregulation of one or more than one of these steps, (B) increased growth factors, (C) increased cell growth factor receptors, (D) increased signal transduction, (E) increased transcription.

Pathology Pearls: Inactivation/Biallelic Loss of Tumor Suppressor Genes and Cancer

- Tumor suppressor gene encodes protein that acts to regulate cell division, keeping it in check. Even one allele of tumor suppressor gene regulates cell division, keeping it in check.
- Tumor suppressor genes encode proteins that prevent malignant tumor by reliably controlling cell division in check, promoting apoptosis and suppressing metastasis.
- Oncogenic loss-of-function (recessive) mutations in tumor suppressor genes act recessively. Inactivation/biallelic loss of tumor suppressor gene induce unrestricted cell division and thus malignant tumor.
- TP53 tumor suppressor gene mutation is associated with 50% of human cancer cases. TP53 tumor suppressor gene induces the transcription of other tumor suppressors, such as **p21** and **p16**, triggers DNA repair and initiates cell apoptosis.
- Germline mutation in **RB** tumor suppressor gene is linked to hereditary retinoblastoma in both eyes as an autosomal dominant trait in children.
- Germline mutation in **BRCA1** tumor suppressor gene is linked to breast carcinoma. Germline mutation in **APC** tumor suppressor gene is implicated in inducing colorectal carcinoma.
- Loss-of-function mutations in tumor suppressor genes have been identified in many human cancers in ovary, breast, lung, colorectal region, head and neck region, pancreas, uterus, and urinary bladder.
- A cell containing one normal and one mutant allele of a tumor suppressor gene is generally phenotyping normal.
- Loss of heterozygosity (**LOH**) of tumor suppressor genes occurs by two mechanisms: mitotic recombination and chromosome mis-segregation.

CELL SIGNALING

The cells of our body are constantly receiving and interpreting signals from our environment. Cell signals are most often chemical molecules found in the extracellular fluid around cells, essential for normal cellular functions, cell division and differentiation. Signaling chemical molecules can trigger cellular responses, including altered cell metabolism, gene expression (transcription) within the nucleus of the cell by endocrine, paracrine and autocrine signaling mechanisms. In 'endocrine signaling', the signaling chemical molecules (hormones) are secreted by endocrine cells and carried through bloodstream to act of target cells at distant body sites. In 'paracrine signaling', the chemical signaling molecule released by one cell acts on neighboring target cells. In 'autocrine signaling', the chemical signaling molecules released from the cell and bind on the same cell membrane to induce cellular response by forming autocrine loop. Cell signaling can be divided into three stages: (a) reception of signaling chemical molecules, (b) signal transduction and (c) specific cellular response.

- **Reception of signaling chemical molecules:** Cell detects a signaling chemical molecule (also known as a ligand) from the outside of the cell, that binds to a receptor protein on the cell surface or inside the cell.
  - **Cell membrane receptors:** Cell membrane receptors function by binding the signal molecule (ligand) and causing the production of second signaling messenger molecule, that induces a cellular response. Cell membrane receptors transmit signal from the extracellular environment to inside the cell by changing shape or by joining another protein once a specific growth factor ligand binds to it. Examples of cell membrane receptors include G protein-coupled receptors and receptor tyrosine kinases (RTKs).
  - **Intracellular receptors:** Intracellular receptors are found inside cells, either in cytoplasm or in the nucleus of the target cell receiving signal. Chemical messengers that are very small or hydrophobic (steroid hormones) can pass through the plasma membrane without assistance and bind theses intracellular receptors. Once bound and activated by signaling chemical molecule, the activated intracellular receptor can initiate a specific cellular response, such as a change in gene expression.
- **Signal transduction:** Signaling chemical molecules bind to cell membrane receptor protein, and initiate the process of multi-step signal transduction pathway, that transmits the signal quickly and amplifies the signal to numerous next signaling molecules at each step. Steps in the signal transduction signaling pathway often involve the addition or removal of phosphate groups which results in the activation of proteins.
  - **Protein kinases:** Enzymes that transfer phosphate groups from ATP to the protein are called protein kinases, which often act on other protein in the signaling pathways, and create phosphorylation cascade, leading to chain reaction.
  - **Protein phosphatases:** Protein phosphatases are important enzymes involved in phosphorylation process, that can rapidly remove phosphate groups from proteins by dephosphorylation process and inactivate protein kinases. Protein phosphatases act as switch, which 'turn off' the transduction pathway. Dephosphorylation process also makes protein kinases available for further use, and enables the cell to respond again, when another signal is received within cell.

- **Secondary messengers:** Other than protein kinases, small non-protein, water-soluble molecules called 'secondary messengers' also transmit signals received by receptors on the cell surface to target molecules in the cytoplasm or the nucleus. The **examples** of second messengers include cyclic AMP (cAMP) and calcium ions.

- **Specific cellular response:** Finally, signaling chemical molecule triggers a specific cellular response through cell membrane receptors such as G protein-coupled receptors, and receptor tyrosine kinases (RTKs). Cell signaling pathway regulates cellular processes, i.e. cell division or apoptosis by 'turning on' or 'turning off' transcription of specific genes. Signaling pathway also regulate the activity of a protein, for example, opening and closing an ion channel in the plasma membrane or promoting a change in cell metabolism such as catalyzing the breakdown of glycogen.

GROWTH FACTORS

Growth factors are relatively small secreted or membrane bound polypeptide ligands, which enable constant communication between nearby and distant cells. Binding of growth factor to cognate specific cell surface receptors, which initiates the intracellular signaling cascade events, that regulate cell cycle, cell proliferation, growth, differentiation, survival, angiogenesis, inflammation, tissue healing and repair. Oncogenes (mutated proto-oncogenes) synthesize aberrant growth factors that enhance unrestricted CSC proliferation, growth, survival, angiogenesis and development of malignant phenotype. Mutated cells with oncogenic activity become self-sufficient to synthesize growth factors to which they respond by creating 'autocrine loop'. The increase in growth factors alone does not lead to cancer but can cause rapid cell growth that increases the chance of gene mutations.

- **Growth factors classification:** Growth factors can be classified according to their roles involved in biological processes, which include: (a) embryogenesis and development, i.e. EGF and IGF, (b) adult labile cells with high turnover rates such as hematopoietic cells and epithelial cells, and (c) wound healing and repair such as PDGF and TNF-α.
- **Growth factors regulate cell cycle:** The transition from G0 to G1 phase of cell cycle is regulated by different growth factors, which include epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), placental growth factor (PGF), hepatocyte growth factor (HGF), insulin growth factor (IGF), tumor necrosis factor-α (TNF-α), granulocyte-colony stimulating factor (G-CSF), nerve growth factor (NGF) and keratinocyte-derived growth factor (KGF) also called FGF-7.
- **Growth factor signaling mechanisms:** Growth factors can act as initiators of signaling cascade in cells by paracrine–endocrine, or autocrine interactions.
  - Paracrine signaling allows cells to communicate each other by releasing molecules, that bind and activate surrounding cells such as platelet-derived growth factor (PDGF), that stimulates proliferation of vascular muscle cells. Paracrine communication maximizes cellular response fidelity in wound signaling to activate wound healing process in the surrounding cells.
  - Endocrine signaling uses circulatory system to transport ligands (hormones synthesized by endocrine cells) to communicate with distant target cells far away from the site of synthesis.
  - Autocrine signaling is characterized by the ability of a cell to respond to its own synthesized growth factors to initiate signal transduction.
- **Aberrant growth factors signaling and cancer:** Mutated cells with oncogenic potential become self-sufficient to synthesize the growth factors to which they respond. The increase in growth factors alone does not lead to development of malignant tumor but can cause rapid cell growth, that increases the chance of gene mutations.
  - Mutations in proto-oncogenes (oncogenes) result in the increased production of aberrant growth factors, e.g. EGF, PDGF, VEGF, TNF-α, TGF-α, TGF-β, FGF, PGF, HGF, IGF, G-CSF, NGF and KGF also called FGF-7 that enhance unrestricted CSC proliferation development of malignant tumor.
  - Proto-oncogenes may be altered to become oncogenes by various mechanisms, i.e. gene amplification, point mutation, chromosomal translocation, chromothripsis ('chromosome shattering of large stretches undergoing rearrangements in a single catastrophic event), and viral activation. Oncogenes (mutations in proto-oncogenes) result in the excess production of aberrant growth factor, that enhances unrestricted CSC proliferation and carcinogenesis.
  - Cancer stem cells (CSCs) often acquire the ability to become self-sufficient to synthesize their own growth factors (e.g. PDGF) to which they respond by forming '**autocrine loop**'. Autocrine signaling in CSC is shown in Fig. 6.109.

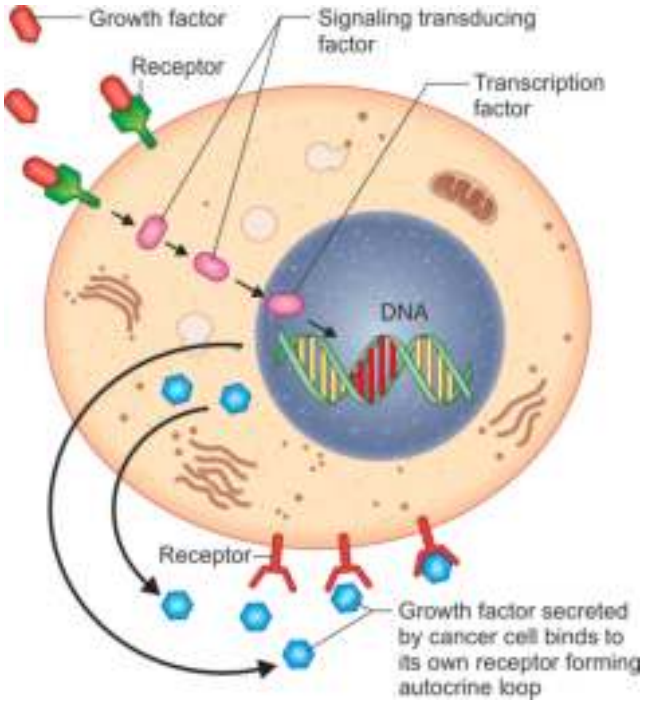


Fig. 6.109: Autocrine signaling in CSC. CSCs become self-sufficient to synthesize excessive aberrant growth factors to which they respond by forming an autocrine loop that enhances unrestricted cell division and induces malignant tumor.

Pathology Pearls: Four Classes of Growth Factors

- Class I growth factors bind to their cognate cell surface receptors.
- Class II growth factors bind to cytoplasmic domain of receptor tyrosine kinase (RTK).
- Class III growth factors consist of a group of intracellular signal transducers (e.g. RAS, SRC).
- Class IV growth factors are nuclear regulatory proteins that bind to DNA segments such as promoters, leading to either initiation of mRNA transcription or suppression of DNA transcription.
  - Fos, Myc, N-Myc and Myb initiate DNA transcription.
  - Tumor suppressors, i.e. TP53 gene (p53 protein) and RB gene (pRB) inhibit cell division.

Epidermal Growth Factor (EGF) Family

Epidermal growth factor (EGF) is synthesized by macrophages and platelets. EGF is also present in saliva, urine, milk and plasma. EGF binds to ErbB family of receptor tyrosine kinases such as ErbB1 (HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). The binding between EGFR and ligand triggers series of downstream intracellular signaling.

Physiologic State

Epidermal growth factor (EGF) stimulates proliferation of normal cells (e.g. fibroblasts and epithelial cells), cell growth, differentiation, and granulation tissue formation. EGF plays an essential role in wound healing through stimulation of regeneration of epidermal and dermal cells.

Pathologic State

Aberrant EGF signaling can induce angiogenesis, unrestricted CSC proliferation malignant tumor growth, survival, invasion, metastasis and resistance to chemotherapy. EGFR mutation is implicated in development of lung adenocarcinoma, glioblastoma multiforme, and colon carcinoma.

Platelet-derived Growth Factor Family

Platelet-derived growth factor (PDGF) is a polypeptide synthesized by macrophages, smooth muscle cells, endothelial cells, and keratinocytes, and stored in platelets α-granules and released on platelet activation upon clotting process. PDGF binds to cognate cell receptor tyrosine kinase (i.e. PDGFRA or PDGFRB), and induces angiogenesis in healthy tissue and malignant tumor stroma.

Physiologic State

Platelet-derived growth factor (PDGF) plays a pivotal role in cell proliferation of connective tissue, migration, maturation of blood vessels and recruitment of pericytes by angiopoietins through the four isomers of PDGF (PDGFA, PDGFB, PDGFC, PDGFD). PDGF is a potent attractant for fibroblasts, which swam into wound site in order to reconstruct the tissue by wound contraction in tissue healing and repair within 48 hours. PDGF stimulates the production of matrix metalloproteinases (**MMPs**), and inhibits platelets aggregation and integrin expression.

Pathologic State

Certain PDGF receptor-bearing CSCs produce isoforms, which stimulate cell growth and survival by forming autocrine loop.

- Aberrant PDGF signaling plays a key role in tumorigenesis, unrestricted CSC proliferation, angiogenesis, evasion of apoptosis, migration, invasion, metastasis and chemoresistance in glioblastoma multiforme, breast carcinoma, lung carcinoma, gastric carcinoma, colon carcinoma, prostatic carcinoma, leukemia and other cancers.
- Overexpression of PDGF ligands and their cognate receptors on CSCs of malignant ovarian tumors is associated with ascites and poor prognosis. On contrary, paracrine aberrant signaling has been found in human cancers that originate from epithelial cells, where it may be involved in stromal cell recruitment, metastasis and epithelial–mesenchymal transition (**EMT**). Inhibition of PDGF receptor signaling has

proven useful for treatment of patients with certain rare cancers.

Vascular Endothelial Growth Factor Family

Vascular endothelial growth factor (VEGF) is a potent essential proangiogenic growth factor for vascular endothelial cells involved in angiogenesis during embryogenesis and postnatal life after sustaining an injury and notably in malignant tumors.

- VEGF family comprises VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). Some VEGF family members can also bind to NRPs, which function as coreceptors.
- VEGF and PlGF can interact with a combination of many VEGF receptors (VEGFR-1, VEGFR-2, VEGFR-3), which are part of the receptor tyrosine kinase superfamily.
- VEGF-A, VEGF-B, and PlGF ligands bind to VEGFR-1. VEGFR-2 interacts with VEGF-A, VEGF-B, and VEGF-C ligands. VEGFR-3 can bind to VEGF-C and VEGF-D.
- VEGF-VEGFR system is crucial for angiogenesis in healthy tissue and cancer stroma. Angiopoietin I and II stabilize the mature newly formed blood vessels.

Physiologic State

Vascular endothelial growth factor (VEGF) plays key role in angiogenesis in healthy tissue. Angiopoietin I and II stabilize mature newly formed blood vessels. VEGF signaling is induced by the binding of VEGF ligand to its cognate transmembrane receptor tyrosine kinase, which results in the activation of multiple downstream signaling pathways, which include: (a) RAS/RAF/MEK/ERK (MAPK) signaling pathway regulates vascular endothelial cell proliferation, survival, and gene expression, (b) PI3K/AKT/mTOR signaling pathway regulates cell survival, (c) phospholipase-C-γ (PLCγ) signaling pathway regulates vascular permeability, and (d) FAK/paxillin signaling pathway is involved in the rearrangement of cytoskeleton in vascular endothelial cells.

Pathologic State

Angiogenesis is prerequisite for continued growth of malignant tumor beyond 1–2 mm, but also for invasion and metastasis. Tumor angiogenesis supplies nutrients and oxygen to promote malignant tumor growth, invasion and metastasis. VEGF is overexpressed in a variety of solid malignant tumors and certain hematologic malignancies associated with poor prognosis.

- The production of VEGF and other growth factors by the malignant tumor results in the angiogenic **switch** '**turning on**', where new vasculature is formed within and around the malignant tumor, allowing it to grow exponentially.
- Tumor vasculature formed under the influence of VEGF is structurally and functionally abnormal. Newly formed tumor vasculature is irregularly shaped, tortuous, leaky and hemorrhagic having dead ends, and not organized into venules, arterioles and capillaries, which result in high interstitial pressure, suboptimal blood flow, and further VEGF production.

Fibroblast Growth Factors

Fibroblast growth factors (FGFs) are potent regulators of cells proliferation chemotaxis, migration and differentiation of fibroblasts and keratinocytes. FGFs are synthesized by macrophages, T cells, mast cells, endothelial cells, and fibroblasts. FGFs have a high affinity for heparan sulfate proteoglycans, leading to activation of one of four cell surface FGF receptors.

Physiologic State

In human beings, 22 members of the FGFs family have been identified, all of which are structurally related signaling molecules, which include: FGF1 (acidic FGF), FGF2 (basic FGF), and FGF7 (keratinocyte growth factor). FGFs bind to fibroblast growth factor receptor family, that has four members: FGFR1, FGFR2, FGFR3 and FGFR4.

- FGF signaling pathway is mediated by RAS/RAF/MEK/ERK (MAPK) signaling pathway, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT/mTOR signaling pathway and phospholipase C-γ (PLC-γ), signal transducers and activators of transcription (STAT), which intersects and synergizes with Wnt/β-catenin of signaling pathways.
- FGFs are critically important in normal development of tissue, maintenance, angiogenesis in somatic stem cells, complete regeneration of a tissue, tissue repair, wound contraction, and matrix deposition in response to injury in postnatal life. Recent studies have revealed that FGF can act synergistically with VEGF to amplify angiogenesis.

Pathologic State

Aberrant expression of some FGFs can contribute to carcinogenesis, FGFs exert autocrine and paracrine functions on tumor cells and stromal cells. Aberrant FGFs play key role in unrestricted cell proliferation, migration, angiogenesis, invasion, metastasis, chemoresistance, and recurrence associated with poor survival.

- **FGF1 gene mutation:** FGF1 mutation is linked to urothelial carcinoma, solid malignant tumors, multiple myeloma, anaplastic astrocytoma, anaplastic oligodendroglioma, glioblastoma multiforme, endometrial carcinoma, cholangiocarcinoma, gastric

carcinoma, and squamous cell lung carcinoma. FGF1 also induces resistance to chemotherapy in ovarian granulosa cell tumor through inhibition of p53 transcriptional activities.

- **FGF2 gene mutation:** FGF2 gene mutation and its overexpression is linked to esophageal carcinoma, renal cell carcinoma, non-Hodgkin's lymphoma, chronic lymphocytic leukemia (CLL), hairy cell leukemia (HCL), multiple myeloma (MM), pancreatic carcinoma, hepatocellular carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, breast carcinoma, prostatic carcinoma, colorectal carcinoma, Ewing sarcoma, hepatocellular carcinoma, ovarian carcinoma, urothelial carcinoma, gastric carcinoma and Kaposi sarcoma.
- **FGF3 gene mutation:** FGF3 gene mutations such as amplification, missense mutations, silent mutations and frameshift insertion have been observed in endometrial carcinoma, gastric carcinoma, colon carcinoma, invasive ductal and lobular breast carcinoma, urothelial carcinoma, lung carcinoma and skin melanoma.

Transforming Growth Factor-α

Transforming growth factor-α (TGF-α) is a protein encoded by the TGF-α gene synthesized by macrophages, T cells, vascular endothelial cells, platelets and keratinocytes. Binding of TGF-α to epidermal growth factor receptor (EGFR) activates a signaling pathway involved in regulation of cell proliferation, differentiation, migration and development.

Physiologic State

TGF-α participates in cell proliferation and migration of keratinocytes involved in granulation tissue formation. TGF-α and its cognate EGF receptor are involved in numerous developing adult epithelial and mesenchymal tissues including gastrointestinal mucosa, lung, liver, kidney, mammary gland, dermis, gonads, skeletal muscle, and nerve cells in central peripheral nervous systems.

Pathologic State

Aberrant TGF-α autocrine and paracrine signaling has been implicated in many human cancers including primary breast carcinoma (50–70%), colorectal carcinoma, hepatocellular carcinoma, urothelial carcinoma, neuroblastoma, astrocytoma, cervical carcinoma, and vulvar carcinoma.

Transforming Growth Factor-β

Transforming growth factor-β (TGF-β) is a multifunctional cytokine belonging to the TGF superfamily, that include TGF-β1, TGF-β2 and TGF-β3. TGF-β is synthesized by all white blood cell lineages, vascular endothelial cells, smooth muscle cells, fibroblasts and keratinocytes. TGF-β inhibits synthesis of matrix metalloproteinases (MMPs) and keratinocytes. TGF-β regulates integrin expression and other cytokines.

Physiologic State

Transforming growth factor-β (TGF-β) cytokine plays critical roles in embryonic stem cell self-renewal and differentiation, homeostasis of differentiated cells, suppression of immune system, and inhibition of cell cycle progression.

Pathologic State

Transforming growth factor-β (TGF-β) cytokine plays dichotomous role in tumor progression. In normal and premalignant cells, TGF-β promotes cell-cycle arrest and apoptosis via induction of tumor suppressors (CDK inhibitors $p15^{INK4B}$ and $p21^{CIP1}$), and c-Myc suppression.

- In the late-stage, upregulation of TGF-β activates downstream oncogenes resulting in tumor initiation and promotion, epithelial–mesenchymal transition (EMT), unrestricted proliferation of CSCs, metastasis and chemoresistance.
- Due to its potent tumor suppressor effects in early stage, TGF-β signaling is lost in human cancers, which is a hallmark of cancer. Mutations or deletions of genes encoding components of the signaling pathway frequently occur in a variety of human cancers of colon, gastric region and pancreas. Taking TGF-β/SMAD4 as an example, its mutations or deletions are demonstrated in 50% of pancreatic cancer patients.

Insulin-like Growth Factor Family

The insulin-like growth factors (IGFs) are proteins with high sequence, similarity to insulin. IGF family has two members: IGF1 and IGF2. IGF1 gene encodes growth hormone. IGF2 gene provides instructions for making a protein called IGF2.

Physiologic State

Binding of IGF1 or IGF2 proteins with specific cell surface receptors activate various intracellular signaling pathways, and regulate cellular functions such as skeletal muscle growth and differentiation.

- IGF1 plays an essential role in brain growth and development, and tissue repair in response damaged to central and peripheral nervous systems during postnatal life.
- IGF2 regulates cell growth, cell proliferation in various tissues, apoptosis, and embryonic development. IGF2 gene is highly active during fetal development, but it is much less active after birth. IGF2 induces expression of the cyclin-dependent kinase inhibitor p21 to promote cell cycle exist.

Pathologic State

Aberrant insulin-like growth factor 1 (IGF-1) promotes development of malignant tumor growth by stimulating unrestricted CSC proliferation, angiogenesis and inhibition of apoptosis. Aberrant insulin-like growth factor 1 (IGF-1) signaling pathway is linked to breast carcinoma, colorectal carcinoma, and prostatic carcinoma. Therapy is aimed to block IGF-IR signaling, that inhibits downstream signaling pathways in these cancers.

Hepatocyte Growth Factor

Hepatocyte growth factor (HGF)/scatter factor (SF) is synthesized by mesenchymal stem cells (e.g. fibroblasts, vascular endothelial cells, and hepatocyte mesenchymal cells).

Physiologic State

HGF/SF plays key roles in regulation of cell growth, motility, morphogenesis of epithelial cells, and hematopoietic stem cells through its receptor c-Met. On binding of HGF to its cognate cell surface receptor c-Met activates signaling pathways resulting in angiogenesis, development of organs, and other morphogenesisprocesses.

Pathologic State

Increased baseline activity of HGF oncogene product and its receptor Met activation triggers RAS/RAF/MEK/ERK (MAPK) signaling pathway, PI3K/AKT/mTOR signaling pathway, and JAK/STAT signaling pathway, which promote angiogenesis, epithelial–mesenchymal transition (EMT), inhibition of E-cadherin expression, cell scattering, unrestricted CSC proliferation, cell survival, evasion of apoptosis, invasion and metastasis of various human cancers (e.g. colon carcinoma, hepatocellular carcinoma, and thyroid carcinoma).

Neurotrophic Factor Family

Neurotrophic factor family members include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/-5 (NT-4/-5). These are small, basic secretory proteins that allow the survival of specific neuronal populations during embryo development and postnatal life.

Physiologic State

Binding of neutrophins to their cognate Trk (tropomyosin-receptor kinase) tyrosine kinase receptors (TRKA, TRKB, TRKC), activate RAS/RAF/MEK/ERK mitogen-activated protein kinases (MAPKs) signaling pathway, phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway, and phospholipase C-γ (PLC-γ) signaling pathway, which are involved in cell survival, proliferation, differentiation, and apoptosis.

Pathologic State

Overexpression or mutation of Trk and receptor tyrosine kinases (RTKs) has been demonstrated in CNS tumors (neuroblastoma and medulloblastoma), thyroid carcinoma, breast carcinoma, lung carcinoma and prostatic carcinoma. Recent studies revealed that neurotrophic factors as well as neurotrophin receptors are present within the normal human prostate gland, benign nodular hyperplasia of prostate and prostatic carcinoma.

CELL SURFACE RECEPTORS

Cell surface receptors are membrane-anchored proteins either inside cell or its cell surface, which receive a signal. Majority of cell surface growth factor receptors are transmembrane receptor tyrosine kinases (RTKs). Growth factor binding to transmembrane receptor kinases leads to phosphorylation of tyrosine residues on several intracellular signaling molecules, which transmit signal to inside the cell, and involved in numerous cellular processes such as cell migration, proliferation, differentiation and survival. Oncogenes (mutated proto-oncogenes) result in the increased activation of aberrant transmembrane receptor tyrosine kinases. Oncogenes can induce transmembrane receptor tyrosine kinases to become constitutively active without need for growth factors (e.g. EGFR, PDGFR, VEGFR, TGF-αR, TGF-βR, FGFR, HGFR, IGFR) on the surface of cells to which growth factors bind.

- **Cell surface receptor components:** Each cell surface receptor has three main components: (a) external ligand-binding domain (extracellular domain), (b) hydrophobic membrane spanning region, and (c) intracellular domain inside the cell. The size and extent of each of these domains of cell surface receptors vary widely, depending on the type of receptors such as PDGFR, EGFR (EGFR-A, EGFR-B, EGFR-C, EGFR-D)/ErbB1, HER2/neu/ErbB2, FGFR2/ErbB1, FGFR3, c-Met, VEGFR, IGFR, RET (rearranged during transfection), KIT, Ret, FLT-3, ErbB3 and ErbB4.
- **Three general categories of cell surface growth factor receptors:** Three general categories of cell surface receptors include: G protein-linked receptors (seven-transmembrane receptors), enzyme-linked receptors, and ion channel-linked receptors. Signal transducing cell surface receptors involved in carcinogenesis are given in Table 6.87.
  - **G protein-coupled cell surface receptors:** G protein-coupled linked cell surface receptors (GPCRs), also called seven-transmembrane receptors are present in the cell membrane.

Table 6.87 Signal transducing cell surface receptors involved in carcinogenesis

Cell Receptor Category	Prototypical Ligands
Receptor tyrosine kinases (RTKs)	EGF, IGF-1
G protein-coupled receptor (GPCR)	Prostaglandins, RANTES, SDF1
Serine/threonine kinases	TGF-β
Kinase associated receptors	GH, TCR, IL-2
Nuclear receptors	Androgen, estrogen, other steroid hormones
Extracellular matrix (ECM) receptors	Fibronectin. collagen, laminin

EGF: Epidermal growth factor; IGF-1: Insulin-like growth factor 1, RANTES: Regulated upon activation, normal T cell expressed and secreted, also known as CCL5; SDF1: Stromal cell-derived factor-1, also known as CXCL12; TGF-β: transforming growth factor-β, GH: Growth hormone; TCR: T cell receptor, IL-2; interleukin-2.

G protein-linked cell surface receptors bind to cognate growth factor ligands on cell surface receptors and transmit signals into intracellular G protein (guanine nucleotide-binding protein), molecule including peptide hormones and neuropeptides. G proteins have three subunits: α, β, and γ. When a signaling molecule binds to G protein-linked receptor in the plasma membrane, GDP molecule associated with the α subunit is exchanged for GTP. The β, and γ subunits dissociate from the α subunit, and a cellular response is triggered either by α subunit or the dissociated β pair. Hydrolysis of GTP to GDP terminates the signal. Aberrant expression and activation of G proteins, and G protein-linked receptors are frequently associated with carcinogenesis.

- **Enzyme-linked cell surface receptors:** Enzyme-linked cell surface receptors bind to cognate growth factor with intracellular domains that are associated with an enzyme such as receptor tyrosine kinases (RTKs), receptor serine/threonine kinases, receptor-like tyrosine phosphatases, histidine kinase associated receptors and receptor guanylyl cyclases.
- **Ligand-gated ion channel cell surface receptors:** Ion channel-linked cell surface receptors have an extensive membrane spanning region, which bind a ligand and open a gated channel through the membrane that allow specific ions to inflow into the cell or outflow from the cell. Ion channel-linked receptors are involved in the cellular response to toxins and venoms in various cells such as cardiac, skeletal and smooth muscle contraction, T cell activation and hormone release.

- **Integrin receptors:** Integrins are heterodimeric transmembrane receptors bind to extracellular matrix (ECM) glycoproteins (i.e. laminin, collagen in the basement membrane and fibronectin), and the actin cytoskeleton.
- **Deregulated firing of cell surface receptors:** Normally functioning cell surface receptors displayed on the plasma membrane of a cell emit signals into the cell interior only when the extracellular domain of the cell surface receptor has bound appropriate growth factor ligand.
  - Excessive numbers of normal structured cell surface receptor molecules can also drive ligand-independent signaling into cell interior.
  - In contrast, in many types of human cancers, CSCs acquire the ability to make a ligand for a cell surface receptor that they also display. This creates an auto-stimulatory or autocrine signaling loop.
  - An example of autocrine signaling is seen in these successive sections of an invasive breast carcinoma, in which islands of CSCs are surrounded by non-staining stroma.
  - However, if the extracellular domain of certain cell surface receptors is deleted because of a mutation in gene encoding cell surface receptor or alternate splicing of the receptor pre-mRNA or subtle alterations in protein structure, such as amino acid substitutions, the resulting truncated extracellular domain receptor protein then emits signals into the cell ligand-independent firing without binding its cognate growth factor ligand leading to unrestricted proliferation of cancer stem cells. Mutant growth factors and receptor tyrosine kinases linked to human cancers are given in Table 6.88.
- **Receptor tyrosine kinase (RTK) activation in normal cells:** Receptor tyrosine kinases can be further categorized into RTKs and nonreceptor tyrosine kinases (NRTKs). RTKs are transmembrane cell proteins, that act as signal transducers, which regulate cell proliferation, differentiation, survival apoptosis and metabolism.
  - **Structure of receptor tyrosine kinases:** All the RTKs possess similar structures except platelet-derived growth factor receptor (PDGFR): (a) ecto-domain protrudes into the extracellular space that binds to extracellular ligands (e.g. Sis, Hst), and (b) cytoplasmic domain transmits signals within the cell.
  - **Classification of receptor tyrosine kinases family:** Receptor tyrosine kinases are classified by according to family, which include: EGFR, PDGFR, VEGFR, FGFR, HGFR, NGFR, EPHR, TIE, DDR, RET, ROS, LTK, ROR, MUSK, and LMR.

Table 6.88 Mutant growth factors and receptor tyrosine kinases (RTKs) linked to human cancers

Growth Factor Main Ligand	Growth Factor Receptor	Cells Responding to Growth Factor	Type of Genetic Alteration	Type of Human Cancer
PDGF (A, B, C, D) (platelet-derived growth factor)	PDGFR	Endothelial cells, vascular smooth muscle cells, other mesenchymal cells, glial cells	Translocation	Chronic myelomonocytic leukemia
EGF (epidermal growth factor)	EGFR-A, EGFR-B, EGFR-C, EGFR-D	Epithelial cells, some mesenchymal cells	Overexpression	▪ Non-small cell lung carcinoma ▪ Breast carcinoma ▪ Colorectal carcinoma ▪ Esophageal carcinoma ▪ Head and neck carcinoma ▪ Prostatic carcinoma ▪ Renal cell carcinoma ▪ Urinary bladder carcinoma ▪ Ovarian carcinoma ▪ Glioblastoma multiforme
No ligand	EGFR/ERBB1	Not applicable	Truncation of ectodomain	▪ Lung carcinoma ▪ Glioblastoma multiforme
NRG (neuregulin), EGF	HER2/neu/ErbB2	Neurons	Overexpression	Breast carcinoma (30%)
FGF (fibroblast growth factor)	FGFR2/ErbB1	Mesenchymal cells, neuroectodermal cells	Amino acid substitutions	▪ Breast carcinoma ▪ Gastric carcinoma ▪ Endometrial carcinoma
FGF (fibroblast growth factor)	FGFR3	Endothelial cells, hematopoietic cells	▪ Overexpression ▪ Amino acid substitutions ▪ Translocations	▪ Urinary bladder carcinoma ▪ Cervical carcinoma ▪ Acute myelogenous leukemia (AML) ▪ Multiple myeloma
HGF/SF (hepatocyte growth factor/scatter factor)	c-Met	Epithelial cells	Overexpression	▪ Osteosarcoma ▪ Glioblastoma multiforme ▪ Breast carcinoma ▪ Prostatic carcinoma ▪ Lung carcinoma
VEGF (vascular endothelial growth factor)	VEGFR	Endothelial cells lining capillaries and lymphatic channels	Overexpression	▪ Breast carcinoma ▪ Angiosarcoma
IGF (insulin growth factor)	IGFR	Wide variety of cell types	Overexpression	▪ Breast carcinoma ▪ Prostatic carcinoma ▪ Lung carcinoma ▪ Colorectal carcinoma
GDNF (glial cell line-derived neurotrophic factor)	RET (rearranged during transfection)	Neuroectodermal cells	Overexpression	▪ Breast carcinoma ▪ Multiple endocrine neoplasia 2 (MEN-2)
SCF (stem cell factor)	KIT	Hematopoietic cells, mesenchymal cells	Amino acid substitutions	Gastrointestinal stromal tumors

Contd...

Table 6.88 Mutant growth factors and receptor tyrosine kinases (RTKs) linked to human cancers (*Contd...*)

Growth Factor Main Ligand	Growth Factor Receptor	Cells Responding to Growth Factor	Type of Genetic Alteration	Type of Human Cancer
GFL	Ret	Thyroid follicular cells	Fusion with other proteins	▪ Papillary thyroid carcinoma ▪ Multiple endocrine neoplasia 2A and 2B
FL (FLT-3)	FLT-3	Myeloid precursors	Tandem duplication	Acute myelogenous leukemia (AML)
Various ligands	ErbB3, ErbB4	Oral epithelial cells	Overexpression	Oral squamous cell carcinoma

Once converted from proto-oncogenes, oncogenes function by synthesizing mutant forms of cellular proteins such as growth factors, cell surface growth factor receptors, signal transducing proteins, nuclear regulatory proteins (transcription factors), chromatin remodeler proteins, cell cycle regulatory proteins, apoptosis regulatory proteins and DNA repair proteins resulting in carcinogenesis.

- **Activation of receptor tyrosine kinases:** RTK activation occurs through the binding of a ligand to the cell surface receptor, which then induces cell surface receptor dimerization. Once ligand-induced dimerization occurs, it activates the intracellular receptor tyrosine kinase domain through the transmembrane domain. RTK activation occurs when this *cis*-autoinhibition is released after ligand binding and dimerization.

- **Receptor tyrosine kinase (RTK) activation in cancer stem cells:** Oncogenes may activate or increase expression of cellular receptors to which growth factors bind. Mutant cellular receptors deliver continuous mitogenic signals to the cell, even in the absence of growth factor in the environment allowing cell surface receptor to signal downstream unrestricted CSC proliferation. For example, an oncogene can code for an incomplete EGF receptor that no longer requires ligand binding for activation.
  - **Mechanisms of receptor tyrosine kinase activation:** Increased receptor tyrosine kinase (RTK) activity occurs by several mechanisms: overexpression, gain-of-function mutations, autocrine activation and chromosomal translocation/chimeric fusion protein. RTK activation can be influenced by tyrosine kinase domain duplications, microRNAs, alterations in tumor microenvironment, and negative RTK signaling regulators and deregulation of RTK. Genetic alterations of RTKs deliver continuous mitogenic signals to the cell, even in the absence of growth factor in the extracellular environment leading to unrestricted CSC proliferation and initiation of carcinogenesis. For example, an oncogene can code for an incomplete EGF receptor that no longer requires ligand binding for activation.
  - **Tyrosine kinase inhibitors in clinical oncology:** Tyrosine kinase inhibitors (TKIs) influence RTK activation and their downstream signaling pathways resulting in increased apoptosis, decreased CSC proliferation and migration in malignancies.

Epidermal Growth Factor Receptor (EGFR) Family Members: Receptor Tyrosine Kinase

Epidermal growth factor receptor (EGFR) family has four members, which include ErbB1, ErbB2 (HER2/neu), ErbB3 and ErbB4. Mutations in epidermal growth factor receptor (EGFR) family are implicated in development of many human cancers.

Physiologic State

Epidermal growth factor receptor (EGFR) binds at least seven different ligands, which include: EGF, TNF-α, heparin-binding EGF-like growth factor (HBEGF), β-cellulin (BTC), amphiregulin (AREG), epiregulin (EREG) and epigen (EPGN). Binding of EGFR to cognate EGF ligand activate multiple signaling pathways, i.e. RAS/RAF/MEK/ERK (MAPK/ERK) signaling pathway, phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway, and JAK/STAT signaling pathway resulting in cell migration, proliferation and survival respectively.

Pathologic State

Epidermal growth factor receptor (EGFR) family members are activated in many high-grade human cancers. Point mutation, amplification and overexpression of EGFR family members (ErbB1, ErbB2 [HER2/neu], ErbB3, and ErbB4) are linked to high-grade human cancers.

- **Point mutation in EGFR gene:** Point mutation in EGFR gene results in constitutive activation of epidermal growth factor receptor tyrosine kinase leading to unrestricted CSC proliferation.
- **Amplification in EGFR gene:** Amplification of EGFR family members such as ErbB1, ErbB2 (HER2/neu) and ErbB3 genes has been implicated in many human cancers. EGFR gene amplification causes sustained activation of EGFR receptor tyrosine kinase activity that stimulates unrestricted CSC proliferation, angiogenesis, cell survival, cell migration and metastasis.

Table 6.89 Gene amplifications in ErbB family of cell surface receptors associated with human cancers

Gene Amplification	Human Cancers
ErbB1 gene	Lung adenocarcinoma
ErbB2 gene (HER2/neu)	Breast carcinoma, ovarian carcinoma, adenocarcinoma lung cancers (adenocarcinoma, squamous cell carcinoma) and salivary gland cancers
ErbB3 gene	Breast carcinoma. prostatic carcinoma and urinary bladder carcinoma

- **ErbB1 gene amplification** is implicated in development of lung adenocarcinoma, breast carcinoma, prostatic carcinoma and urinary bladder carcinoma.
- **ErbB2 gene (HER2/neu) gene amplification** is linked to breast carcinoma, ovarian carcinoma, lung adenocarcinoma, squamous cell lung carcinoma and salivary gland cancers.
- Gene amplifications in ErbB family of cell surface receptors associated with human cancers are given in Table 6.89.

- **Overexpression of EGFR gene:** Overexpression of EGFR gene in human cancers is indicative of high-grade malignant tumor, invasion of lymph nodes with high rate of recurrence and fatal outcome. EGFR family member ErbB proteins can be demonstrated by immunohistochemistry technique by application of monoclonal antibody on the cell membrane, or using fluorescent *in situ* hybridization (FISH) technique.

Targeted Therapy

Monoclonal antibodies and tyrosine kinase inhibitors (TKIs) are currently used to target EGFR mutations in human cancers. Monoclonal antibodies (e.g. cetuximab and panitumumab) bind to extracellular domain of EGFR to prevent ligands from activating the EGFR. TKIs bind to the cytosolic domain of EGFR, and prevents activation process. Anti-EGFR antibodies are targeted to the external ligand binding domains while the small inhibitors or TKIs target its cytoplasmic receptor tyrosine kinase domains. Genetic mutations in K-RAS, BRAF, and ALK translocation predict poor response to EGFR therapy.

Vascular Endothelial Growth Factor Receptor

Vascular endothelial growth factor receptor is receptor tyrosine kinase (RTK) expressed on the plasma membrane of endothelial cells. There are three types of vascular endothelial growth factor receptors (VEGFRs), namely VEGFR1, VEGFR2, VEGFR3, whereas there are five structurally related VEGF ligands including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor. There are also co-receptors involved in ligand binding called neuropilins (NRPs).

Physiologic State

Normally, VEGF binding to cognate VEGFR and transmit signals via intracellular signaling pathways, i.e. RAS/RAF/MEK/ERK (MAPK) pathway, PI3K/AKT/mTOR pathway and phospholipase C-γ (PLC-γ) pathway to other signal transduction effectors via autophosphorylation of specific residues in its structure leading to induction of angiogenesis and lymphangiogenesis during embryonic development and adult life.

Pathologic State

Binding of VEGF ligand to mutated VEGFR activates angiogenic switch signal, which remains constantly 'turning on' without 'urning off' leading to formation of structurally and functionally new blood vessels in and around malignant tumor growth, before a malignant tumor can grow beyond 1–2 mm. The newly formed blood vessels are irregularly shaped, tortuous, leaky, hemorrhagic, having dead ends, and not organized into venules, arterioles and capillaries. Tumor angiogenesis plays a key role in malignant tumor growth, invasion and metastasis.

- Amplification of vascular endothelial growth factor receptor (VEGFR) gene is linked to breast carcinoma, lung carcinoma, urothelial carcinoma, colorectal carcinoma, ovarian carcinoma, gastric carcinoma, pancreatic carcinoma, glioblastoma multiforme, renal cell carcinoma and multiple myeloma.

Targeted Therapy

Targeted therapy works in two different ways to inhibit VEGFR: (a) therapeutic agent prevents a VEGF ligand from binding to VEGFR outside the cell, or (b) therapeutic agent prevents signal from VEGFR from reaching other intracellular proteins inside the cell, that inhibit growth of malignant tumor.

Fibroblast Growth Factor Receptor

Fibroblast growth factor receptor (FGFR) family consists of four members (FGFR1, FGFR2, FGFR3, and FGFR4) encoded by different genes. FGFRs are receptor tyrosine kinases (RTKs). All of them have two different isomers produced by alternative splicing except FGFR4.

Physiologic State

Fibroblast growth factor receptors (FGFRs) are receptor tyrosine kinases expressed on the cell membrane. FGFR binds to FGF ligand and activates downstream signaling pathways, which play essential role in cellular processes such as cell growth, proliferation, differentiation, angiogenesis, and wound healing during embryonic development and adult life.

Pathologic State

In malignant tumors containing genetically altered FGFRs, the most frequent genetic alteration in FGFR1 (most common), FGFR2, FGFR3 and FGFR4 (least common) can be observed in decreased frequency. Genetic alterations in FGFRs occur due to gene amplification, point mutation and formation of oncogenic gene fusions.

- **FGFR gene amplification:** The most common genetic alteration is the amplification of FGFR genes.
  - FGFR1 gene amplification is linked to lung carcinoma and breast carcinoma.
  - FGFR2 gene amplification is linked to gastric carcinoma.
- **FGFR gene point mutation:** Point mutations in FGFRs are found in breast carcinoma, colon carcinoma, lung carcinoma, head and neck squamous cell carcinoma, and glioblastoma multiforme.
- **Oncogenic FGFRs-TCCC3 gene fusions:** FGFR2 and FGFR3 are commonly involved in the formation of oncogenic fusion gene. FGFR3-TACC3 oncogenic fusion gene has been observed in lung carcinoma, cervical carcinoma, urinary bladder carcinoma, nasopharyngeal carcinoma, and glioblastoma multiforme.

Targeted Therapy

FGFRs have been considered as promising drug targets for therapy of various human cancers. Multiple small molecule inhibitors targeting FGFRs family of receptor tyrosine kinases have been developed. Pan-FGFR inhibitor erdafitinib has been recently approved for the treatment of metastatic or unresectable urothelial carcinoma.

Platelet-derived Growth Factor Receptor Family

Platelet-derived growth factor receptor (PDGFR) is cell surface receptor tyrosine kinase (RTK). PDGFR has four members, which include: PDGFRA, PDGFRB, PDGFRC, and PDGFRD. Mutations in PDGFR family are linked to human cancers.

Physiologic State

Platelet-derived growth factor receptor (PDGFR) is essential for regulation of embryonic development, cell proliferation, migration, and angiogenesis.

Pathologic State

Mutation in PDGFR is linked to cancer progression by stimulating proliferation of CSCs, angiogenesis, epithelial–mesenchymal transition (EMT), evasion of apoptosis, migration, invasion, metastasis, and chemoresistance of certain human cancers.

- **Mutations in PDGFR genes:** Mutations in PDGFR receptor genes have been found in certain human cancers. Increased PDGF signaling has a key role in cancer progression, unrestricted CSC proliferation of angiogenesis, evasion of apoptosis, migration, invasion, metastasis and chemoresistance.
- **Autocrine PDGFR signaling:** Autocrine PDGFR signaling has been implicated in the development of glioblastoma multiforme and leukemia.
- **Paracrine PDGFR signaling:** In contrast, paracrine signaling due to overexpression of PDGFR has been observed in human cancers that originate from epithelial cells (e.g. breast carcinoma, lung carcinoma, gastric carcinoma, colon carcinoma, prostatic carcinoma and other cancers), where it may be involved in epithelial–mesenchymal transition (EMT), invasion and metastasis.

Insulin-like Growth Factor-1 Receptor

Insulin-like growth factor-1 receptor (IGF1R) is transmembrane receptor tyrosine kinase (RTK), that binds insulin-like growth factor-1 (IGF1) with high affinity and IGF2 with a lower affinity. IGF1R plays a key role in cellular transformation events. Cleavage of the precursor generates α and β subunits of IGF1R.

Physiologic State

Insulin-like growth factor-1 (IGF1) binding to receptor tyrosine kinase results in its activation and autophosphorylation as well as phosphorylation of multiple substrates, that function as adaptor proteins, including insulin receptor substrates (IRS1/IRS2), SHC1 transforming protein (apoptosis protein) and 14-3-3-signaling proteins such as kinases, phosphatases and transmembrane receptors.

- Phosphorylation of IRS1/IRS2 proteins activate RAS/RAF/MEK/ERK (MAPK/ERK) signaling pathway, and phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway.
- Activated RAS/RAF/MEK/ERK (MAPK) pathway regulates cell proliferation, and phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway stimulates protein synthesis, and inhibits apoptosis.

Pathologic State

Insulin-like growth factor-1 receptor (IGF1R) is overexpressed in most human cancers, where it inhibits apoptosis of CSCs, and thus enhances their survival. Mutations in IGF1R (i.e. missense mutations, nonsense mutations, silent mutations, frameshift deletions) are linked to development of colon carcinoma, gastric carcinoma, breast ductal carcinoma, lung adenocarcinoma, and cutaneous melanoma. IGF1R is a membrane

protein that binds SHC/ERa. Overexpression of SHC proteins is associated with malignant tumor development, invasion and metastasis.

Hepatocyte Growth Factor Receptor

The hepatocyte growth factor receptor (HGFR), also known as MET or c-Met is a receptor tyrosine kinase (RTK) encoded by MET gene, which binds to its ligand hepatocyte growth factor/scatter factor (HGF/SF) and its splicing isoform (NK1, NK2) involved in cell proliferation, migration and morphogenesis.

Physiologic State

Normally, HGF-induced Met receptor tyrosine kinase is tightly regulated by paracrine ligand delivery, ligand activation on the target cell surface, and the ligand activated Met receptor tyrosine kinase internalization and degradation.

- Hepatocyte growth factor and its HGF receptor tyrosine kinase (RTK) encoded by c-Met proto-oncogene are essential for embryonic development, organogenesis, and wound healing. The ligand/receptor system regulates essential cellular processes such as cell proliferation and motility, morphogenesis and differentiation.
- Hepatocyte growth factor (HGF) messenger RNA is expressed primarily is mesenchymal cells, but not in epithelial cells, while its receptor (HGFR) is predominantly expressed in epithelial cells. This pattern of HGH and HGFR gene expression in combination with the unique biological effects of HGF on its target cells has suggested that HGF mediates crosslink between the epithelial and stromal components of a given tissue/organ.

Pathologic State

Hepatocyte growth factor (HGF)-induced Met receptor tyrosine kinase signaling contributes to oncogenesis and tumor progression in many human cancers and promotes tumor angiogenesis, invasion and metastasis. Expression of HGF and HGFR genes is orchesterized in stromal and epithelial respectively by extracellular signals derived from steroid hormones and cytokines such as IL-1, IL-6 and TNF-α.

- Overexpression of HGF and HGFR genes is linked to development of breast carcinoma, non-small cell lung carcinoma and associated with lymph node invasion mediated by **rho overexpression**, ovarian carcinoma, gastric carcinoma, colon carcinoma, lung carcinoma, skin carcinoma and hereditary or sporadic papillary renal cell carcinoma.
- Met exon mutations predominantly occur in elderly patients with lung adenocarcinoma.
  - Patients with advanced-stage non-small cell lung carcinoma have c-Met mutations, and also concurrent Met gene amplification and gene fusions at exon 15 such as HLA-DRB1-Met, KIF5B-Met, METUBE2H and MET-ATXNL7L1.
  - Overexpression of c-Met is correlated to distant metastasis, large tumor size and high histologic grade in breast carcinoma.
- Autocrine activation of HGF/c-Met signaling has been observed in colorectal carcinoma and acute myelogenous leukemia (AML) involving β-catenin and co-activation of FGFR1, and also development of chemoresistance against multikinase inhibitors in hepatocellular carcinoma.

RET (Rearranged during Transfection) Receptor Tyrosine Kinase

RET (rearranged during transfection) proto-oncogene located on chromosome 10q11.2 encodes a RET receptor tyrosine kinase involved in glial cell-derived neurotrophic factor (GDNF) of extracellular signaling molecules. RET protein dimerization results in autophosphorylation of several intracellular transduction proteins tyrosine kinase.

Physiologic State

RET receptor tyrosine kinases are present on normal cells. Upon binding of GDFN family ligand to RET receptor tyrosine kinase activates RAS/RAF/MEK/ERK (MAPK) signaling pathway, phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway induce cell proliferation, differentiation, neuronal navigation and cell migration.

Pathologic State

Point mutation in RET receptor tyrosine kinase is linked to development of human cancers such as familial or sporadic medullary thyroid carcinoma, and papillary thyroid carcinoma; and multiple endocrine neoplasia 2A, 2B (MEN-2A, MEN-2B).

Targeted Therapy

Targeted cancer therapies block RET receptor tyrosine kinase by two mechanisms: (a) outside the cell, chemotherapeutic agent can prevent a ligand from binding RET receptor, and (b) inside the cell, chemotherapeutic agent can prevent the signal from RET receptor from reaching other proteins, which promote cell growth and progression of malignant tumor.

c-KIT (CD117) Proto-oncogene Encodes Receptor Tyrosine Kinase

c-KIT (CD117) proto-oncogene mapped on chromosome 4q12 encodes a transmembrane protein with

intrinsic tyrosine kinase activity, which functions as the receptor for stem cell factor (SCF). It is expressed on mast cells, hematopoietic progenitor cells (myeloid, lymphoid, erythroid, and megakaryocytic progenitor cells), gastrointestinal pacemaker cells, melanocytes and germ cells. SCF assists in the recovery of cardiac function following myocardial infarction by increasing the number of cardiomyocytes and vascular channel.

Physiologic State

c-KIT (CD117) through interactions with its ligand stem cell factor (SCF) induces receptor tyrosine kinase (RTK) dimerization, activation of receptor tyrosine kinase activity and initiation of several signal transduction pathways that regulate cell proliferation, differentiation, mobilization of hematopoietic stem cells and progenitor cells (myeloid, lymphoid, erythroid and megakaryocytic cells).

Pathologic State

c-KIT (CD117), a receptor tyrosine kinase is involved in intracellular signaling. Overactivation of receptor tyrosine kinase c-KIT (CD117) induces alterations in the signaling pathways leading to unrestricted CSC proliferation, differentiation, migration and survival. Gain-of-function or point mutations in c-KIT (CD117) receptor tyrosine kinase have been linked to several human cancers such as gastrointestinal stromal tumors (GISTs), seminoma and acute myelogenous leukemia (AML).

Targeted Therapy

Targeting receptor tyrosine kinase c-KIT mutations in solid tumors are scientific rationale and novel therapeutic options.

Anaplastic Lymphoma Kinase (ALK) Proto-oncogene Encodes ALK Receptor Tyrosine Kinase

Anaplastic lymphoma kinase (ALK) proto-oncogene encodes ALK receptor tyrosine kinase (RTK), that binds to growth factors such as pleiotrophin (PTN) and midkine (MK); and transmits signals from the cell surface to within the cell through a process called transduction. ALK protein dimerization results in autophosphorylation of several intracellular ALK receptor tyrosine kinase.

Physiologic State

Anaplastic lymphoma kinase (ALK) receptor tyrosine kinase plays a pivotal role in cellular communication, normal development and function of the nervous system.

Pathologic State

Translocation/amplification of ALK (anaplastic lymphoma kinase) receptor tyrosine kinase is demonstrated in neuroblastoma.

- Point mutation of ALK receptor tyrosine kinase is demonstrated in lung adenocarcinoma.
- Constitutive activation of receptor tyrosine kinases (RTKs) via chromosomal translocation or somatic mutations can lead to aberrant stimulation of signaling pathways resulting in development of malignant tumor, invasion and metastasis.
- Hybrid NPM-ALK gene formed due to chromosome t(2;5) is linked to development of anaplastic large cell lymphoma.

ROS1 Proto-oncogene Encodes Receptor Tyrosine Kinase

ROS1 is a proto-oncogene located on chromosome 6q22.1 that encodes a type 1 integral membrane protein with receptor tyrosine kinase (RTK) activity.

Physiologic State

ROS1 is a member of the insulin receptor tyrosine kinase family related to ALK, that activates downstream signaling pathways involved in cell growth, proliferation and differentiation. ROS1 has been described as an 'orphan' RTK and has no known ligand.

Pathologic State

Mutation in ROS1 proto-oncogene encoding aberrant receptor tyrosine kinase is linked to development of non-small cell lung carcinoma (NSCLC), glioblastoma multiforme, renal oncocytoma, gastric carcinoma, colorectal carcinoma, cholangiocarcinoma and chronic myelomonocytic leukemia. Novel hybrid genes (CD74-ROS1, SLC32A-ROS1 and FIG-ROS1) have been demonstrated in non-small cell lung carcinoma. ROS1-GOPC/FIG1 novel fusion gene product has been detected in hepatic angiosarcoma.

Targeted Therapy

ROS1 proto-oncogene inhibition may be an effective treatment strategy for the subsets of patients with non-small cell lung carcinoma (NSCLC) which express CD74-ROS1, SLC32A-ROS1 and FIG-ROS1 novel fusion genes.

FMS-like Receptor Tyrosine Kinase 3

FMS-like tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase that is expressed almost exclusively in the hematopoietic progenitor cells (CD34+/c-KIT+).

Physiologic State

Binding of receptor FMS-like tyrosine kinase 3 (FLT3) to its ligand protein induces dimerization, and activation of receptor tyrosine kinase activity through RAS/RAF/MEK/ERK (MAPK) signaling pathway and phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway resulting in proliferation of hematopoietic progenitor cells.

Pathologic State

Mutations in FMS-like tyrosine kinase 3 (FLT3) gene lead to constitutive ligand independent autophosphorylation of the FMS-like receptor tyrosine kinase resulting in development of malignant tumor through signaling pathways involved in unrestricted CSC proliferation, differentiation and survival. Point mutations in codon 835 or deletions of codon 836 in FMS-like tyrosine kinase 3 (FLT3) gene are demonstrated in acute myelogenous leukemia (AML) in 25–30% of patients.

Targeted Therapy

Multiple small molecule inhibitors are under development to target aberrant FLT3 activity that confers a poor prognosis in AML patients.

AKT1 Proto-oncogene Encodes Putative Receptor Serine/Threonine Protein Kinase

AKT1 proto-oncogene mapped on chromosome 14q32–33 encodes putative AKT1 serine/threonine protein kinase found in variety of cells. AKT1 serine/threonine kinase plays a key role in many signaling pathways involved in cell growth, proliferation, differentiation, survival, glycogen metabolism, and apoptosis.

Physiologic State

ATK1 serine/threonine protein kinase via PI3K/AKT/mTOR signaling pathway appears to be essential for the normal development of nervous system, cell-to-cell communication among neurons, neuronal survival, and formation of memories.

Pathologic State

AKT1 gene mutation has oncogenic potential to transform normal cells into CSCs. Amplification of AKT1 gene encoding aberrant serine/threonine kinase is linked to lung adenocarcinoma, gastric carcinoma, colon carcinoma, endometrioid carcinoma, and breast invasive ductal carcinoma.

AKT2 Proto-oncogene Encodes Putative Receptor-like Serine/Threonine Protein Kinase

AKT2 proto-oncogene mapped on chromosome 19q13.2 encodes a putative receptor-like serine/threonine protein kinase, which is capable of phosphorylating several intracellular proteins, that is an effector molecule in the signaling pathway linked to insulin's metabolic effects.

Physiologic State

AKT2 serine/threonine protein kinase plays important role in cell metabolism, proliferation, survival, growth and angiogenesis.

Pathologic State

Amplification of AKT2 serine/threonine kinase is implicated in development of ovarian carcinoma, pancreatic carcinoma, breast carcinoma, and prostatic carcinoma.

BTAK Proto-oncogene Encodes Putative Receptor-like Serine/Threonine Protein Kinase

BTAK proto-oncogene mapped on chromosome 20q13 encodes a putative receptor-like serine/threonine protein kinase involved in cellular transformation activity. Amplification of BTAK gene is linked in development of breast carcinoma, ovarian carcinoma and gastric carcinoma.

RAF1 Proto-oncogene Encodes Putative Receptor-like Serine/Threonine Protein Kinase

RAF1 proto-oncogene is a cellular homolog of viral RAF gene (v-RAF) located on chromosome 3p25.2 encodes a putative receptor-like serine/threonine kinase.

Physiologic State

Putative receptor-like serine/threonine kinase acts as a regulatory link between the membrane-associated RAS GTPase, and RAS/RAF/MEK/ERK/MAPK signaling pathway leading to sequential phosphorylation of the dual-specific protein kinases MEK1 and MEK2, which in turn phosphorylate to activate serine/threonine specific protein kinases, ERK1 and ERK2. This regulatory link functions as a switch determining cell fate decisions including cell growth, proliferation and differentiation, survival, apoptosis and angiogenesis in wound healing.

Pathologic State

Point mutations in RAF1 gene are involved in the development of lung carcinoma, colorectal carcinoma and ovarian carcinoma.

Phosphatidylinositol 3-Kinase Catalytic Subunit Alpha Proto-oncogene Encodes p110α Subunit with Kinase Domain

Phosphatidylinositol 3-kinase catalytic subunit alpha (PIK3CA) proto-oncogene located on chromosome 3q26.3 encodes p110α protein, which is a major catalytic subunit of an enzyme phosphatidylinositol 3-kinase

(PIK3). Somatic mutations in the PIK3CA gene are linked to lung carcinoma, squamous cell carcinoma of skin, breast carcinoma and ovarian carcinoma.

GTP-BINDING PROTEINS (G PROTEINS)

G protein-coupled receptors (GPCRs) are cell surface receptors associated with GTP-binding proteins (also called G proteins).

- Guanosine triphosphate (GTP) binding proteins transmit signals outside the cell and induce changes within the cell.
- RAS family genes encoding proteins are small guanosine triphosphatases (GTPases), which exist in equilibrium between guanosine diphosphate (GDP) and guanosine triphosphate (GTP) bound forms.
- GTP-binding proteins act as molecular switches and induce conformational changes in molecular regions when RAS transitions from inactive GDP-bound state (turning off) to active GTP-bound state '**turning on**'. Nonreceptor protein kinases transmit the signal from the cell surface receptor to the nucleus.
- Proteins can be activated or inactivated by altering protein activity is the addition of phosphate group to one or more sites on the protein, a process is called **phosphorylation**.

Physiologic State

RAS family of genes (i.e. K-RAS, N-RAS and H-RAS) encode GTP-binding proteins, that act as molecular switches which can '**turn on**' when RAS protein is bound to GTP (guanosine triphosphate) to form complex in the active state of signal transduction, and '**turn off**' when bound to GDP (guanosine diphosphate) form complex in inactive state of signal transduction-bound conformation of RAS protein shows high affinity interactions with effector proteins that propagate downstream signaling from the receptor to the nucleus via nonreceptor protein kinases, and regulate cell growth, proliferation, differentiation, and apoptosis.

Pathologic State

The oncogenic mutation in RAS gene encodes aberrant RAS protein that loses its GTPase activity, so remains activated, resulting in continual promotion of transcription thus leading to development of malignant tumors in 30% cases.

- K-RAS is linked to non-small cell lung carcinoma, colorectal carcinoma and pancreatic carcinoma.
- N-RAS is linked to colorectal carcinoma and melanoma.
- H-RAS is linked to breast carcinoma. RAS gene mutations also make cells resistant to chemotherapy.

SIGNAL TRANSDUCTION PROTEINS

Signal transduction defines the series of molecular events, that occur to convert an external stimulus into cellular response by involving phosphorylation of target molecules by enzymes with protein kinase activity. A signal transduction pathway is initiated, when a ligand binds to its cellular receptor resulting in a conformational change. The ability of cells to sense external signals and respond to external signal is essential for tissue development and repair, immunity and homeostasis during embryogenesis and postnatal life.

- **Signal transduction pathways:** The most important signaling pathways have been shown to be implicated in human cancers include: (a) RAS/RAF/MEK/ERK (MAPK/ERK) signaling pathway, (b) phosphatidylinositol 3-kinase (P13K)/AKT/mTOR signaling pathway, (c) JAK/STAT signaling pathway, (d) Hedgehog signaling pathway, (e) NOTCH signaling pathway, (f) Wnt/β-catenin signaling pathway, (g) NF-κB signaling pathway, (h) receptor tyrosine kinases (RTKs) signaling pathway and (i) integrin pathway.
  - Out of these signaling pathways, the most common JAK/STAT and NF-κB signaling pathways have been shown to be disrupted and implicated in human cancers.
  - Cell signaling pathways are shown in Fig. 6.110. Cell signaling paradigms in cell division are shown in Fig. 6.111.
- **Signal transduction pathway and crosstalk mechanisms:** Signaling crosstalk can occur via different mechanisms: (a) the molecule in one signaling transduction pathway can affect the rate of activation of signaling molecules, (b) two signaling pathways can compete for common component, and (c) growth factor receptors can have altered ability to recognize ligands.
- **Mutations in signal transduction molecules and cancers:** Components of signaling molecules can be mutated or overexpressed leading to development of malignant tumor. Some oncogenes encode aberrant signal-transduction proteins involved in transmitting signals from the growth factor receptor tyrosine kinase (RTK) of the cell to the nucleus.
  - If cell surface receptors (e.g. HER2/neu) are overexpressed, signaling can happen in the absence of ligand (receptor function crosstalk). Non-receptor protein kinases are also included in the signaling cascade that transmit the signal from the cell receptor to the nucleus.
  - Individual signaling pathways could have opposite effects on transcription factor activation (gene expression crosstalk). Ligand availability can be

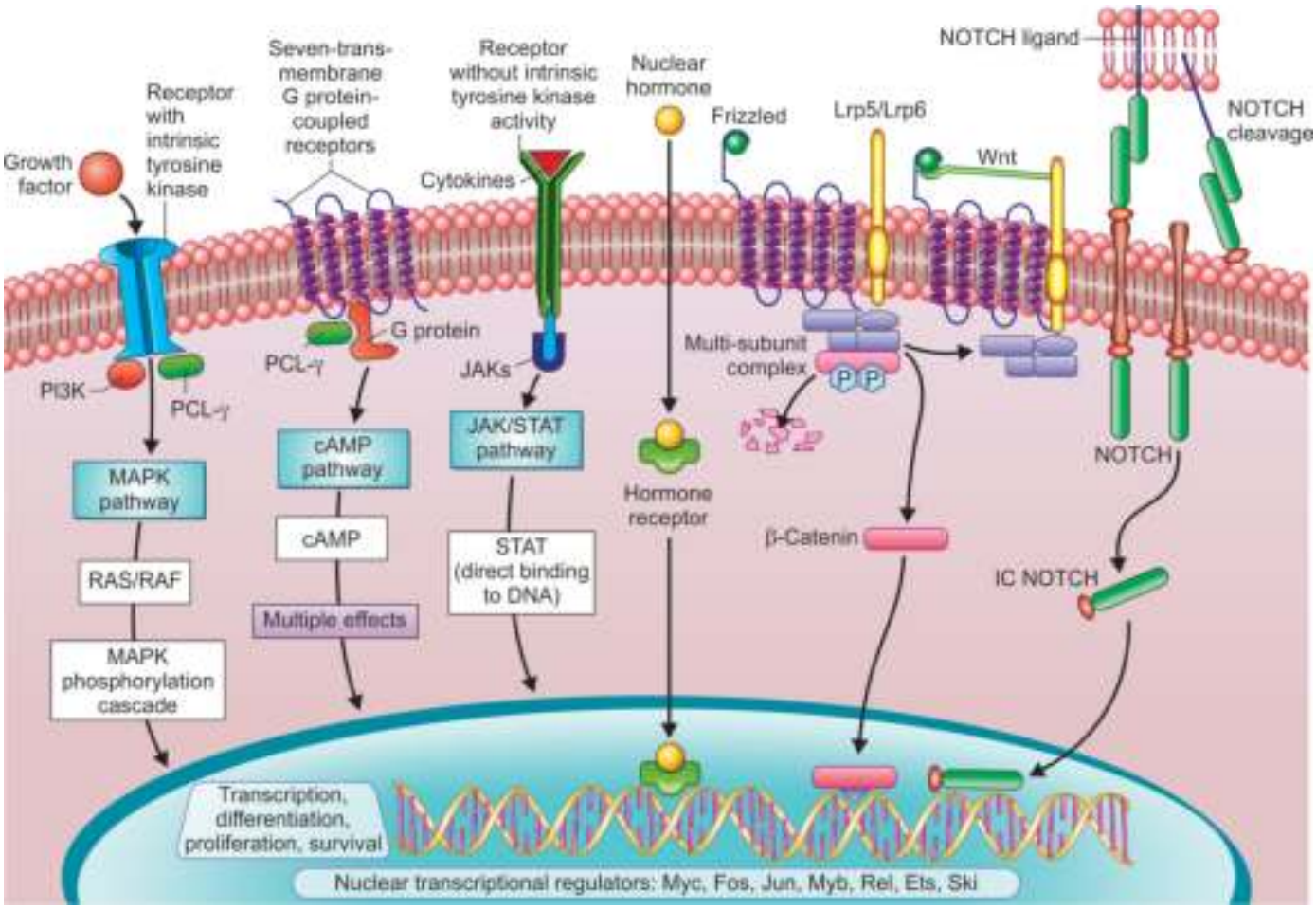


Fig. 6.110: Cell signaling pathways: Cell signaling pathways are the key biological mechanisms that transduce extracellular signals to affect transcription-mediated gene regulation within cells. Cell signaling pathways regulate cell proliferation and survival and are therefore important for maintaining homeostasis and development, which include: phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR, RAS/RAF/MEK/ERK (MAPK/ERK), JAK/STAT, Wnt/β-catenin, NOTCH, Hedgehog, NF-κB, TGF-β and ABL nonreceptor pathways.

altered due to different mechanisms, but often occurs in response to gene expression changes (intracellular crosstalk).

- Selected cellular oncogenes (mutated proto-oncogene) associated with many human cancers include HER2/neu (also called ErbB2), RAS, Myc, SRC, hTERT, and BCL-2.
- BCR-ABL1 oncogene (Philadelphia chromosome) caused by translocation of segments of chromosome 9 and chromosome 22. When the protein synthesized by BCR-ABL1 oncogene, a receptor tyrosine kinase (RTK), is continually synthesized that results in continuous transmission of signal responsible for cell growth and cell division of leukemia cells in chronic myelogenous leukemia (**CML**), and acute lymphoblastic leukemia (**ALL**). Status of signaling pathways with oncogenic potential linked human cancers are given in Table 6.90.
- **Analysis of signal transduction pathways and targeted therapy:** It is essential to analyze key signaling pathway including their communication with other signaling pathway (crosstalk) involved in development of malignant tumor, progression, and targeted therapy.

RAS/RAF/MEK/ERK (MAPK/ERK) Signaling Pathway

RAS/RAF/MEK/ERK (MAPK—mitogen-activated protein kinase) signaling cascade is essential for cell-to-cell and intracellular communication, which regulates cell adhesion, proliferation, differentiation, migration and survival.

Physiologic State

RAS/RAF/MEK/ERK (MAPK/ERK) signaling pathway plays an integral part in transducing signals from growth factors and cytokines through receptor tyrosine kinase (RTK).

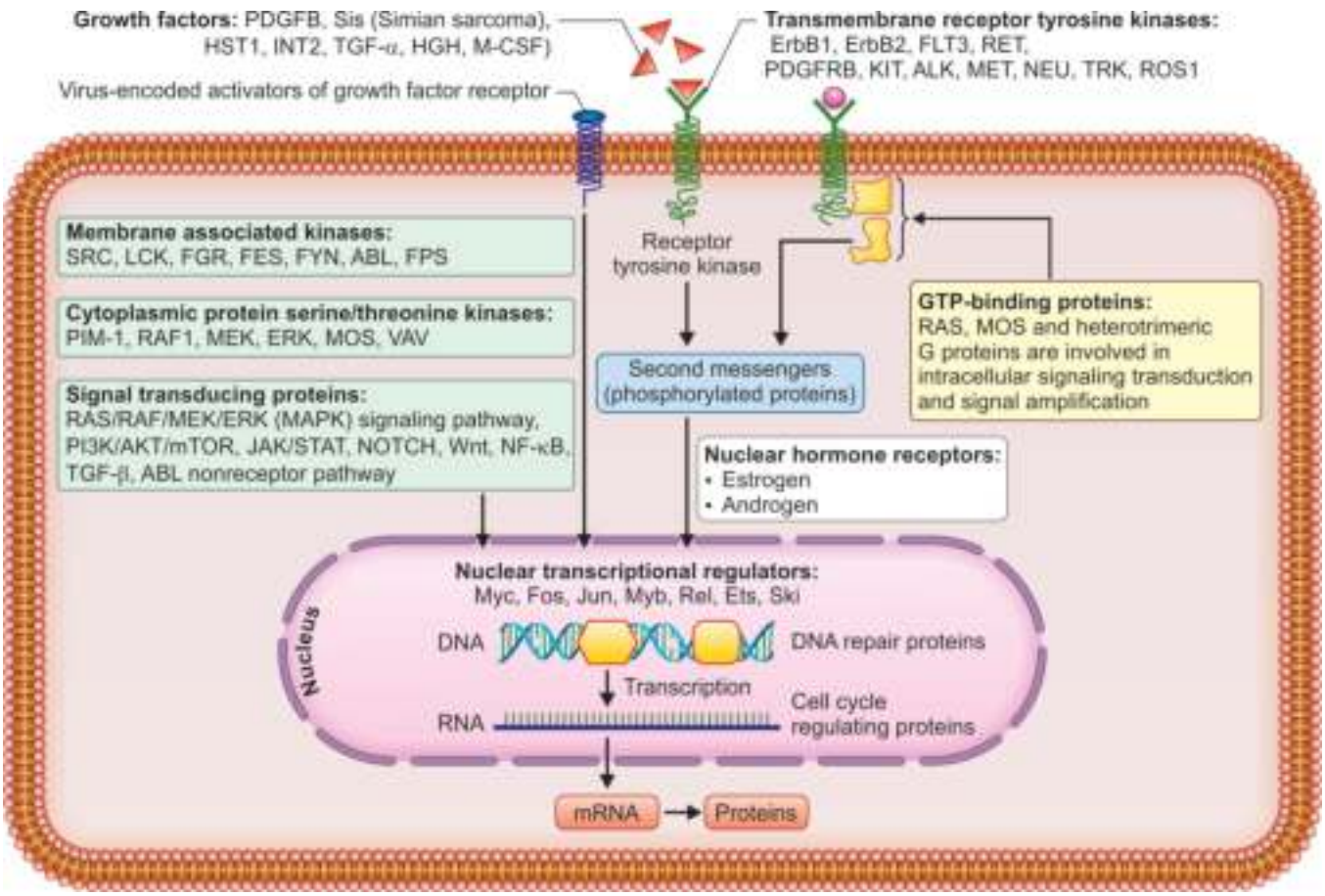


Fig. 6.111: Cell signaling paradigms in cell division. Extracellular ligands (e.g. Sis, HST) bind to cell membrane receptors (EGFR, ErbB2, KIT). One of several signaling pathways is then activated. The receptor may be a G protein-coupled receptor (e.g. RAS, MOS, heterotrimeric G proteins), which stimulates guanine nucleotide-related signaling, or the ligand may traverse the cell membrane to activate receptors within the cytosol, without a cell membrane intermediate. Cellular intermediates of many cells are activated. The end result for all signaling pathways is activation of transcription, particularly of proteins that help take the cell through the cell cycle.

Table 6.90 Status of signaling pathways with oncogenic potential linked human cancers

Signaling Pathways	Target Gene Expression	Human Cancers
RAS/RAF/MEK/ERK (MAPK/ERK) signaling pathway with oncogenic potential	↑K-RAS (point mutation) GTPase	▪ Colon carcinoma ▪ Lung carcinoma ▪ Pancreatic carcinoma
	↑H-RAS (point mutation) GTPase	▪ Renal cell carcinoma ▪ Urinary bladder carcinoma
	↑N-RAS (point mutation) GTPase	▪ Melanomas ▪ Hematologic malignancies
	↑GNAO (point mutation)	Uveal melanoma
	↑GNAS (point mutation)	▪ Pituitary adenoma ▪ Other endocrine tumors
	↑B-RAF (point mutation/translocation)	▪ Hairy cell leukemia (100%) ▪ Melanoma (60%) ▪ Colon carcinoma ▪ Dendritic cell tumor ▪ Benign nevi

Contd...

Table 6.90 Status of signaling pathways with oncogenic potential linked human cancers (*Contd...*)

Signaling Pathways	Target Gene Expression	Human Cancers
PI3K/AKT/mTOR signaling pathway with oncogenic potential	↑AKT	▪ Breast carcinoma ▪ Ovarian carcinoma ▪ Thyroid carcinoma
	↓PTEN	▪ Glioblastoma multiforme ▪ Melanoma ▪ Lung carcinoma
JAK/STAT signaling pathway with oncogenic potential	↑STAT	▪ Lung carcinoma ▪ Head and neck cancers ▪ ALL
NOTCH signaling pathway with oncogenic potential	↑NOTCH (point mutation/translocation/gene rearrangement)	▪ T-ALL ▪ Lymphomas ▪ Breast carcinoma
Wnt/β-catenin signaling pathway with oncogenic potential	↑β-Catenin	▪ Small intestine adenocarcinoma ▪ Gastric polyps
	↓APC	▪ Colon carcinoma ▪ Gastric adenomas
NF-κB signaling pathway with oncogenic potential	↑Rel	▪ Hodgkin's disease ▪ NK/T cell lymphoma
TGF-β signaling pathway with oncogenic potential	↓SMAD	Pancreatic carcinoma
	↑BMP2	Lung carcinoma
Non-receptor tyrosine kinase signaling pathway with oncogenic potential	↑ABL-BCR	CML

ALL: Acute lymphoblastic leukemia; T-ALL: T cell acute lymphoblastic leukemia; CML: Chronic myelogenous leukemia.

- Central to this signaling cascade is RAS, a small membrane-bound GTPase switch protein that shuttles between two conformational states: active GTP bound and inactive GDP-bound. It is activated by guanine exchange factor usually found in the cytoplasm of the cell.
- Activated GTP bound RAS activates the serine/threonine RAF kinase, the latter proceeds to phosphorylate and activate MEK. Then MEK phosphorylates and activates ERK-1 and ERK-2 (MAPKs). ERK-1 and ERK-2 can phosphorylate kinases in the cytoplasm that regulate translation of transcription factors and translocation into the nucleus leading to cell proliferation.

Pathologic State

Some components of RAS/RAF/MEK/ERK (MAPK/ERK) signaling cascade (e.g. RAS, BRAF) are mutated or aberrantly expressed leading to unrestricted CSC proliferation, development of malignant tumor and chemoresistance.

- Mutations can also occur at genes encoding upstream receptors (e.g. EGFR and FLT-3) and chimeric chromosomal translocations (e.g. BCR-ABL1), which transmit their signals through these signaling cascades.
- Hyperactivation of RAS/RAF/MEK/ERK (MAPK) signaling is associated with acute myelogenous leukemia (AML) in >50%, acute lymphoblastic leukemia (ALL), breast carcinoma, colon carcinoma, pancreatic carcinoma, melanoma and prostatic carcinoma associated with poor prognosis.
- Consequently, RAS/RAF/MEK/ERK (MAPK/ERK) signaling pathway is a formidable target for therapeutic intervention, which has received tremendous attention.

JAK (Janus Kinase)/STAT Signaling Pathway

JAK/STAT signaling pathway in response to growth factors (e.g. EGF, PDGF), and cytokines (e.g. IL-6) plays key role in mediating cell proliferation, differentiation, migration, and survival, angiogenesis stem cell maintenance, apoptosis, hematopoiesis, immune system, and embryological processes (mammary gland development). There are four members of the JAK family and seven members of STATs.

Physiologic State

The binding of extracellular ligand results in activation of JAK/STAT signaling pathway via changes to the transmembrane receptor tyrosine kinase family

that permits the intracellular JAKs associated proteins with them to phosphorylate one other. Transphosphorylated JAKs can phosphorylate downstream substrates, such as receptors, and STATs. Activated STATs enter the nucleus and bind target genes, thus regulate their transcription.

Pathologic State

Aberrant JAK/STAT signaling pathway is implicated in development of malignant epithelial tumors in various organs (breast, stomach, liver and pancreas).

- Aberrant cytokine production, mutations of receptor tyrosine kinases (RTKs), and rearrangements and mutations in both JAK (JAK1, JAK2 and JAK3) and STAT proteins lead to development of malignant tumors.
- Gain-of-function of JAK1 and JAK3 is linked to leukemia and lymphoma. STAT3 is constitutively activated in over 50% of human cancers such as lung carcinoma, breast carcinoma, head and neck carcinomas, and hematologic malignancies.

NF-κB Signaling Pathway

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor family consists of five distinct proteins as follows: RelA, RelB, Relc, p100 and p50.

Physiologic State

Rel gene encodes NF-kB transcription factor that regulates transcription of DNA and cytokines production, i.e. IL-1 and TNF-α involved in cell proliferation, oxidative stress response, innate immune response, inflammation and apoptosis.

- In response to injurious stimuli, NF-κB transcription factor undergoes phosphorylation on serine 32 and 36 by IκB kinase (IKK).
- Phosphorylated NF-κB is ubiquitinated by the E3 ubiquitin ligase complex and targeted for degradation by 26S proteasome. The released NF-κB dimers can then translocate into the nucleus and activate transcription of target genes.

Pathologic State

Mutations of NF-κB signaling pathway have been linked to human cancers. Amplification of Rel gene encoding mutated NF-κB transcription factor is linked to Hodgkin's disease, B cell non-Hodgkin's lymphoma and natural killer/T cell non-Hodgkin's lymphoma. Immunohistochemical analysis of B cell-derived lymphoma samples with Rel amplifications have established nuclear Rel expression in several cases.

Phosphatidylinositol 3-Kinase (PI3K)/AKT/mTOR Signaling Pathway

Phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway is a major prosurvival intracellular network, that regulates protein synthesis, growth, proliferation, migration, survival, metabolism, angiogenesis and cell cycle progression.

Physiologic State

Phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway is activated by receptor tyrosine kinases, integrins, B cell and T cell receptors, G protein-coupled receptors and other stimuli, that induce production of phosphatidylinositol-3,4,5-triphosphates (PIP3) by phosphoinositol 3-kinase (PI3K).

Pathologic State

Aberrant activation of PI3K/AKT/mTOR signaling pathway has a significant role in carcinogenesis, unrestricted CSC proliferation, angiogenesis, epithelial–mesenchymal transition (EMT), and chemoresistance in various organs such as breast, endometrium, colon, lung and head and neck regions.

- **Genetic alterations in the PI3K gene:** The p100-α (100α) subunit encoded by PI3K gene is the most prevalent altered catalytic subunit of PI3K isoform in human cancers. Binding of transmembrane receptor tyrosine kinases, i.e. ErbB2, EGFR, Met, Ret and VEGFR to PI3K signaling pathway activates RAS proteins. After RAS proteins being activated, PI3K-100α transforms its lipid substrate phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), which active AKT/mTOR signaling pathway.
- **Genetic alterations in the mTOR gene:** The mTOR protein encoded by mTOR gene belongs to a serine/threonine kinase that regulates cellular responses to stressors such as DNA damage and nutrient deprivation. The mTOR activation mutations increase threonin kinase activity of mTOR leads to overactivated downstream signaling overactivity and unrestricted cell proliferation resulting in development of malignant tumors of endometrium, esophagus, stomach, colon, kidney, urinary bladder, and skin melanoma.

NOTCH Signaling Pathway

NOTCH signaling pathway consists of membrane tethered proteins, that upon binding to cognate NOTCH ligand in transmembrane region undergoes proteolytic cleavage, and releases transcription factor that shuttles to the nucleus.

Physiologic State

NOTCH signaling pathway plays an important role in cell proliferation, differentiation, and apoptosis including earliest stages of T cell development.

Pathologic State

Mutation is the NOTCH signaling pathway has been detected in human T cell acute lymphoblastic leukemia (T-ALL). Recurrent chromosomal translocation t(7;9)(q34;q34.3) involving human NOTCH 1 gene has been found in a small subset of T cell acute lymphoblastic leukemia (**T-ALL**).

- Aberrant notch signaling possibly increases cell proliferation through activation of c-Myc transcription factor, whose deregulated expression has been linked in the development of T cell acute lymphoblastic leukemia, non-Hodgkin's lymphomas, breast carcinoma, melanoma, medulloblastoma and ovarian carcinoma.
- In the case of human breast carcinoma, amplification of NOTCH receptors and the presence of cognate Jagged 1 ligands, correlate with a more aggressive malignant phenotype.

Wnt/β-Catenin Signaling Pathway

Wnt/β-catenin signaling pathway consists of >30 extracellular Wnt-ligands, which interact with receptors of the '**Frizzled family**', and induces accumulation of β-catenin in the cytoplasm and its eventual translocation into the nucleus, where it interacts with DNA-binding proteins such as TCF/LEF (T cell factor/lymphocyte enhancer binding factor). TCF/LEF DNA binding proteins act as transcriptional activators, which induces cell proliferation via activation of c-Myc, and cyclin D genes.

Physiologic State

The β-catenin is a core component of the cadherin protein complex, whose stabilization is essential for the activation of Wnt/β-catenin signaling pathway involved in cell growth, polarity, migration, and differentiation; neural patterning, and organogenesis during embryonic development. Wnt/β-catenin signaling pathway is essential for intestinal homeostasis.

Pathologic State

Mutation-induced activation of Wnt/β-catenin signaling pathway either activates adenomatous polyposis coli (APC) tumor suppressor gene and stabilizes β-catenin by rendering it unfit for phosphorylation/degradation, through mutations of residues located in its exon 3.

- The aberrant Wnt/β-catenin signaling pathways causes mutation of adenomatous polyposis coli (APC) tumor suppressor gene, and facilitates CSC renewal, unrestricted CSC proliferation and differentiation, thus exerting crucial roles in development of colorectal carcinoma, and chemoresistance.
- In more than 50% of cancers of breast, colorectal region, stomach, liver, endometrium and ovary including skin melanoma, β-catenin accumulates within the nucleus or cytoplasm.

SMAD/Transforming Growth Factor-β Signaling Pathway

The SMAD-dependent transforming growth factor-β (TGF-β) signaling pathway plays important role in embryogenesis and tissue hemostasis. TGF-β superfamily includes a diverse range of structurally and functionally related proteins, such as bone morphogenetic proteins (BMPs), activins, inhibins, growth differentiation factors (GDFs), and glial-derived neurotrophic factors (GDNFs). These proteins transmit signal by stimulating formation of specific heteromeric complexes of type 1 and type 2 serine/threonine kinase receptors.

Physiologic State

SMADs are intracellular regulatory proteins that can be categorized based on their functions: (a) **receptor-regulated SMADs** (R-SMAD, e.g. SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8), (b) **common-mediator SMADs** (CO-SMADs, e.g. SMAD4), (c) **inhibitory SMADs** (I-SMADs, e.g. SMAD6 and SMAD7).

- Receptor-regulated SMADs (R-SMADs) bind to membrane-bound serine/threonine receptors, and are activated by their kinase activity. Co-factor SMADs bind to the activated R-SMADs to form a complex that translocates into the nucleus, where they function to regulate gene expression, i.e. transcriptional activation and repression. In addition, nuclear accumulation of active nuclear SMAD complexes is maintained in strict accordance with the degree of receptor activation at any time of signaling. SMAD phosphorylation by active receptors, and constitutive SMAD dephosphorylation by nuclear phosphates, are coupled through nucleocytoplasmic shuttling of SMADs.
- Inhibitory SMADs counteract the effects of R-SMADs, thus exerting an inhibitory effect on TGF-β superfamily signaling by various mechanisms. The SMAD2, SMAD3, and SMAD4 stable complexes activate transcription of cell cycle suppressors ($p15^{INK4b}$, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$) and repressors transcription of the proliferation activator c-Myc.

Pathologic State

SMAD/transforming growth factor-β (SMAD/TGF-β) signaling pathway has dichotomous roles. It has been observed that TGF-β acts as a tumor suppressor, and apoptosis in the early stage of the disease, and as a tumor promoter in its late stages.

- **In the initial stage**, SMAD/TGF-β signaling pathway inhibits the proliferation of normal cells and promotes apoptosis of premalignant cells by increasing expression of cyclin-dependent kinase inhibitors (CDKIs) such as $p15^{INK4b}$, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$.
- **In the late-stage**, mutations in constituent proteins of the SMAD/TGF-β signaling pathway impair the antiproliferative and proapoptotic activities of TGF-β, and activates downstream oncogenes leading to unrestricted CSC proliferation, cell growth, epithelial–mesenchymal transition (EMT), metastasis and chemoresistance. Mutations in TGF-β receptors, and SMADs are linked to colorectal carcinoma and pancreatic carcinoma.

Hedgehog Signaling Pathway

Hedgehog (Hh) signaling pathway plays an essential role in cell differentiation of embryonic cells during embryogenesis and tissue homeostasis. Aberrant Sonic Hedgehog (**Shh**) signaling pathway plays key role in evolution of chemoresistance and radioresistance of several human cancers.

Physiologic State

Hedgehog (Hh) signaling pathway is composed of three specific proteins: Hh signal peptide (i.e. Shh, Dhh, Ihh), transmembrane receptors (patched—PTCH, smoothened—Smo), and downstream transcription factor (Gli).

- Hedgehog (Hh) signaling pathway is initiated at the plasma membrane, where Hh signal peptide interacts with its 12 transmembrane protein receptors (patched—PTCH, smoothened—Smo). PTCH normally inhibits another transmembrane protein is called 'smoothened' (Smo), which itself prevents specific genes from transcribed RNA.
- When Hh signal peptide ligand binds to PTCH protein, it activates the Smo protein. The Smo protein interacts with other molecules through the cell's cytoplasm and into the nucleus. Smo transmembrane protein regulates gene transcription through the downstream transcription factor Gli.

Pathologic State

Hedgehog (Hh) signaling pathway is disrupted in diverse types of human cancers such as basal cell carcinoma, small cell lung carcinoma, pancreatic carcinoma, prostatic carcinoma, medulloblastoma and gastrointestinal malignancies.

Integrin Signaling Pathway

Integrin belongs to the family of cell adhesion molecules. Integrins are heterodimeric receptor proteins used by cells to bind to extracellular matrix (ECM), and actin cytoskeleton.

Physiologic State

An integrin molecule is composed of two noncovalently associated glycoprotein subunits α and β, which binds to induce intracellular signals leading to cellular responses such as cell-to-cell interaction (adhesion), cell growth, proliferation, migration and apoptosis.

Pathologic State

In human cancers, aberrant expression with normal functioning, rather than dominant genetic variations of genes encoding integrins has been observed. The aberrant expression is mediated through integrin signaling pathway, that leads to unrestricted CSC proliferation, migration, angiogenesis, evasion of apoptosis, interactions between CSCs and extracellular matrix, extracellular matrix remodeling, invasion and metastasis. Interaction between extracellular matrix glycoproteins and CSC integrins is given in Table 6.91.

Targeted Therapy

A number of molecules targeting integrins have been developed for treatment of many human cancers such as prostatic carcinoma and melanoma.

Receptor Tyrosine Kinase (RTK) Signaling through RAS Proteins

RAS, a small GTP-binding protein, is essential component of signal transduction pathway used by growth factors binding to receptor tyrosine kinases (RTKs), which initiates cell proliferation, growth, modulation of metabolism and differentiation.

Physiologic State

GTP-binding proteins are small GTPases, which act as '**molecular switches**' in signaling pathways to regulate functions of other proteins.

- GTPase liberates free inorganic phosphates (P). Cell activation with growth factors such as epidermal growth factor (EGF) induces RAS to move from an

Table 6.91 Interaction between extracellular matrix glycoproteins and cancer stem cell integrins

Binding Integrin Molecules on Tumor Cell	Extracellular Matrix Glycoproteins
VLA-2 (α_2, β_1)	Collagen and laminin
VLA-4 (α_4, β_1)	Fibronectin variant
VLA-5 (α_5, β_1)	Fibronectin
VLA-6 (α_6, β_1)	Laminin
α_6, β_4	Laminin
αv, β_3	Vitronectin

VLA: Very late activation antigen

inactive GTP bound (**switch turning off**) to an active GTP bound state (**switch turning on**).
- After binding of EGF ligand to cognate EGF receptor tyrosine kinase (RTK) leads to receptor autophosphorylation residues, which induce multiple signaling cascades.
- Activation of RAS/RAF/MEK/ERK (MAPK) signaling pathway plays essential role in cell proliferation, survival and differentiation.

Pathologic State

RAS/RAF/MEK/ERK (MAPK/ERK) signaling pathway transmits extracellular signals to specific intracellular targets.
- Aberrant activation of RAS/RAF/MEK/ERK/MAPK signaling pathway is linked to transformation of normal cell to CSCs and development of malignant tumor.
- About 30% of malignant tumors harbor RAS mutations, while 8% of malignant tumors are driven by RAF mutations.

TRANSCRIPTION FACTORS

In molecular biology, nuclear regulatory proteins are sequence-specific DNA binding '**transcription factors**' excluding RNA polymerase, synthesized in the cytoplasm, and have to be transported into the nucleus of the target cells. Transcription factors bind nearby specific DNA sequence and help specific target genes to 'turn on' (gene activation) or 'turn off' (gene silencing). Hence, transcription factors can either act as activators (enhancers) or repressors (silencers), that boost a gene's transcription. Hence, transcription factors regulate the rate of transcription of genetic information from DNA to messenger RNA.

Pathology Pearls: Transcription Factor—Terminology

- **Human gene:** Human gene contains 5'→3' untranslated, promoter, enhancer and silencer regions.
- **Gene expression:** Gene expression is the process by which information from a gene is used in the synthesis of a unique set of functional proteins and RNA molecules in each cell type of our body.
- **Promoter sequences:** Promoter sequences are regions on DNA, where transcription of a gene is initiated with the help of enzyme RNA polymerase by interacting with different transcription factors.
- **TATA box:** A TATA box is a conserved DNA sequence that indicates, where a genetic sequence can be read and decoded. TBP gene encodes the TATA box binding protein.
- **DNA bending:** DNA bending is essential in transcription regulation, which brings distal promoter and enhancer regions into its close proximity.
- **Transcription:** Transcription is the process, in which DNA sequence is transcribed into RNA molecule essential for protein synthesis with the help of enzyme RNA polymerase.
- **Transcription factor:** Gene expression requires transcription factor (sequence-specific DNA binding factor), that binds to DNA and regulates gene expression by promoting or suppressing transcription.
- **Transcription regulation:** Transcription regulation is defined as rate of gene transcription by helping or hindering RNA polymerase binding to the DNA. Increase in the rate of transcription is called upregulation, activation or promotion. Decrease in the rate of gene transcription is known as downregulation, repression or suppression.
- **Coactivator:** Coactivator is small protein molecule, that works with transcription factors to increase the rate of gene transcription.
- **Corepressor:** Corepressor is a small protein molecule, that works with transcription factors to decrease the rate of gene transcription.
- **Response elements:** Response elements are short sequences of DNA within a gene promoter or enhancer region, that are able to bind specific transcription factors and regulate gene transcription.

Transcription Factors: Domains and Motifs

Transcription factors consist of two types of structural components: (a) **domain**—an independent tertiary structural unit of a protein assumed to fold independently of the rest of the protein possessing autonomous function (e.g. leucine zipper motif, helix-loop-helix), and (b) **motif**—a short conserved region formed by the spatial arrangement of amino acids in a protein sequence (e.g. helix-turn-helix motif, zinc finger motif, homeodomain motif).
- **DNA binding domain:** DNA binding domain is usually a basic α-helix, which recognizes a specific sequence (response element), mainly located in the major grove of DNA.
- **Transactivation domain:** Transactivation domain interacts with RNA polymerase II transcription initiation complex, and activates transcription, or repress transcription in some cases.
- **Dimerization domain:** Many of the transcription factors bind to DNA as homodimer/heterodimer domains.
- **Ligand binding site:** When an effector binds to ligand binding site, it produces a 3D conformational change, thus activating or inactivating the transcription factors.

Transcription Factors: Functions

Transcription factors (TFs) are regulatory proteins possessing domains mainly located in the major groove of DNA, which bind to specific upstream 5'→3' regulatory DNA, sequences, i.e. promoter or enhancer

regions of specific target genes without breaking the hydrogen bonds. Target genes, which include Fos-related genes (Fos-B, Fra-1, Fra-2), egr-1, egr-2, Nur77, Srf-1), JunB gene, EGR-1 gene, Nur77 gene, Srf-1 gene and Myc (c-Myc, L-Myc, N-Myc).

- Transcription factors also possess a domain that interacts with RNA polymerase II or other transcription factors and consequently regulates transcription of DNA into RNA. Their function is to recruit or inhibit the binding of RNA polymerase to the promoter region of DNA. Other nuclear regulatory proteins can work with transcription factors to either promote or inhibit the transcriptional activity of a target gene being expressed.
- Hence, transcription factors regulate the rate of transcription of genetic information from DNA to messenger RNA (mRNA) to synthesize unique set of functional proteins, thus acting as 'nuclear messengers' by involving multiple intracellular signal transduction pathways stimulated by cell surface receptors.
- Other proteins (e.g. coactivators, corepressors, chromatin modelers, histone acetyltransferases, histone deacetylases, kinases and methylases) are also essential in regulation of gene expression, but lack DNA binding domains, and therefore are not transcription factors.
- Transcription factors can act as either activators (enhancers) or repressors, that can **turn on/turn off** genes and their transcription respectively.
  - Binding of enhancer (activator) transcription factors causes the DNA to bend, bringing them near a gene promoter, even though they may be thousands of nucleotide base pair away.
  - Other transcription factors join the enhancer (activator) transcription factors, forming a protein complex, which bind to the gene promoter. This protein complex makes it easier for RNA polymerase to attach to the promoter and start transcription of gene.
- An insulator can stop the enhancer (activator) transcription factors from binding to the gene promoter region of zinc finger protein is called CTCF transcription factor repressor (named from the sequence CCCTC, which occurs in all insulators) binds to it.
- DNA methylation, a process of adding a methyl group to nucleotide base pairs done by a DNA methyl transferase enzymes is a epigenetic alteration, which '**turns off**' tumor suppressor gene leading to development of cancers. Modulation of transcription process is shown in Fig. 6.112.

Transcription Factors with Altered Activity Mechanisms

Transcription factor activity is altered by two mechanisms: (a) **direct mechanism** through chromosome translocation, gene amplification, gene overexpression, point mutation, and (b) **indirect mechanism** through noncoding DNA mutations that affect binding of transcription factor. Altered activity of transcription factors is linked to many human cancers, which include: c-Myc (Burkitt's lymphoma), L-Myc (small cell lung carcinoma), N-Myc (neuroblastoma), JunB (breast carcinoma, prostatic carcinoma), Fos-B (promotion of CSC-like properties), serum response factor 1 (SRF-1: **hepatocellular carcinoma**), and NUR-77 (**melanoma**).

CHROMATIN REMODELER PROTEINS

In eukaryotes, DNA is tightly wrapped into a complex called 'chromatin' consisting of DNA, RNA and protein. DNA carries the cell's genetic instructions. The histone octamer proteins in the chromatin help to organize DNA into 'bead-like' structures are called '**nucleosomes**' by using energy of ATP by providing a base on which the DNA is wrapped around in compact form that fits in the nucleus.

- Rearrangement of chromatin from a condensed state to a transcriptionally accessible state, allows transcription factors or other DNA binding proteins to access DNA and regulate gene expression.
- Chromatin is essential for all DNA-dependent biological processes (i.e. DNA replication, transcription, DNA repair, genetic recombination and cell division).
- Mutated chromatin remodular proteins have now been detected in most human cancers, which are regarded a novel therapeutic target.

Chromatin: Major Proteins and their Functions

The major proteins in the chromatin are histone octamer proteins, that help to organize DNA into 'bead-like' structures are called 'nucleosomes' by providing a base on which the DNA is wrapped around in compact form that fits in the nucleus.

- A nucleosome consists of 146 nucleotide base pairs of the DNA that is wrapped around a set of 8 histones called histone octomer proteins. Histones H2A, H2B, H3 and H4 are known as histone octamer core proteins, and they come together to form one nucleosome. The nucleosome is formed by two H2A-H2B dimers and an H3-H4 tetramer.
- Extensive studies revealed that chromatin plays a critical role in regulation of transcription. There are two key players in regulation of chromatin dynamics: (a) chromatin remodelers that alter DNA–histone interactions by energy harnessed through ATP hydrolysis, and (b) nucleosome-modifying enzymes

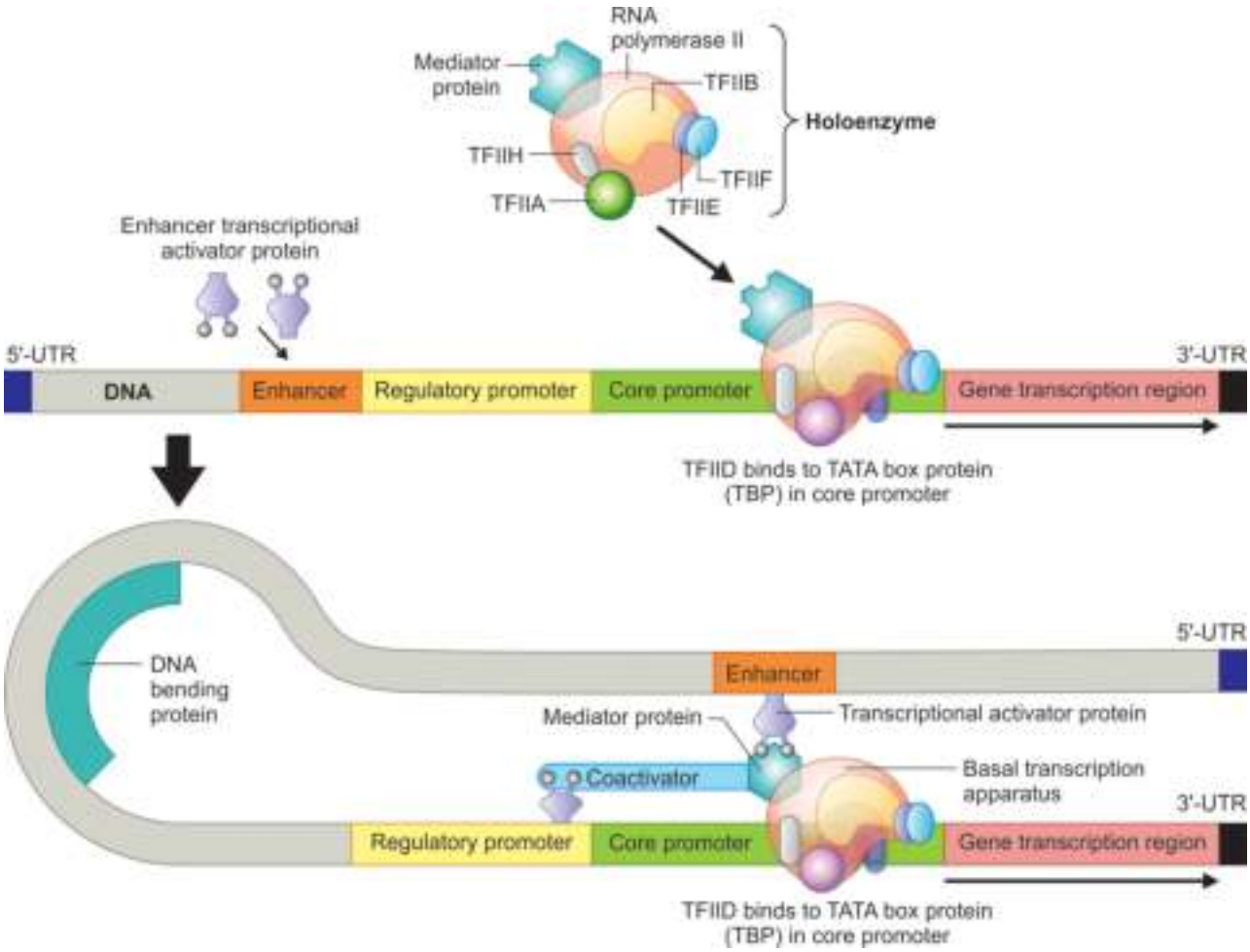


Fig. 6.112: Modulation of transcription process. Transcription is the process in which DNA sequence is transcribed into RNA molecule essential for protein synthesis with the help of enzyme RNA polymerase positioned on DNA with the help of TATA binding proteins. TATA binding proteins bind the TATA box, a DNA sequence that compromises part of the promoter. DNA can loop around itself to cause interaction between an activator protein and other proteins that mediates the activity of RNA polymerase.

(histone acetylases or deacetylases) that modulate DNA and histone residues by specifically adding or removing covalent modifications.

- Specific DNA-binding transcription factors recruit histone acetylases and histone deacetylases to promote activation or repression of transcription respectively. These findings strongly support that transcription is regulated by histone acetylation and deacetylation. ATP-dependent chromosome-remodeling activity enhances the histone deacetylase activity. Therefore, these two mechanisms are involved in regulation of transcription. Both DNA methylation and histone modification are involved in establishing patterns of gene repression during embryonic development.
- DNA methylation should not be confused with histone methylation. DNA methylation leads to stable long-term repression due to conversion of cytosine nucleotide base pairs of DNA to 5-methyl-cytosine, whereas histone octamer methylation causes local formation of heterochromatin, which is a reversible event. DNA methylation and histone octamer modification pathways are dependent on one another, and this crosslink is mediated by biological interactions between DNA methyltransferases and SET domain histone methyltransferases.
- Relationship between DNA methylation and histone octamer modification is involved in normal development as well as somatic cell reprogramming and malignant tumor.

Gene Mutations Encoding Chromatin Structure Linked to Human Cancers

Genes encoding proteins regulate chromatin structure. DNA and histone octamer harbor recurrent mutations (e.g. point mutations, amplification, deletion, fusion) in most human cancers. These gene mutations lead to modifications in chromatin and DNA, and an altered epigenetic state that contribute to tumorigenesis.

- Deregulation of chromatin remodelers transform normal cell to CSC. Mutated chromatin remodular proteins have now been detected in most human cancers, which are regarded a novel therapeutic target.
- ALL1 gene (also called MLL) encodes chromatin remodular protein under physiologic state. ALL1 gene mutation encodes abnormal chromatin remodular protein, which is linked to acute lymphoblastic leukemia (ALL).
- In addition, recurrent gain-of-function point mutations in histone variants HIST1 H3A (H3.1) and H3F3A (H3.3) result in critical epigenetic alterations leading to tumorigenesis in pediatric glioblastoma multiforme, chondroblastoma, and undifferentiated sarcoma.
- Genetic alterations in components of ATP-dependent chromatin remodeling complexes are quite common in human cancers.

Pathology Pearls: Gene Expression—Mechanisms

Nucleosome is fundamental subunit of chromatin composed of 146 nucleotide base pairs around histone octamer. Nucleosome can slide over along DNA. Chemical modifications to DNA and histone octamer affect nucleosome spacing.

- Gene silencing and repression of transcription methylation of DNA and histone octamer tail causes nucleosomes to pack tightly together due to condensation of heterochromatin, which inhibits binding of transcription factors to DNA sequence; hence DNA becomes inaccessible to transcription factors resulting in gene silencing (turning off) and transcription repression.
- On the contrary, acetylation of DNA and histone octamer results in loose packing of nucleosomes due to relaxation of euchromatin, which enhances binding of transcription factors to DNA sequence, hence DNA becomes accessible to transcription factors leading to gene activation (turning on), induction of transcription and production of protein.

CELL CYCLE REGULATORY PROTEINS

Cell cycle regulatory proteins are products of proto-oncogenes commonly represented by cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors and some of their substrates interacting partners and upstream regulators, that can affect the cell cycle in several different ways.

- Cell cycle is the ordered sequence of events that occur in a somatic cell in preparation for cell division into two identical daughter cells.
- Cell cycle has interphase (G1, S and G2 phases), followed by the mitotic phase (mitosis and cytokinesis), and G0 phase.
- For the cell to move past each of the cell cycle checkpoints, all positive regulatory molecules must be '**turned on**' and all negative regulatory molecules must be '**turned off**'.

Cell Cycle Regulation under Physiologic State

Cell cycle is regulated by positive and negative regulatory proteins. Disruption of normal regulation of the cell cycle can lead to unrestrained CSC proliferation and accumulation of genetic errors resulting in development of malignant tumor.

Positive Regulation of Cell Cycle

Positive cell cycle regulatory proteins include cyclins and cyclin-dependent kinases (CDKs) responsible for the progression of the cell cycle through various checkpoints (i.e. one near the end of G1, a second at the G2/M transition, and the third at metaphase). Other positive cell regulatory proteins are maturation/mitosis promoting factor, anaphase-promoting complex, E2F/DP1 complex, MDM2 protein, and Myc protein.

- Cyclins regulate the cell cycle only when cyclins are tightly bound to CDKs to form cyclin/CDKs complex, that causes the cell to enter S phase by phosphorylation of RB protein. The integrity of the DNA and proper chromosome duplication are assessed at the G1 checkpoint. Attachment of each kinetophore to a specific chromatin fiber is assessed at the M checkpoint.
- Positive cell cycle regulatory proteins allow the cell cycle to progress to the next phase. The concentrations of cyclin proteins fluctuate throughout the cell cycle in a predictive pattern. There is direct correlation between accumulation of cyclin, and three major cell cycle checkpoints.
- Increase in the concentration of cyclin proteins is triggered by both external and internal signals. After cell progresses to the next phase of the cell cycle, there is sharp decline of cyclin levels following each cell cycle checkpoint as a result of degradation by cytoplasmic enzymes.

Negative Regulation of Cell Cycle

Negative cell cycle regulatory proteins include pRB, p53 protein, INK family of proteins (p27, p21 and p57) and CIP/KIP family of proteins (p16, p15, p18 and 19) involved in monitoring the DNA damage and restriction of cell cycle progression from G1/S phase until damage is repaired. If DNA of cell is not repaired, cell undergoes apoptosis. Defect in G1/S checkpoint is linked to tumor cancers.

- TP53 tumor suppressor gene encodes p53 encodes, that arrest the cell cycle in G1 phase by inhibiting CDK4. The p53 also prevents pRB phosphorylation that may provide time for repair of DNA in the cell.

- Two most important tumor suppressor genes (e.g. RB and TP53) encode proteins that inhibit G1/S progression. RB protein halts the cell cycle in the G1 phase. RB binds to E2F transcription factor, which cannot bind the DNA, and transcription is inhibited. Cell growth triggers the phosphorylation of RB protein. Phosphorylated of pRB releases E2F, which binds to DNA, and '**turns on**' gene expression, thus advancing the cell cycle.

Cell Cycle Dysregulation and Cancer

Cell cycle dysregulation is a hallmark of cancer. The proteins involved in cell division events no longer appropriately drive cell cycle progression from one phase to the next.

- The ability of normal cells to undergo cell cycle halt after damage to DNA is crucial for the maintenance of genomic integrity. The biochemical pathways that halt the cell cycle progression in response to cellular stressors are called cell cycle checkpoints.
- There are three major checkpoints in the cell cycle; one near the end of G1/S transition, second at G2/M transition, and the third at metaphase. The integrity of the DNA is assessed at the G1 checkpoint. Proper chromosome duplication is assessed at the G2 checkpoint. Attachment of each kinetophore to a specific chromatin fiber is assessed at the M checkpoint.
- Defective checkpoint function resulting in genetic modifications lead to tumorigenesis. The regulation of cell cycle checkpoint signaling also has clinical implications, because the abrogation of checkpoint function can alter sensitivity of CSCs to chemotherapeutic agents.
- Dysregulation of cyclin D1 gene expression contributes to the loss of normal cell cycle control during tumorigenesis. Amplification of cyclin D1 is linked to breast carcinoma, esophageal squamous cell carcinoma and multiple myeloma. Amplification of cyclin E is implicated in gastric carcinoma.
- The family of cyclin-dependent kinases (CDKs) has critical role in cell cycle regulation and controlling transcriptional elongation.
- Dysregulated CDKs have been linked to tumorigenesis, invasion and metastasis.
  - Amplification of CDK4 gene (12q13) is linked to glioblastoma multiforme, melanoma and sarcoma.
  - Amplification of CDK6 gene (19q21–22) is implicated in breast carcinoma, colon carcinoma, glioblastoma multiforme and esophageal adenocarcinoma.

APOPTOSIS REGULATORY PROTEINS

Proto-oncogenes may also synthesize gene protein products that regulate apoptosis process. Caspases, a family of cysteine proteases, are the '**central regulators of apoptosis**'.

- Apoptosis is a programmed cell death, and characterized by nuclear condensation, cell shrinkage, cell membrane blebbing, intact, and preserved cytoplasmic organelles, swelling of the endoplasmic reticulum, chromatin condensation (pyknosis), and chromosomal DNA fragmentation (karyorrhexis), DNA laddering, and the eventual formation, and phagocytosis of apoptotic bodies by macrophages and surrounding tissue cells within an hour, rendering their appearance very transient. Phagocytic cell synthesizes cytokines (TGF-β, IL-10), which inhibit inflammation in apoptosis.
- Apoptotic signals contribute to safeguarding the genomic integrity, while defective apoptosis can cause genetic alterations, that deregulate cell proliferation, interfere with differentiation, promote angiogenesis, evasion of apoptosis, escape immune destruction by CD8+ cytotoxic T cells and natural killer cells, invasion, metastasis and resistance to chemotherapy and/or radiotherapy.

Regulation of Apoptotic Signaling in Normal Cells

Apoptosis is programmed cell death regulated by pro-apoptotic protein (BAX, BAK, DIVA, BCL-Xs, BIK, BIM, BID, BAD, EGL) and anti-apoptotic protein (BCL-2, BCL-XL, MCL-1, CED-9, A1 summate) molecules, and mediated by intrinsic (mitochondrial) pathway and extrinsic (death receptor pathway), that maintains cell hemostasis.

- Caspases, a family of cysteine proteases, are the central regulators of apoptosis, which cleave cellular components (i.e. structural proteins in the cytoskeleton and nuclear DNA).
- Both extrinsic and intrinsic pathways initiated by initiator caspases (2, 8, 9, 10, 11, 12), which can flow independently until the last step of DNA degradation by executioner caspases 3, 6 and 7.
- The perforin/granzyme apoptosis pathway activates executioner caspase 3.

Aberrant Apoptotic Signaling Associated Cancers

Aberrant apoptotic signaling results in cancers, which occurs by several mechanisms: (a) upregulation of anti-apoptotic proteins (e.g. BCL-2 protein present in integral outer mitochondrial membrane encoded by BCL-2 proto-oncogene), (b) downregulation of pro-apoptotic proteins (e.g. BAX), (c) TP53 tumor suppressor gene inactivation/biallelic loss, (d) loss of apoptotic peptidase activating factor 1 (APAF1), and (e) inactivation of death-induced signaling complex FADD.

- BCL-2 is human proto-oncogene located on chromosome 18, that encodes BCL-2 anti-apoptotic protein in the integral outer mitochondrial membrane, that works to prevent apoptosis by intrinsic apoptotic pathway. Aberrant anti-apoptotic BCL-2 (B cell lymphoma 2) protein signaling induces genetic alterations leading to transformation of normal cell to unrestricted CSC proliferation, evasion of apoptosis, angiogenesis, escape immune destruction by CD8+ cytotoxic T cells and natural killer cells, invasion, metastasis, chemoresistance, and resistance to radiotherapy in certain human cancers.
- B cell lymphomas with concurrent IgH-BCL-2 and Myc rearrangements are aggressive neoplasms, which exhibit chromosomal translocations t(8;14)(q24;q32) involving IgH gene resulting in juxtaposition and overexpression of BCL-2 anti-apoptotic protein. This chromosomal translocation is a hallmark of B cell lymphoma and is also found in 20–30% of *de novo* diffuse large B cell lymphoma (DLBCL), chromosomal translocations involving Myc at 8q24 and immunoglobulin partners, including IgH and kappa (κ) and lambda (λ) chains are characteristic of Burkitt's lymphoma.
- BCL-2 inhibitors are being developed to down-regulate BCL-2. BCL-2 consists of four conserved domains (BH4, BH3, BH1 and BH2), which differentiate it from other BCL-2 family of members (e.g. BIM, BID, PUMA, NOVA, BAD, HRK, BMF and BIK). Among these homologies, motifs, BH3, BH1 and BH2 are the most common targeted therapy in clinical oncology.

DNA REPAIR PROTEINS MAINTAINING GENOME INTEGRITY

DNA repair mechanisms maintain the integrity of genome, and thus prevent carcinogenesis. DNA glycosylases remove the damaged nucleotide base pairs by cutting them out of the DNA strand through cleavage of the covalent bonds between the nucleotide base pairs and the sugar phosphate backbone. The resulting gap is then filled by a specialized repair polymerase enzyme and sealed by ligase.

- XPA is DNA repair protein that recognizes damaged DNA and forms complexes with other proteins such as XPB and XPD, which are involved in DNA repair process. XPB and XPD proteins act as helicases that unwind the damaged DNA.
- Most DNA damage gets repaired by DNA repair proteins. But if the DNA damage persists, cell has less ability to repair itself and hence, errors will build up in other genes overtime and allow development of malignant tumor.

PROTO-ONCOGENES, ONCOGENES AND VIRAL ONCOGENES

Our cells contain many important normal proto-oncogenes that regulate cell growth and division. The mutated forms of proto-oncogenes are called **oncogenes**. When the viral oncogene infects host cell, the reverse transcriptase enzyme generates cDNA, which is then integrated into the genome. Currently, there are seven recognized human oncogenic viruses, which include Epstein-Barr virus (EBV), human papillomavirus (HPV), hepatitis B and C viruses (HBV and HBC), human T cell lymphotrophic virus 1 (HTLV-1), human herpesvirus 8 (HHV-8) and Merkel cell polyomavirus.

- **Proto-oncogenes:** Proto-oncogenes are normal cellular genes, which perform different functions in the cells.
  - Each proto-oncogene codes for growth factor, cell surface receptor and specific intracellular regulatory proteins responsible for providing positive signals, that lead to cell growth, cell division, apoptosis and maintenance of normal cell.
  - Proto-oncogenes can be classified into various groups based on the functional and biochemical properties of protein products, which include: (a) growth factors, (b) cell surface receptors, (c) members of signal transduction pathways such as guanosine triphosphate (GTP)-binding proteins and nonreceptor protein kinases, (d) nuclear regulatory proteins, i.e. transcription factors, (e) cell cycle regulatory proteins, (f) apoptosis regulatory proteins, and (g) DNA repair proteins. Cellular compartments in which proto-oncogene or oncogene products reside involved in cell division are shown in Fig. 6.113.
  - Examples of proto-oncogene encoding growth factors include EGF, PDGF, VEGF, TGF-α, TGF-β, FGF, HGF, IGF, G-CSF, GM-CSF, KGF, NGF, BMP, erythropoietin and thrombopoietin, which bind to their cognate cell surface receptors, which span the plasma membrane and bridge communication between the extracellular environment and inside of the cell.
  - In general, extracellular growth factors bind cell surface receptors, which activate intracellular downstream signal transducers, which initiate DNA transcription of target genes. Many proto-oncogenes play an important role in growth of fetus during embryogenesis.
  - Proto-oncogenes are '**turned off**' once the developmental cellular processes are completed. Examples

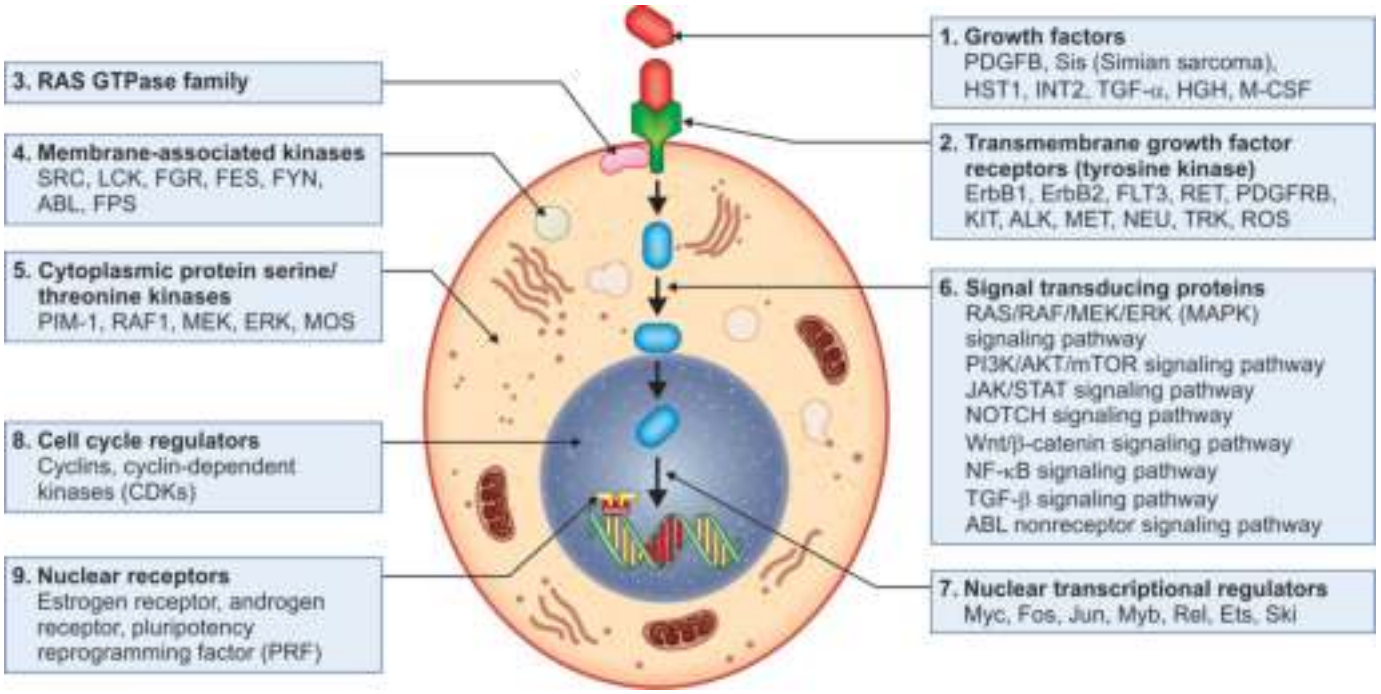


Fig. 6.113: Cellular compartments in which proto-oncogene or oncogene products reside involved in cell division: (1) growth factors, (2) membrane receptor tyrosine kinase, (3) RAS GTPase family, (4) membrane-associated kinases, (5) cytoplasmic protein serine/threonine, (6) cell signaling pathways, (7) nuclear transcriptional regulators, (8) cell cycle regulators and (9) nuclear receptors. Categories of signaling receptors including receptors that utilize a receptor tyrosine kinase (RTK), a nonreceptor tyrosine kinase, a nuclear receptor that binds its cognate ligand.

of proto-oncogenes to convert into oncogenes include HER2/neu (also called ErbB2), RAS, Myc, SRC, hTERT, BCL-2 and EGFR.

- **Oncogenes:** Mutated forms of proto-oncogenes, which occur by various genetic mechanisms: insertional mutagenesis, point mutation, amplification, and chromosomal rearrangements. Oncoproteins are the products of oncogenes leading to transcription, and translation to RNA and abnormal protein.
  - Mutations altering the structure or expression of proteins (e.g. growth factors, cell surface receptors, signal transducing proteins, transcription factors, DNA repair proteins, chromatin remodelers, cell cycle proteins and apoptosis regulators), generally give rise to dominantly active oncogenes resulting in carcinogenesis.
  - HER2 oncogene can result in increased expression of HER2/neu protein on cell surface of CSCs of breast carcinoma. EGFR overexpression is observed in non-small cell lung carcinoma. Oncogenes, mode of activation and associated malignant tumors are given in Table 6.92.

Table 6.92 Oncogenes, mode of activation and associated tumors

Oncogene Category	Proto-oncogene	Mode of Activation	Associated Malignancies
Growth factors			
PDGFB chain	PDGFB (Sis—Simian sarcoma)	Overexpression	▪ Astrocytoma ▪ Osteosarcoma
Fibroblast growth factors (FGFs)	▪ HST1 ▪ FGF3	▪ Overexpression ▪ Amplification	▪ Osteosarcoma ▪ Osteosarcoma ▪ Gastric carcinoma ▪ Urinary bladder carcinoma ▪ Breast carcinoma ▪ Melanoma
TGF-α	TGFA	Overexpression	Astrocytoma

Contd...

Table 6.92 Oncogenes, mode of activation and associated tumors (*Contd...*)

Oncogene Category	Proto-oncogene	Mode of Activation	Associated Malignancies
HGF (hepatocyte growth factor)	HGF	Overexpression	▪ Hepatocellular carcinoma ▪ Thyroid carcinoma
Growth factor receptors			
EGF-receptor family	▪ ErbB1 (EGFR), ErbB2 (avian erythroblastosis) ▪ ErbB2 (HER2/neu)	▪ Amplification ▪ Amplification	▪ Lung adenocarcinoma ▪ Breast carcinoma (marker of aggressiveness)
FMS-like tyrosine 3 kinase	FLT3	Point mutation	Leukemia
Receptors for neurotrophic factors	RET	Point mutation	Multiple endocrine neoplasia: MEN-2A. MEN-2B familial thyroid medullary carcinoma
PDGF receptor	PDGFRB	Overexpression or translocation	▪ Gliomas ▪ Leukemias
Receptor for KIT ligand	KIT	Point mutation	▪ Gastrointestinal stromal tumors (GISTs) ▪ Seminomas ▪ Leukemias
ALK receptor	ALK	▪ Point mutation ▪ Translocation/ amplification ▪ NPM/ALK fusion gene encodes hybrid NPM-ALK protein	▪ Lung adenocarcinoma ▪ Neuroblastoma ▪ Diffuse large B cell lymphoma (DLBCL)
Signal-transducing proteins			
GTP-binding proteins (G proteins)	▪ K-RAS ▪ H-RAS ▪ N-RAS ▪ GNAP ▪ GNAS	▪ Point mutation ▪ Point mutation ▪ Point mutation ▪ Point mutation ▪ Point mutation	▪ Colon carcinoma ▪ Lung carcinoma ▪ Pancreatic carcinoma ▪ Urinary bladder carcinoma ▪ Renal cell carcinoma ▪ Melanomas ▪ Hematologic malignancies ▪ Uveal melanoma ▪ Pituitary adenoma ▪ Other endocrinal tumors
Non-receptor tyrosine kinase	ABL (Abelson mouse leukemia)	▪ Chromosomal translocation (9;22) ▪ Point mutation	▪ CML ▪ ALL
RAS signal transduction	BRAF	▪ Point mutation ▪ Translocation	▪ Melanoma ▪ Leukemia ▪ Colon carcinoma
NOTCH signal transduction	NOTCH1	▪ Point mutation ▪ Translocation	▪ Breast carcinoma ▪ Leukemia/lymphomas
JAK/STAT signal transduction	JAK2	Translocation	▪ ALL ▪ Myeloproliferative disorders

Contd...

Table 6.92 Oncogenes, mode of activation and associated tumors (*Contd...*)

Oncogene Category	Proto-oncogene	Mode of Activation	Associated Malignancies
Transcription factors (nuclear regulatory proteins)			
Myc (myelocytomatosis) transcriptional factor	▪ c-Myc	▪ Translocation (8;14) involving IgH	▪ Burkitt's lymphoma
	▪ N-Myc	▪ Amplification	▪ Neuroblastoma
	▪ L-Myc	▪ Amplification	▪ Small cell lung carcinoma
Cyclins and cyclin-dependent kinases (cell cycle regulators)			
Cyclins	CCND1 (cyclin D1)	▪ Translocation t(11;14) involving IgH	▪ Mantle cell lymphomas
		▪ Amplification	▪ Multiple myeloma ▪ Breast carcinoma ▪ Esophageal carcinoma
Cyclin-dependent kinase	CDK4	Amplification or point mutation	▪ Glioblastoma multiforme ▪ Melanoma ▪ Sarcoma

ALL: Acute lymphoblastic leukemia; CML: Chronic myelogenous leukemia

- **Viral oncogenes:** Viral oncogenes play important role in carcinogenesis, when viral oncogene infects human cell, and the reverse transcriptase enzyme generates cDNA, which is then integrated into the human genome.
  - Viral oncogenesis can be defined as the feature of oncogenic viruses that induces benign and malignant proliferation of infected cells.
  - Oncogenic viruses code different oncoproteins, which can override growth suppressor signals, interfere with cellular receptor-mediated signal transduction pathways, and target cellular genes to immortalize and/or transform virus infected cell to CSC leading to unrestricted cell proliferation and development of malignant tumor growth.

PROTO-ONCOGENES FUNCTION DURING PHYSIOLOGIC AND PATHOLOGIC STATE

Proto-oncogene plays a role in regulating normal cells division. An oncogene is a mutated proto-oncogene that has potential to induce cancer. Proto-oncogene can become activated by several genetic mechanisms inclucing transduction, insertional mutagenesis, amplification, point mutations, and chromosomal translocations. Proto-oncogenes function during physiologic and pathologic state are being discussed here.

HER2/neu (ERBB2) Proto-oncogene

HER2/neu (also called ERBB2) proto-oncogene encodes the transmembrane receptor tyrosine kinase (RTK) called human epidermal growth factor receptor 2 (EGFR-2) on breast tissue cells, which are involved in cell growth, DNA repair and cell division.

Physiologic State

HER2/neu encodes transmembrane growth factor receptors, which include epidermal growth factor receptor 2 (EGFR-2), HER2/neu, HER3 and HER4. HER2/neu consists of an extracellular domain, which interacts with other EGFR family members, and an intracellular tyrosine kinase domain. HER2/neu forms heterodimers with other EGFR family members, induced by binding of EGF to EGFR. Hence, HER2/neu serves as a coreceptor of EGFR.

Pathologic State

There are two copies of HER2/neu gene in the normal breasts. HER2/neu gene amplification, i.e. extra copies (i.e. >2 copies) results in development of breast carcinoma in 30% of women as a result of unrestricted CSC division than normal cell division in normal breasts.

- HER2/neu amplification can alter the response to chemotherapeutic agents, and associated with aggressive clinical course of breast cancer patients.
- HER2/neu positive breast carcinoma cases are treated by humanized 'Trastuzumab' (**Herceptin**) monoclonal antibody, that binds to the HER2/neu protein, and block its activity, preventing excessive cell proliferation of breast carcinoma.

RAS Proto-oncogene Family

RAS proto-oncogene family includes K-RAS, H-RAS, and N-RAS. RAS gene encodes a 21-kDa guanosine

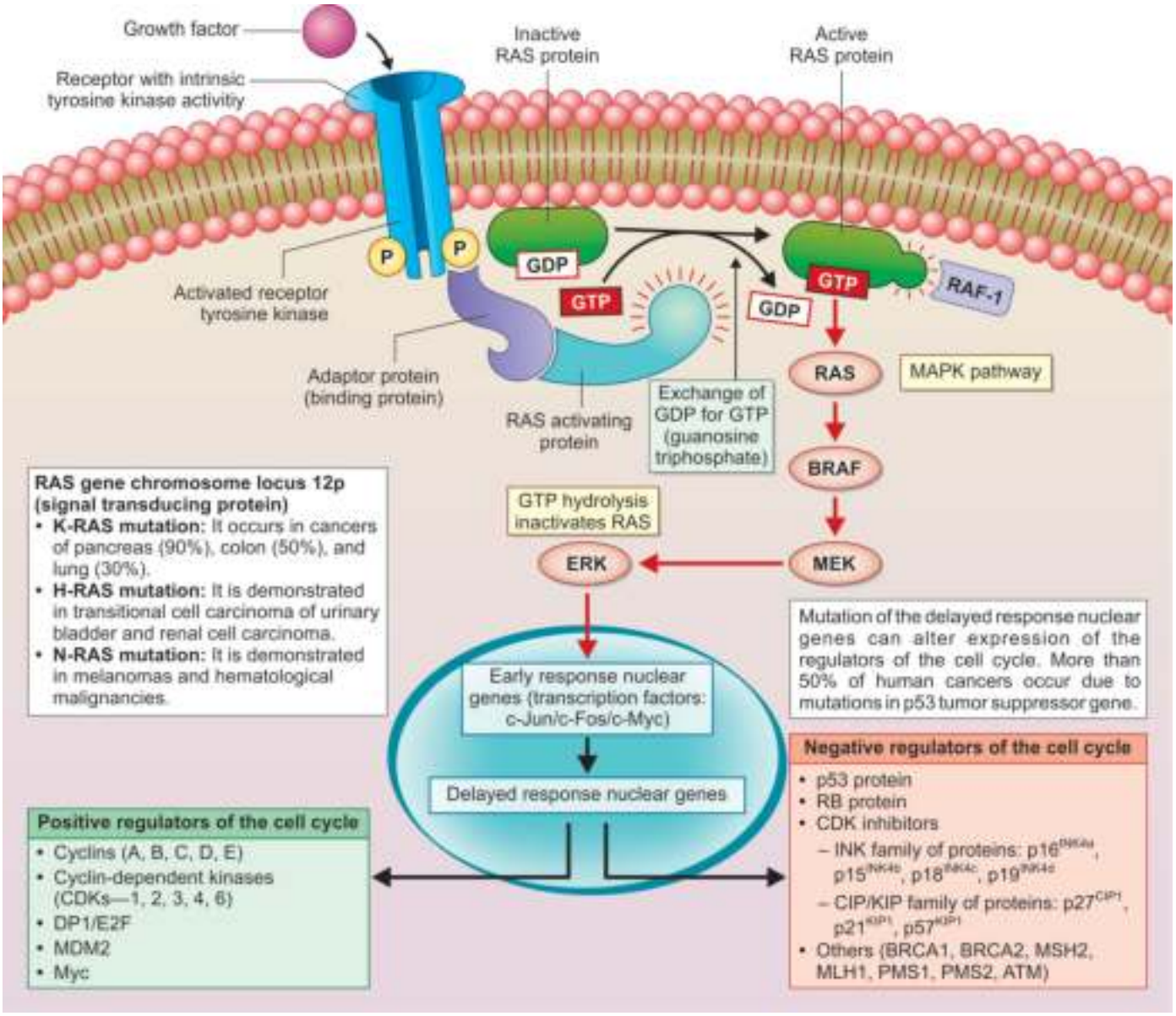


Fig. 6.114: RAS signaling pathway. RAS family proteins (K-RAS, H-RAS, and N-RAS) belong to superfamily of small GTP-binding and hydrolyzing proteins (GTPases), which regulate intracellular signal transduction networks such as mitogen-activated protein kinases (MAPK: RAS → BRAF → MEK → ERK) and phosphoinositol 3-kinase (PI3K) pathways, and induce cell growth, cell differentiation, migration and cell survival. In a normal cell, binding of a ligand to the tyrosine kinase receptor (RTK) stimulates autophosphorylation of specific tyrosine on the tyrosine kinase receptors, inactive (GDP-bound) RAS is activated to a GTP-bound state through GTPase activity of RAS that keeps RAS-mediated signaling in check. Mutations in RAS family of genes are linked to cancers.

triphosphate (GTP)-binding protein that plays key role in many cellular processes including actin cytoskeletal integrity, cell proliferation, differentiation, migration, cell adhesion, and apoptosis. RAS signaling pathway is shown in Fig. 6.114.

Physiologic State

RAS protein is the one of the on/off switches in a series of steps. GTP-binding protein binds to its cognate receptor [EGFR receptor tyrosine kinase (RTK) activates RAS/RAF/MEK/ERK/MAPK signaling pathway and phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway.

- GTP-binding protein activity is regulated by growth factors (e.g. EGF), that control its ability to bind and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP).
- Cell activated by growth factors induces RAS from an active GTP-bound state to inactive GDP-bound state. To turn the 'switch on' of the signaling pathway, RAS protein must bind to guanosine triphosphate (GTP) in the cells. To turn 'switch off' the signaling pathway,

RAS gene product must break up (hydrolyze) the GTP molecule to GDP. GTP proteins belong to the larger group of enzymes called GTPase.

Pathologic State

RAS gene mutation encodes RAS oncoprotein that causes an uncontrolled growth-promoting signal. RAS gene amplification is implicated in many human cancers. Point mutations of RAS gene at codons 12, 13, 59 and 61 are linked to carcinogenesis, and hence are targets for novel approaches for cancer treatment.

- K-RAS mutation is demonstrated in pancreatic carcinoma (90%), lung carcinoma, colon carcinoma (50%), and thyroid carcinoma (50%). Identification of RAS gene mutations in the DNA of pancreatic carcinoma shed into the feces may enable clinicians to differentiate between chronic pancreatitis and pancreatic carcinoma.
- H-RAS mutation is found in renal cell carcinoma and urinary bladder carcinoma. Mutations in N-RAS and BRAF (MAPK signaling pathway) are demonstrated in melanoma. All the cancer therapies are targeted toward anti-RAS modalities.

Myc Proto-oncogene

Myc is a family proto-oncogene (c-Myc, L-Myc and N-Myc genes) encodes transcription factors, which binds and regulates the transcription of specific targeted genes regulating biological processes such as cell proliferation, cell growth, stem cell self-renewal, survival, metabolism, protein synthesis, cell differentiation and apoptosis.

Physiologic State

Myc proto-oncogene plays important roles in stem cells including pluripotent stem cells. In neural stem cells, N-Myc promotes a rapidly proliferative stem cell and precusor-like state in the developing brain while inhibiting differentiation.

Pathologic State

Myc gene mutation induces tumorigenesis by evading multiple tumor suppressor checkpoint mechanisms. Myc gene amplification is linked to human cancers (e.g. Burkitt's lymphoma, diffuse large B cell lymphoma (DLBCL), acute lymphoblastic leukemia (ALL), breast carcinoma, lung carcinoma, colon carcinoma, and other carcinomas).

- Chromosomal translocation t(8;14) (q24;q32) involves the c-Myc gene (8q24), and the immunoglobulin heavy chain (IgH) locus (14q32) results in fusion of BCL-2 gene located near to IgH locus (14q32) leading to overexpression of BCL-2 anti-apoptotic protein. These findings are characteristic of B cell malignancies such as Burkitt's lymphoma (90%), diffuse large B cell lymphoma (2–5%), and acute lymphoblastic leukemia (ALL).
- Long distance polymerase chain reaction (LD-PCR) assays can detect oncogene/Ig gene rearrangement and fusion genes. L-Myc gene amplification is demonstrated in small cell lung carcinoma. N-Myc gene amplification is characteristic of neuroblastoma.

SRC Family of Nonreceptor Tyrosine Kinase Proto-oncogene

SRC proto-oncogene is a nonreceptor tyrosine kinase distributed in different locations within the cell such as plasma membrane, perinuclear membrane and endosomal membrane that plays key roles in cell signaling. SRC proto-oncogene can also be present in the cytoplasm, and between cells at adherens junctions, where it performs different functions.

Physiologic State

SRC proto-oncogene nonreceptor tyrosine kinase coordinates embryonic development, cell adhesion, cell growth, cell migration and cell differentiation.

- SRC proto-oncogene encodes SRC protein, that transfers phosphate groups to the target molecules. Addition/removal of phosphate groups acts like switch, which can turn on/turn off switch and alter several target biomolecules resulting in the transmission of signals to the nucleus, that help in regulation of cells.
- SRC protein present on plasma membrane, which can transduce signals from immune cell receptors, integrin receptors, G protein-coupled receptors and cytokine receptors to intracellular signaling pathways that transmit signals to the nucleus, cytoskeleton and other cellular components; and regulate gene transcription, immune response, cell adhesion, cell cycle progression, migration, and apoptosis.
- SRC proto-oncogene located within the nucleus, regulates cell cycle progression, and cell division by its interaction with other cellular proteins. For example, SRC can interact with Sam68, and regulate gene expression.
- SRC proto-oncogene regulates the respiratory enzyme '**cytochrome c**' oxidase (Cox) in bone osteoclasts, where SRC-induced phosphorylation of Cox is required for maintaining high levels of ATP to meet high energy requirements of cells.

Pathologic State

SRC proto-oncogene belongs to SRC family of kinases, which is similar to v-SRC gene of Rous sarcoma virus. SRC oncogene (mutated SRC proto-oncogene gene) has been suggested to play a key role in tumorigenesis,

where it may facilitate malignant tumor progression through angiogenesis, invasion and metastasis to distant organs.

- Overexpression of c-SRC gene leads to the constitutive activation of STAT3, increased DNA binding of STAT3 at DNA consensus elements, and the cell cycle, thus resulting in unrestricted CSC proliferation.
- SRC proto-oncogene nonreceptor tyrosine kinase protein activity is increased in colon carcinoma, breast carcinoma pancreatic carcinoma and brain tumors. SRC is a promising target for cancer therapy.

Telomerase Reverse Transcriptase Proto-oncogene

Telomerase reverse transcriptase (TERT), also known as human telomerase reverse transcriptase (hTERT), is a proto-oncogene located on short arm of chromosome 5 in the region of 5p15.33, that codes for the catalytic subunit of telomerase enzyme. TERT promoter mutations are demonstrated in 80–90% of human cancers, and can be used as biomarker in diagnosis and outcome prediction.

Physiologic State

Telomerase is ribonucleoprotein enzyme; whose mechanism is to add nucleotide base pairs to telomeres, which maintain chromosomal ends. In normal cells, telomerase enzyme activity is only present during fetal development. In adults, normal somatic cells lack telomerase activity.

Pathologic State

Evasion of replicative senescence and CSC proliferation without restriction, sometimes designated as immortalization, is one of the hallmarks of cancer that may be attained through reactivation of telomerase in somatic cells in adults.

- Telomerase enzyme is abnormally active in most CSCs, which undergo cell growth and unrestricted CSC proliferation. TERT gene mutation can occur by various mechanisms: mutation within promoter region, gene amplification, and rearrangements in 4% of human cancers.
- TERT gene mutations within promoter region enhance TERT transcription activity in urothelial carcinoma (68%), central nervous system tumors (28%), thyroid cancers—follicular thyroid carcinoma, papillary thyroid carcinoma and differentiated/anaplastic thyroid carcinoma (15%), prostatic carcinoma, endometrial carcinoma, rhabdomyosarcoma (2%), neuroblastoma, lung carcinoma, colorectal carcinoma, ovarian carcinoma, breast carcinoma, adrenocortical carcinoma, and acute myelogenous leukemia (AML).
- Rearrangements in the TERT gene within promoter region have been implicated in neuroblastomas. Mutations in TERT gene have been demonstrated in familial idiopathic pulmonary fibrosis, and dyskeratosis congenita, e.g. aplastic anemia or malignancies.

BCL-2 Proto-oncogene

BCL-2 (B cell lymphoma 2) proto-oncogene located on chromosome 18 encodes an integral outer mitochondrial membrane protein (called BCL-2), that works to prevent apoptosis via intrinsic apoptotic (mitochondrial) pathway.

Physiologic State

BCL-2 proto-oncogene prevents BAX/BAK oligomerization, which would otherwise lead to the release of several apoptogenic molecules from the mitochondrion. BCL-2 binds to and inactivates BAX and other pro-apoptotic proteins, thereby inhibiting apoptosis.

Pathologic State

BCL-2 gene overexpression can contribute to metastasis in certain **human hematolymphoid malignancies**. For examples, follicular lymphoma, Burkitt's lymphoma, diffuse large B cell lymphoma, chromosomal translocation t(8;14) (q24;q32) involves the c-Myc gene (8q24) and the immunoglobulin heavy chain (IgH) locus (14q32) results in fusion of BCL-2 gene located near to IgH locus (14q32) resulting in overexpression of BCL-2 anti-apoptotic protein and thus evasion of apoptosis of malignant cancer stem cell (CSC). BCL-2 inhibitors are being developed to downregulate BCL-2.

- BCL-2 consists of four conserved domains (BH1, BH2, BH3 and BH4), which belongs to BCL-2 family members (e.g. BIM, BID, PUMA, NOVA, BAD, HRK, BMF and BIK).
- Among these homologies, motifs, BH3, BH1 and BH2 are the most commonly targeted therapy for cancers in clinical oncology.

Epidermal Growth Factor Receptor Proto-oncogene

Epidermal growth factor receptor (EGFR) is a transmembrane protein encoded by EGFR gene, which spans the cell membrane so that one end of the spanning protein remains inside the cell's cytosol, and the other end of the protein projects outer surface of the cell. EGFR is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinase (RTK): EGFR (ErbB-1), HER2/neu (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4).

Physiologic State

Epidermal growth factor receptor (EGFR) binds at least seven different ligands: epidermal growth factor (EGF), transforming growth factor-α (TGF-α), heparin

binding EGF-like growth factor (HBEGF), β-cellulin (BTC), amphiregulin (AREG), epiregulin (EREG) and epigen (EPGN).

- Binding of EGF to EGFR induces two EGFR proteins to stick to each other. The joined EGFR proteins are called a dimer, that activates cell surface receptor leading to a process of autophosphorylation of two copies of the EGFR protein. EGFR activation increases the activity of multiple signaling pathways.
- In particular RAS/RAF/MEK/ERK/MAPK signaling pathway, PI3K/AKT/mTOR signaling pathway and JAK/STAT signaling pathway downstream signals into nucleus (e.g. SP1, Myc, Jun, Fos, Elk, ERG1, STAT), and ultimately evokes a variety of biological responses such as cell proliferation, differentiation, migration, adhesion and apoptosis.

Pathologic State

Epidermal growth factor receptor (EGFR) gene is mutated or upregulated in many human cancers (e.g. breast carcinoma, lung carcinoma, and head and neck squamous cell carcinoma). Upregulation of EGFR leads to angiogenesis, invasion, unrestricted CSC proliferation survival and metastasis.

- Monoclonal antibodies and tyrosine kinase inhibitors (TKIs) are currently used to target EGFR mutations in human cancers.
- Monoclonal antibodies (e.g. cetuximab and panitumumab) bind to extracellular domain of EGFR to prevent ligands from activating the EGFR. TKIs bind to the cytosolic domain of EGFR and prevents activation process.

CCND1 Proto-oncogene Encodes Cyclin D Protein

CCND1 proto-oncogene located on chromosome 11q13.3 encodes cyclin D1 protein that regulates cell proliferation, metabolism, growth and differentiation.

- During G1 phase of cell cycle, cyclin D1 is synthesized rapidly, that accumulates in the nucleus, and undergoes degradation as the cell enters the S phase of cell cycle.
- Cyclin D1 is a regulatory subunit of cyclin-dependent kinases CDK4 and CDK6. The protein dimerizes with CDK4/CDK6 to regulate the G1/S phase transition, and entry into the S phase.

Physiologic State

Recent studies revealed that cyclin D1 can also regulate cell cycle via CDK-independent pathway. Cyclin D1 regulates the activity of transcription factors, coactivators and corepressors that govern histone octamer acetylation and chromatin remodeling proteins. Mitogens via RAS-mediated signaling pathways and hormones induce cyclin D1 synthesis, inhibit its proteolysis, and export from the nucleus during the G1 phase of cell cycle. Degradation of cyclin D1 occurs via ubiquitin-mediated proteolysis pathway at the end of S phase of cell cycle.

Pathologic State

Genomic rearrangement or gene amplification of CCND1 proto-oncogene codes for excess of aberrant cyclin D1 leading to unrestricted CSC proliferation, cell growth, angiogenesis via VEGF production, evasion of apoptosis, invasion and metastasis and chemoresistance in many cancers.

- Cyclin D1 overexpression is linked to development of non-small cell lung carcinoma (30–45%), head and neck squamous cell carcinoma (30–50%), pancreatic carcinoma (25%), urothelial carcinoma (15%), breast carcinoma (13%), and parathyroid adenomas. Cyclin D1 overexpression is strongly correlated with ER+ breast carcinoma and chemoresistance.
- Mantle cell lymphoma (MCL) is a hematologic neoplasm characterized by the presence of chromosomal translocation t(11;14) (q13;q32), which extraposes CCND1 gene (also called BCL-1, PRAD-1) at 11q13 with an enhancer of the immunoglobulin heavy chain (IgH) at 14.32q. The resultant aberrant cyclin D1 expression plays a key role in the pathogenesis of mantle cell lymphoma. Chromosomal translocation of cyclin D1 gene locus is also demonstrated in 15–20% of multiple myeloma patients.

TUMOR SUPPRESSOR GENES

Tumor suppressor genes encode proteins that normally function to inhibit cell proliferation and tumorigenesis by regulation of cell cycle, differentiation, signal transduction, cell adhesion, maintenance of genomic integrity, DNA (deoxyribonucleic acid) damage repair, and inducing apoptosis. Loss-of-function mutations in tumor suppressor genes possess oncogenic potential. Since, one copy of a tumor suppressor gene is sufficient to regulate cell proliferation, both alleles of a tumor suppressor gene must be lost or inactivated in order to induce malignant tumor. Thus, oncogenic loss-of-function mutations in tumor suppressor genes act recessively. Deletions or point mutations in tumor suppressor genes prevent production of any protein or enhance production of a nonfunctional protein. Oncogenes resulting from activation of proto-oncogene (i.e. turning on) and inactivation/biallelic loss of tumor suppressor genes (i.e. turning off) may result in unrestricted CSC proliferation, and development of malignant tumors. Tumor suppressor gene in physiologic state and pathologic state is shown in Fig. 6.115.

- Five broad classes of tumor suppressor proteins are generally recognized as being encoded by tumor suppressor genes: (a) intracellular proteins such as

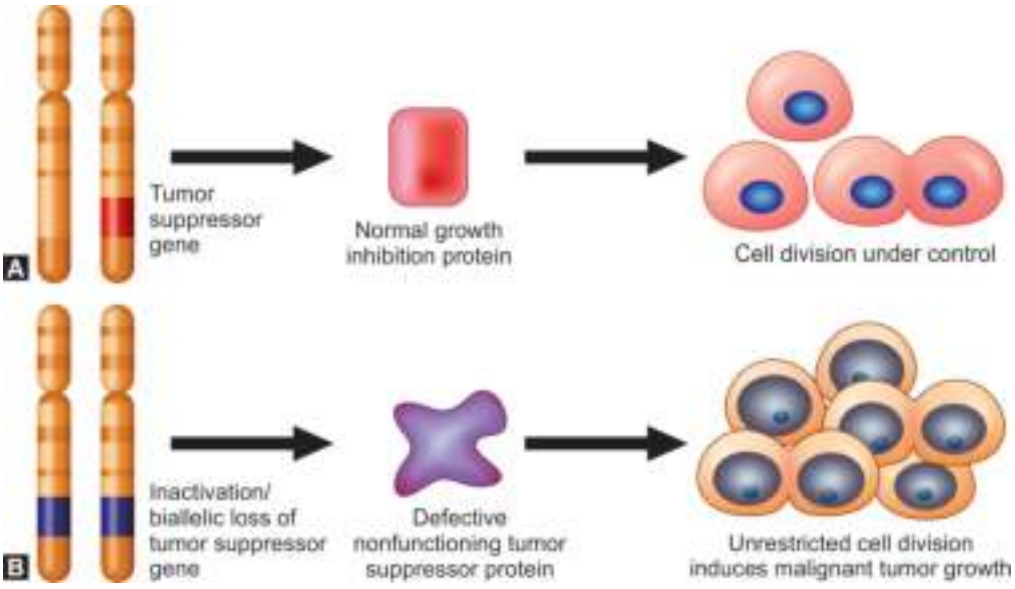


Fig. 6.115: Tumor suppressor gene in physiologic state and pathologic state. (A) Normally, tumor suppressor gene regulates cell division under control, (B) inactivation/biallelic loss of tumor suppressor gene causes unrestricted cell division and induces malignant tumor.

p16 cyclin-dependent kinase inhibitor, that regulate or inhibit progression through a specific stage of the cell cycle, (b) receptors for hormones that function to inhibit cell proliferation, (c) cell cycle checkpoint regulatory proteins, that arrest cell cycle, if DNA gets damaged or abnormalities in chromosomes, (d) proteins that promote programmed cell death (apoptosis), and (e) enzymes that participate in DNA repair. Inherited germline mutations in tumor suppressor genes are responsible for familial cancer syndromes running in families. However, somatic mutations in tumor suppressor genes are most common and acquired, and not inherited. Tumor suppressor genes can be divided into two major categories, known as gatekeepers and caretakers. Inactivation of gatekeeper and caretaker genes contribute to development of malignant tumor, invasion and metastasis.

- **Tumor suppressor gatekeeper genes** encode proteins, that regulate cell proliferation. They act as guards that inhibit cells from passing through cell cycle checkpoints by countering the progression of cellular growth and encouraging apoptosis. Examples of tumor suppressor gatekeeper genes include **TP53, APC,** and **RB1**.
- **Tumor suppressor caretaker genes** encode proteins, that regulate the maintenance of the genetic information integrity in each cell. Examples of tumor suppressor caretaker genes include **DNA repair genes** (e.g. BRCA1, BRCA2), **mismatch repair genes** (e.g. MLH1, MSH2, MSH6, PMS2), CDKN2A and XP. Functional categories of mutations in tumor suppressor genes and associated cancers are given in Table 6.93.

- Tumor suppressor gene has two alleles. If only one allele for the tumor suppressor gene is mutated, but

Table 6.93 Functional categories of mutations in tumor suppressor genes and associated cancers

Gene and Locus	Protein	Function	Germline/Somatic Mutations and Familial/Sporadic Tumors
Tumor suppressor genes function to inhibit Wnt/β-catenin mitogenic signaling activator pathway			
APC gene (5p22.2)	APC protein	APC inhibits Wnt/β-catenin signaling activator pathway, thus prevents nuclear transcription, and also degrades β-catenin	▪ Familial tumors—familial adenomatous polyposis coli and colon carcinoma ▪ Sporadic tumors—colon carcinoma, gastric carcinoma, pancreatic carcinoma, thyroid carcinoma and melanoma
NF1 gene (13p11)	Neurofibromin protein 1	NF1 inhibits RAF/MEK/ERK/MAPK signaling activator pathway	▪ Familial tumors—neurofibromatosis type 1 and malignant peripheral nerve tumors ▪ Sporadic tumors—juvenile chronic myelogenous leukemia, neuroblastoma and schwannoma

Contd...

Table 6.93 Functional categories of mutations in tumor suppressor genes and associated cancers (*Contd...*)

Gene and Locus	Protein	Function	Germline/Somatic Mutations and Familial/Sporadic Tumors
NF2 gene (22q)	Merlin protein	NF2 stabilization of cytoskeleton membrane linkage	▪ Familial tumors—neurofibromatosis type 2 (acoustic schwannoma and meningioma) ▪ Sporadic tumors—schwannoma and meningioma
PTCH-1 gene (9q22.3)	Patched protein	Patched protein inhibits Hedgehog signaling pathway	▪ Familial tumors—Gorlin's syndrome (basal cell carcinoma, medulloblastoma, other benign tumors) ▪ Sporadic tumors—basal cell carcinoma and medulloblastoma
SMAD2 gene (18q21) and SMAD4 gene (18q22)	SMAD2/SMAD4 protein	Both SMA2/SMAD4 inhibit TGF-β signaling activator pathway, repress CDK4 and Myc and induce expression of CDK inhibitor	▪ Familial tumors—juvenile polyposis ▪ Sporadic tumors—colon carcinoma and pancreatic carcinoma
PTEN gene (10q23.3)	Phosphatase and tensin homologue	PTEN protein inhibits AKT/PKB signaling pathway	▪ Familial tumors—Cowden syndrome (cancers of breast, endometrium and thyroid gland) ▪ Sporadic tumors—lymphoid neoplasms, glioblastoma multiforme, endometrial carcinoma, prostatic carcinoma, breast carcinoma and lung carcinoma
Tumor suppressor genes function to inhibit cell cycle progression			
CDKN2A gene (9p2)	▪ $P16^{INK4a}$ protein ▪ $P14^{ARF}$ protein	$P16^{INK4a}$ protein inhibits CDK. $P14^{ARF}$ protein indirectly activates p53 tumor suppressor gene	▪ Familial tumors—familial melanoma ▪ Sporadic tumors—breast carcinoma, pancreatic carcinoma, urothelial carcinoma, lung carcinoma, head and neck squamous cell carcinoma and esophageal carcinoma
RB gene (13p14.2)	RB protein	RB gene codes for pRB protein, master brake on cell cycle, and inhibits G1 to S transition during cell cycle, and also inhibits nuclear transcription factor	▪ Familial tumors—retinoblastoma and osteosarcoma ▪ Sporadic tumors—retinoblastoma, osteosarcoma, breast carcinoma, colon carcinoma, prostatic carcinoma, urinary bladder carcinoma and lung carcinoma
Tumor suppressor genes function to inhibit angiogenesis			
von Hippel-Lindau (vHL) gene (3p25)	von Hippel-Lindau (vHL) protein	vHL inhibits hypoxia-inducible factor 1α (HIF1α) and regulates nuclear transcription	▪ Familial tumors—von Hippel-Lindau syndrome (cerebellar hemangioblastoma, retinal angiomas, bilateral renal cell carcinoma and pheochromocytoma) ▪ Sporadic tumors—renal cell carcinoma
SDH (SDHA, SDHB, SDHC, SDHD) genes (11q23)	Succinate dehydrogenase subunits of B and D	These gene proteins participate in TCA cycle and oxidative phosphorylation	▪ Familial tumors—paraganglioma and pheochromocytoma ▪ Sporadic tumor—paraganglioma
STK11 gene (19q13.3)	Liver kinase B1 (LKB1) or STK11 protein	Gene protein activates AMPK family of kinases and suppresses cell growth when cell nutrients and energy levels are low	▪ Familial tumors—Peutz-Jeghers syndrome (cancers of GIT, pancreas, cervix, ovary, and breast) ▪ Sporadic tumors—non-small cell lung carcinoma, cervical carcinoma, colorectal carcinoma, pancreatic carcinoma and melanoma
CDH1 gene (16q22.1)	E-cadherin	Gene protein participates in cell adhesion. It inhibits cell motility	▪ Familial tumors—gastric carcinoma ▪ Sporadic tumors—gastric carcinoma and breast lobular carcinoma

Contd...

Table 6.93 Functional categories of mutations in tumor suppressor genes and associated cancers (*Contd...*)

Gene and Locus	Protein	Function	Germline/Somatic Mutations and Familial/Sporadic Tumors
Tumor suppressor gene function as genomic stability enabler (most important)			
TP53 gene (17p13.1)	p53 protein	p53 inhibits cell cycle G1 to S phase, repairs DNA and induces cell apoptosis by activating BAX gene	▪ Familial tumors—Li-Fraumeni syndrome (breast carcinoma, adrenocortical carcinoma, osteosarcoma, rhabdomyosarcoma, leukemia, brain tumors) ▪ Sporadic tumors—many human cancers (>50% of human cancers)
Tumor suppressor genes function to repair damaged DNA			
BRCA1 caretaker gene (17q21)	BRCA1 protein	BRCA1 binds RAD51 molecule that mediates repair of double-stranded breaks in DNA	▪ Familial tumors—ovarian carcinoma, breast carcinoma, prostatic carcinoma and fallopian tubal carcinoma ▪ Sporadic tumors—breast medullary carcinoma and metaplastic carcinoma
BRCA2 gene (13q12)	BRCA2 protein	BRCA2 binds RAD51 molecule that mediates repair of double-stranded breaks in DNA	▪ Familial tumors—breast carcinoma, ovarian carcinoma, gastric carcinoma, fallopian tubal carcinoma ▪ Sporadic tumor—Fanconi anemia
MLH1 gene (3p21), MSH2 gene (2p15), MSH3 gene (5q11.12), MSH6 gene (2p16), PMS1 gene (2p32), PMS2 gene (7p22)	MLH1, MSH2, MSH3, MSH6, PMS1, PMS2 proteins	DNA nucleotide excision repair pathway, mutation is associated with accumulation of numerous mismatched errors in DNA replication	▪ Familial tumors—hereditary non-polyposis colorectal cancer—HNPCC (Lynch syndrome) increased risk of development of carcinoma of endometrium (first most common cancer), ovary and right colon carcinoma ▪ Sporadic tumors—colorectal carcinoma, endometrial carcinoma, Muir-Torre syndrome, hepatobiliary carcinoma, genitourinary carcinoma and glioblastoma multiforme
Tumor suppressor genes functions by various mechanisms			
WT1 (11p13.)	WT1 gene protein	Transcription factor	▪ Familial tumors—Wilm's tumor ▪ Sporadic tumors—Wilm's tumor and certain leukemias
MEN1 (11q13)	Menin protein	Transcription factor	▪ Familial tumors—multiple neoplasia syndrome 1 (MEN1) ▪ Sporadic tumors—parathyroid adenoma, pancreatic islet cell tumor, and pituitary tumor
DPC4 (18q21.1)	DPC4 protein	DPC4 encodes relay molecule in cell division inhibitory pathway	▪ Familial tumors—rarely occur ▪ Sporadic tumors—pancreatic carcinoma (most common), breast carcinoma and ovarian carcinoma
ATM (l11p22)	ATM protein	Receptor tyrosine kinase (RTK)	▪ Familial tumors—ataxia-telangiectasia, breast carcinoma, gastric carcinoma and leukemia/lymphoma ▪ Sporadic tumors—unknown
p16 (9p21)	p16 protein	p16 inhibits nuclear cyclin-dependent kinase activity	▪ Familial tumors—familial melanoma ▪ Sporadic tumors—mesothelioma, pancreatic carcinoma, melanoma and astrocytoma

Contd...

Table 6.93 Functional categories of mutations in tumor suppressor genes and associated cancers (*Contd...*)

Gene and Locus	Protein	Function	Germline/Somatic Mutations and Familial/Sporadic Tumors
TGF-β (19q)	TGF-β protein	TGF-β inhibits G1 to S phase	▪ Familial tumors—not occur ▪ Sporadic tumors—pancreatic carcinoma and colorectal carcinoma
CHEK2 (22q12)	CHEK2 protein	CHEK2 induces cell cycle arrest, repairs damaged DNA; and activates BRCA1 and p53 tumor suppressor genes by phosphorylation	▪ Familial tumors—breast carcinoma (5%) ▪ Sporadic tumors—post-radiation induced breast carcinoma, prostatic carcinoma, thyroid carcinoma and renal cell carcinoma

1. Tumor suppressor gatekeeper genes inhibit the cell with damaged DNA proliferation and also promote apoptosis of damaged cells. Tumor suppressor gatekeeper genes include TP53, RB and APC.
2. Tumor suppressor caretaker genes maintain the integrity of the genome by repairing DNA damage.
3. Tumor suppressor caretaker genes include BRCA1, BRCA2, MLH1, MSH2, MSH6, PMS2, FAMC, and Xp.

the other allele can still produce enough of normal protein to retain the appropriate function. This means that mutations in tumor suppressor gene tend to be recessive, and thus both alleles must be mutated in order to allow abnormal cell growth to proceed to induce malignant tumor, invasion and metastasis. On the contrary, mutant oncogene alleles are typically dominant.

- Loss of heterozygosity (LOH) of tumor suppressor genes occurs by mitotic recombination, or chromosome misaggregation in the genomic DNA regions during mitosis process. LOH is defined as the loss of one parent's contribution to the somatic cell. LOH is a common form of allelic imbalance by which heterozygous somatic cells become homozygous somatic cells, because one of the two alleles gets lost.
- Hypermethylation of gene refers to epigenetic alterations due to increase in DNA methylation by addition of cytosine and adenosine residues in the promoter region of gene(s), which can silence gene expression or loss of gene function associated with transcriptional loss in the absence of structural alterations. Aberrant DNA methylation is a common phenomenon in carcinogenesis, and the DNA methylation profiles are altered in various human cancers.

TUMOR SUPPRESSOR GATEKEEPER GENES

Tumor suppressor gatekeeper genes encode proteins, that inhibit cell cycle progression on detection of DNA damage, and also induce apoptosis. When both copies of a tumor suppressor gene are inactivated/lost in a cell, the inhibitory gene product can no longer be produced, and the cells have less control of their growth leading to the unrestricted CSC proliferation, and development of malignant tumor. Examples of tumor suppressor gatekeeper genes are TP53, APC, and RB1.

TP53 Tumor Suppressor Gatekeeper Gene

TP53 tumor suppressor gatekeeper gene is located on chromosome 17p13.1, that encodes a tumor suppressor p53 protein, which acts as a transcription factor and 'guardian of the genome'.

Physiologic State

The p53 protein is activated as a result of DNA damage. The p53 protein activates p21, which in turn inhibits the cyclin/CDKs complexes required for cell cycle progression past G1 phase. Cell cycle arrest allows time for DNA repair to occur. If DNA damage is beyond repair, p53 protein can induce apoptosis via activating proapoptotic BAX gene in the mutated cell, an important function, that is crucial for preventing carcinogenesis. TP53 gene guardian of human genome is shown in Fig. 6.116.

Pathologic State

Somatic mutations in the TP53 tumor suppressor genes are one of the most frequent alterations in human cancers of ovaries, esophagus, colorectal region, head and neck, larynx, lung, cervix, skin (melanoma) and leukemia in >50% of cases.

- Germline heterogenous mutations are the underlying cause of **Li-Fraumeni syndrome**, which predisposes to a wide spectrum of early-onset cancers of breast and adrenal cortex, sarcoma, leukemia, brain tumors. TP53 tumor suppressor gene mutations are also potential prognostic and predictive markers, as well as targets for pharmacological intervention.
- TP53 tumor suppressor gene has two alleles (i.e. wild and mutant types). TP53 gene mutant allele inhibits TP53 wild type allele in CSCs.

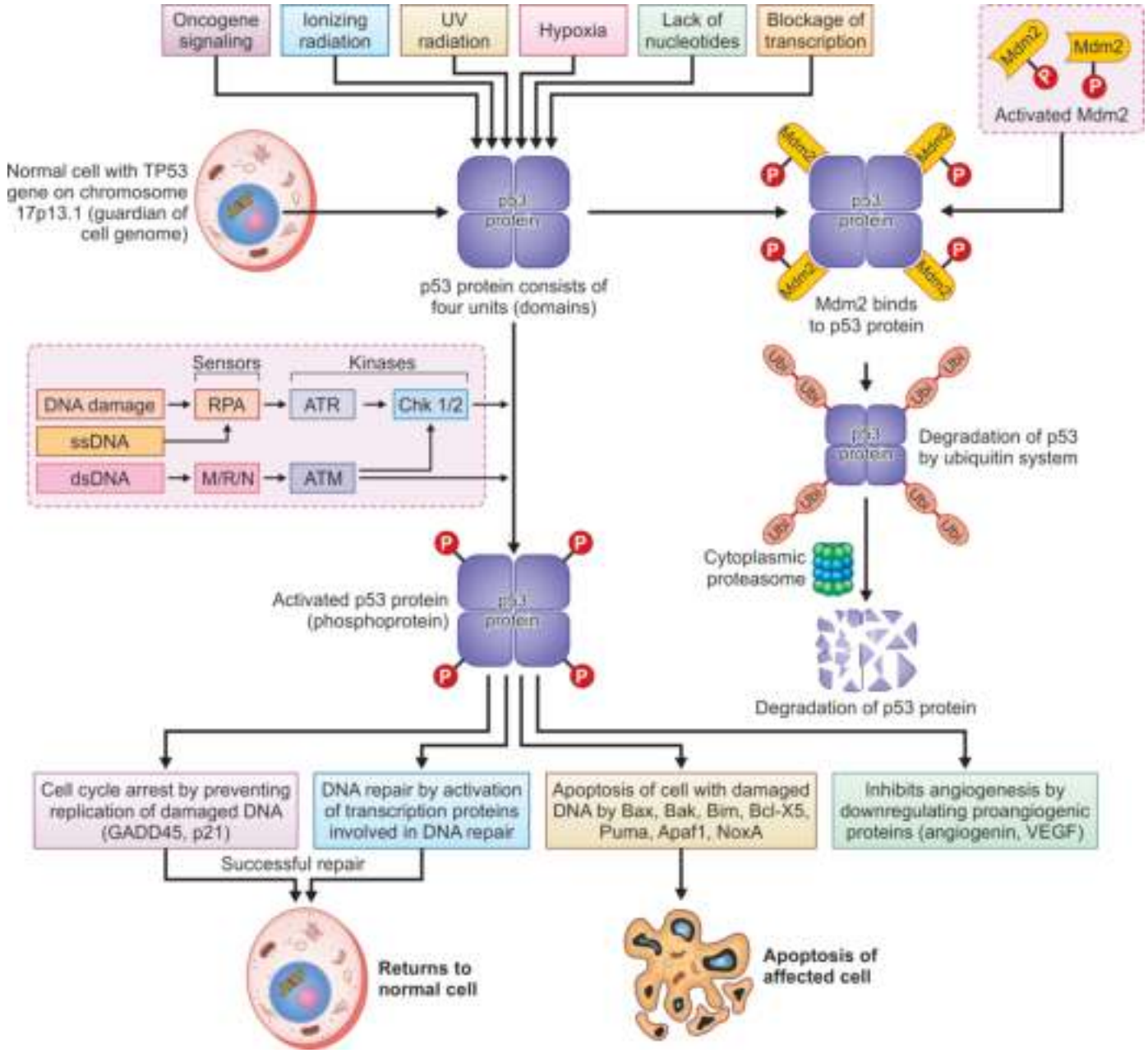


Fig. 6.116: TP53 gene guardian of human genome. TP53 tumor suppressor gene product p53 protein maintains integrity of the human genome. Inactivation/biallelic loss of TP53 tumor suppressor gene causes cell cycle arrest in G1 phase due to upregulation of cyclin-dependent kinase inhibitor CDKN1A and GADD45 genes until either the DNA mutation is repaired in mild damage or undergoing apoptosis in severe damage. If p53 is defective, carcinogen-induced damaged cell proceeds to S phase. Such mutations are propagated to daughter cells, possibly leading to malignant tumor formation.

- Wild-type of TP53 allele occurs in individuals with normal phenotype possessed by majority of the natural population and its p53 protein binds DNA in a sequence-specific manner to mediate its function.
- Mutant type of TP53 allele product p53 is unable to bind wild-type of p53 protein on DNA sequence and may exert 'gain-of-function' activities, that favor development of malignant tumor, invasion and metastasis. TP53 tumor suppressor gene mutation linked to carcinogenesis is shown in Fig. 6.117.

Retinoblastoma 1 Tumor Suppressor Gatekeeper Gene

Retinoblastoma 1 (RB1) is a gatekeeper tumor suppressor gene mapped on chromosome 13q14.2, that encodes a tumor suppressor pRB protein (pRB, i.e. master brake on cell cycle).

- RB protein (pRB) inhibits G1/S phase by inhibiting nuclear transcription factor. Mutation of RB gene (deletion and nonsense) induces loss of regulation of cell cycle activation through sequestration of transcription factors. Dephosphorylated RB gene inhibits cell cycle.

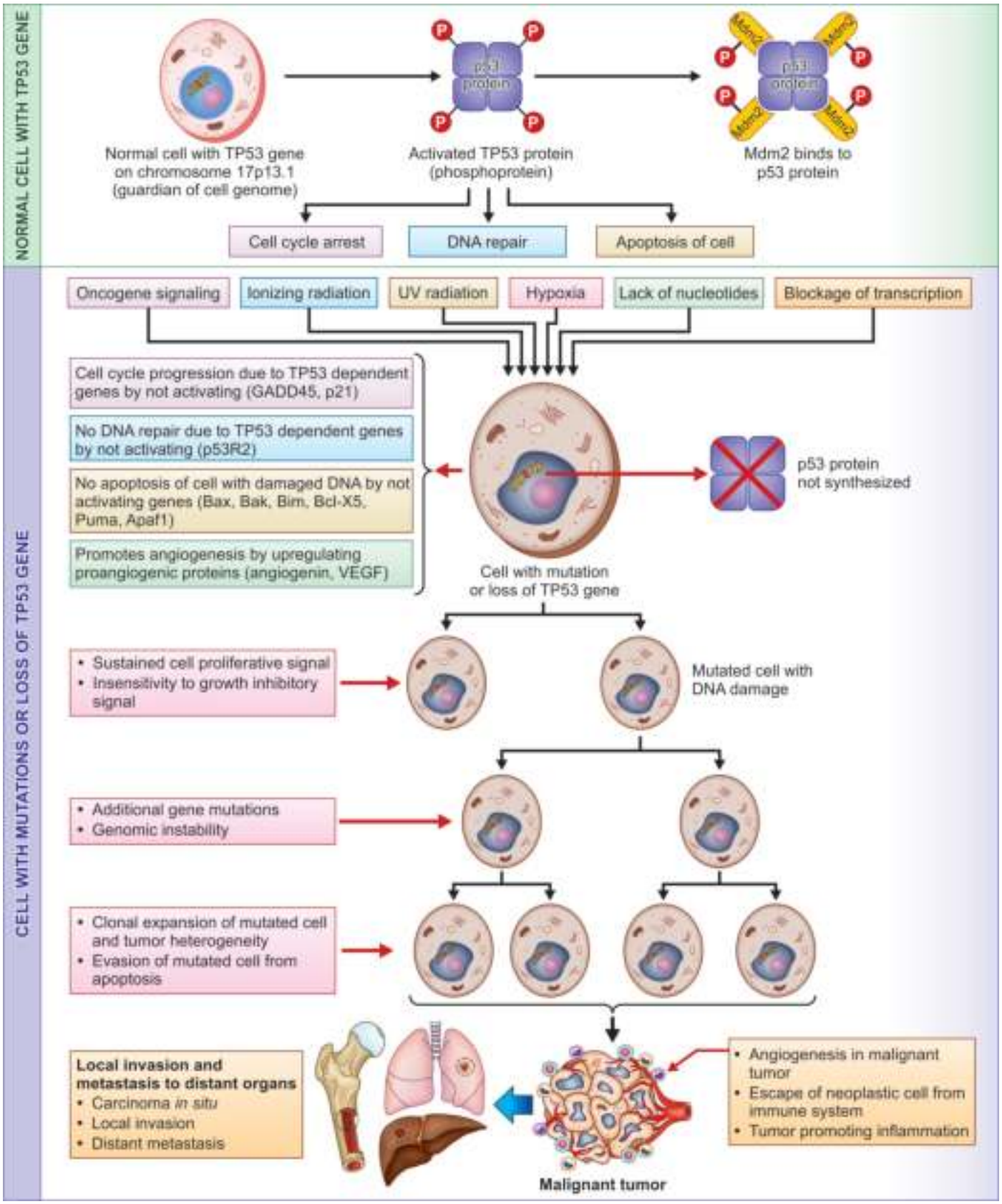


Fig. 6.117: TP53 tumor suppressor gene mutation linked to carcinogenesis. Mutations in TP53 tumor suppressor gene change single amino acid in p53, which impair p53 protein's function. Without normal functioning of p53 protein, DNA damage can accumulate mutations in the cells, which may continue to divide in unrestricted manner and induce malignant tumor.

Table 6.94 pRB tumor suppressor phosphorylation and dephosphorylation in cell cycle

Characteristics	pRB Phosphorylation	pRB Dephosphorylation
Definition	pRB phosphorylation is the addition of a phosphate group to a molecule by protein kinase	pRB dephosphorylation is the removal of a phosphate group to a molecule by phosphatase
Phosphate group	Addition of phosphate group to a molecule	Removal of phosphate group from a molecule
Enzyme involved	Protein kinase	Phosphatase
Consequence	Phosphorylation induces conformational change and provides platform for phosphate-binding proteins	Dephosphorylation induces changes in protein, i.e. stability, activity, interaction and subcellular localization

- When RB gene is phosphorylated, cell division takes place. pRB tumor suppressor phosphorylation and dephosphorylation in cell cycle are given in Table 6.94.

Physiologic State

Retinoblastoma 1 (RB1) tumor suppressor gatekeeper gene provides instructions for making a protein called pRB, that acts as a tumor suppressor, and regulates cell growth by keeping cells from dividing, in an unrestricted manner.

Pathologic State

RB tumor suppressor gatekeeper gene mutations have been demonstrated in familial cancers (retinoblastoma, osteosarcoma) in 40% of cases, and sporadic cancers (retinoblastoma, osteosarcoma, breast carcinoma, prostatic carcinoma, urinary bladder carcinoma, glioblastoma and small cell lung carcinoma) in 60% of cases. Healthy persons have two alleles of the RB gene.

- **Familial retinoblastoma** develops in genetically predisposed in 40% of persons, who have only one active allele of the RB gene. The second allele of RB gene has either lost due to deletion of a portion of the long arm of chromosome 13 or inactivated by a mutation.
- **Sporadic retinoblastoma** occurs only if both alleles of RB gene are lost or inactivated in 60% of persons.

Adenomatous Polyposis Coli Tumor Suppressor Gatekeeper Gene

Adenomatous polyposis coli (APC) is a tumor suppressor gatekeeper gene located on chromosome 5p22.2, that encodes a tumor suppressor APC protein. The APC gene alterations play an important role in the development of malignant tumors in multistep process of carcinogenesis.

Physiologic State

Adenomatous polyposis coli (APC) tumor suppressor gene encodes APC protein that suppresses canonical Wnt/β-catenin signaling, thus prevents accumulation of β-catenin. The β-catenin helps control the expression of particular genes, and promotes the cell growth, cell proliferation and differentiation.

Pathologic State

Adenomatous polyposis coli (APC) tumor suppressor gene mutation is implicated in heritable (e.g. familial adenomatous polyposis (FAP) and colon carcinoma) and nonheritable tumors (e.g. colon carcinoma, gastric carcinoma, pancreatic carcinoma, thyroid carcinoma and melanoma). FAP is autosomal dominant syndrome characterized by germline mutations in APC gene resulting in intestinal adenomatous polyposis and a very high incidence of colorectal adenocarcinoma.

- **Current familial adenomatous polyposis diagnostic criteria:** Current familial adenomatous polyposis (FAP) diagnostic criteria of any one of the mentioned: germline mutation in APC gene or 100–1000 colorectal adenomatous polyps (classic definition) first appear at 10–20 years of age, or colorectal adenomas identical to tubular adenomas under the 30 years of age in patient with a family history of FAP, or intra-abdominal desmoid fibromatosis, osteoma of mandible, or multiple epidermoid cysts in patient with a family history of FAP.
- **Gardner syndrome:** Gardner syndrome is characterized by familial adenomatous polyposis, osteomas especially in mandible, mesenteric and rarely abdominal desmoid fibromatosis, and cutaneous epidermoid cysts.
- **Turcot syndrome:** Turcot syndrome is associated with hereditary colorectal carcinoma. Turcot syndrome includes two distinct syndromes: familial adenomatous polyposis with medulloblastoma or hereditary nonpolyposis colon carcinoma (HNPCC) with glioblastoma multiforme. Some cases of Turcot syndrome can develop hepatoblastoma (≤2%) and rarely pancreatic, biliary and endocrine neoplasms.

TUMOR SUPPRESSOR CARETAKER GENES

Tumor suppressor caretaker genes repair damaged DNA during cell cycle arrest, and maintain genomic stability. Tumor suppressor caretaker genes include DNA repair genes (e.g. BRCA1, BRCA2), mismatch DNA repair genes (e.g. MLH1, MSH2, MSH3, MSH6, PMS1, PMS2), CDKN2A and XP.

BRCA1 and BRCA2 Tumor Suppressor Caretaker Genes

BRCA1 and BRCA2 tumor suppressor caretaker genes encode proteins involved in DNA repair by resolution of DNA crosslinks.

Physiologic State

BRCA1 and BRCA2 tumor suppressor caretaker genes encoding proteins are unrelated, both are normally expressed in the cells and breast and ovarian tissues, where they function to repair damaged DNA, or destroy cells by apoptosis mechanism if DNA cannot be repaired.

Pathologic State

Loss of BRCA1 tumor suppressor caretaker genes result in DNA strands breaks and aneuploidy after cell division. High-penetrance BRCA1 and BRCA2 tumor suppressor caretaker genes are responsible for **hereditary breast carcinoma** and **ovarian carcinoma**.

Mismatch Repair System Tumor Suppressor Caretaker Genes

Mismatch repair (MMR) system genes, i.e. MLH1 gene (3p21), MSH2 gene (2p15), MSH3 gene (5q11.12), MSH6 gene (2p16), PMS1 gene (2p32) and PMS2 gene (7p22) belong to tumor suppressor caretaker genes. Their gene products ensure the genome integrity by proofreading and fixing mismatched nucleotide base pairs during DNA replication.

Physiologic State

The principal function of DNA mismatch repair (MMR) system is to correct DNA polymerase misincorporation errors (i.e. nucleotide base-base mismatches and insertion/deletion mispairs) that arising during DNA replication and recombination leading to maintenance of genomic stability.

Pathologic State

Mutations in one or more mismatch DNA repair system genes induce genomic instability regions of repeating nucleotides called '**microsatellite regions**'.

- While microsatellite instability does not directly affect carcinogenesis, rather it indicates pathogenicity of tumorigenesis likely reflective of underlying defect in DNA repair system.
- Mutations in one or more mismatch repair (MMR) system genes are linked to **hereditary nonpolyposis colon carcinoma (HNPCC)** also known as **Lynch syndrome**. Colorectal carcinoma with high microsatellite instability confers very poor prognosis.

Cyclin-dependent Kinase Inhibitor 2A (CDKN2A) Tumor Suppressor Caretaker Gene

Cyclin-dependent kinase inhibitor 2A (CDKN2A) is a tumor suppressor caretaker gene located on chromosome 9p21.3, that encodes several proteins including well studied $p16^{INK4A}$ and $p14^{ARK}$ proteins. Both proteins inhibit cell growth and cell division in older cells (senescence).

Physiologic State

CDKN2A tumor suppressor caretaker gene provides instructions for making several proteins such as $p16^{INK4A}$ and $p14^{ARK}$ proteins, which function to induce cell cycle arrest in G1 and G2 phases. The $p16^{INK4A}$ inhibits cyclin-dependent kinases (e.g. CDK4 and CDK6) and restricts cell cycle progression. The $p14^{ARF}$ protein protects p53 tumor suppressor protein from being degradation, thus indirectly activates p53 protein.

Pathologic State

Germline mutation in cell cycle-associated CDKN2A tumor suppressor caretaker gene is linked to familial melanoma and pancreatic carcinoma. Somatic mutations in the cell cycle-associated CDKN2A tumor suppressor caretaker gene are found in 25% of head and neck cancers and also in colorectal cancer by promoting invasion and metastasis by inducing epithelial–mesenchymal transition (EMT).

Xeroderma Pigmentosum Tumor Suppressor Caretaker Genes

Xeroderma pigmentosum (XP) gene provides instructions for making a protein that is involved in repairing of damaged DNA. Ultraviolet B (UVB) solar rays, toxic chemical radiation, and unstable oxygen derived free radical molecules can also cause DNA damage.

Physiologic State

Eight of the XP genes make up the nucleotide excision repair pathway (NER) that detects and repairs ultraviolet induced DNA damage. The ninth XP gene acts to bypass unrepaired DNA damage.

Pathologic State

The ultraviolet B (UVB) component of the solar spectrum induces DNA damage, in the absence of error-free DNA repair, may give rise to mutations in caretaker and gatekeeper genes. DNA repair genes are best candidates for caretaker genes as exemplified by autosomal recessive XP-linked to skin carcinogenesis.

TUMOR SUPPRESSOR GENES FUNCTION AS INHIBITORS OF Wnt/β-CATENIN MITOGENIC SIGNALING ACTIVATOR PATHWAY

Gene products of various tumor suppressor genes such as APC, NF1, NF2, PTCH, SMAD2, SMAD4 and PTEN function to inhibit Wnt/β-catenin mitogenic signaling pathway, and thus inhibits transcription of DNA sequence into an RNA molecule with the help of enzyme RNA polymerase.

Adenomatous Polyposis Coli Tumor Suppressor Gene

Adenomatous polyposis coli (APC) tumor suppressor gene is located on chromosome 5q22.2, that encodes a protein. Germline APC gene mutation is associated with familial adenomatous polyposis progressing to colon carcinoma. Somatic APC gene mutation is linked to colon carcinoma, gastric carcinoma, pancreatic carcinoma, thyroid carcinoma, and melanoma.

Physiologic State

Adenomatous polyposis coli (APC) gene product plays a critical role in several cellular processes such as cell adhesion, cell migration, organization of the actin and microtubule networks, spindle formation, and chromosome segregation. APC gene product suppresses canonical Wnt/β-catenin signaling pathway.

Pathologic State

Due to APC gene mutation, aberrant activation of canonical Wnt/β-catenin signaling pathway gives rise to accumulation of β-catenin in the nucleus, and promotes transcription of targeted oncogenes such as c-Myc and cyclin D1. As a result, it contributes to carcinogenesis, and progression of several human epithelial and lymphoid cancers.

- **Germline mutation in APC tumor suppressor gene** is associated with familial malignant tumors (e.g. familial adenomatous polyposis progressing to colon carcinoma). **Turcot syndrome** related to familial adenomatous polyposis associated with medulloblastoma in Ashkenazi Eastern and Central European Jewish heritage.
- **Somatic mutation in APC tumor suppressor gene** is linked to sporadic malignant tumors (e.g. colon carcinoma, gastric carcinoma, pancreatic carcinoma, thyroid carcinoma and melanoma).
- Summary of adenomatous polyposis syndrome, genetics, inheritance and clinical manifestations is given in Table 6.95.

Neurofibromatosis Type 1 Tumor Suppressor Gene

Neurofibromatosis type 1 (NF1) tumor suppressor gene is located on chromosome 17p11, that encodes neurofibromin protein. **Neurofibromin** produced by oligodendrocytes and Schwann cells plays key role in the formation of protective myelin sheaths around nerves.

Table 6.95 Summary of adenomatous polyposis syndrome, genetics, inheritance and clinical manifestations

Adenomatous Polyposis Syndrome	Genetics	Clinical Manifestations
Autosomal dominant inheritance		
Classic familial adenomatous polyposis (FAP)	Germline adenomatous polyposis coli (APC) gene mutations	▪ Adenomatous polyposis in colon (100–1000 polyps), gastric region, duodenum and periampullary region ▪ Colorectal carcinoma ▪ Garner's syndrome (desmoid tumor fibromas, lipomas, sebaceous cyst, epidermoid cyst) ▪ Turcot's syndrome (multiple adenomatous polyps and medullo-blastoma) ▪ Congenital hypertrophy of the retinal pigmented epithelium (CHRPE)
Attenuated familial adenomatous polyposis (AFAP)	Germline adenomatous polyposis coli (APC) gene mutations	▪ Multiple adenomatous polyps (<100) in colon, upper gastrointestinal tract (fundus, duodenum) ▪ Colorectal carcinoma (70% risk) ▪ Hepatoblastoma ▪ Gastric carcinoma ▪ Breast carcinoma

Contd...

Table 6.95 Summary of adenomatous polyposis syndrome, genetics, inheritance and clinical manifestations (*Contd...*)

Adenomatous Polyposis Syndrome	Genetics	Clinical Manifestations
Polymerase proofreading-associated polyposis (PRAP)	Germline POLE or POLD1 gene mutations	▪ Colonic (Number of polyps unknown) ▪ Colorectal carcinoma ▪ Ovarian carcinoma ▪ Endometrial carcinoma ▪ Pancreatic carcinoma ▪ Brain malignancies
Autosomal recessive inheritance		
MUTYH-associated polyposis (MAP)	Biallelic MUTYH gene mutations	▪ Multiple adenomatous polyps (<100) ▪ Colorectal carcinoma ▪ Duodenal carcinoma ▪ Ovarian carcinoma ▪ Urinary bladder carcinoma ▪ Skin malignancies
NTHL1 associated polyposis (NAP)	Germline homozygous or compound heterozygous NTHL1 gene mutations	▪ Colonic (Number of polyps unknown) ▪ Colorectal carcinoma ▪ Endometrial carcinoma ▪ Duodenal carcinoma ▪ Breast carcinoma ▪ Pancreatic carcinoma

Physiologic State

Neurofibromin encoded by NF1 tumor suppressor gene acts as a tumor suppressor protein by '**turning off**' another p21-RAS protein, that inhibits a stimulatory GTPase (RAS) protein of cell growth, and division via RAS/RAF/MEK/ERK (MAPK) signaling pathway.

Pathologic State

NF1 tumor suppressor gene mutation is linked to development of neurofibromatosis type 1, juvenile myelomonocytic leukemia, myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). Patient with neurofibromatosis type 1 (NF1) presents with two or more neurofibromas or one plexiform neurofibroma, 6 café-au-lait macules >5 mm (pubertal) or >15 mm (post-pubertal), ocular iris hamartomas, sphenoid bone dysplasia and first degree relative with neurofibromatosis type 1.

Neurofibromatosis Type 2 Tumor Suppressor Gene

Neurofibromatosis type 2 (NF2) tumor suppressor gene is located on chromosome 22q.12.2 encodes merlin protein, also known as schwannomin, that stabilizes cytoskeleton membrane linkage. Merlin protein is produced by Schwann cells, that wrap round and insulate nerves.

Physiologic State

Merlin encoded by NF2 tumor suppressor gene plays key role in regulation of signaling pathways, which is important for controlling cell shape, cell growth and cell adhesion. Merlin protein functions as a tumor suppressor, that restricts cell growth and cell division.

Pathologic State

Neurofibromatosis type 2 (NF2) tumor suppressor gene mutation (deletion or nonsense) is associated NF2, schwannomatosis and tumors.

- Neurofibromatosis type 2 occurs due to germline mutations present in all the cells of the body. These patients can also develop meningiomas and ependymomas.
  - Diagnostic criteria of neurofibromatosis type 2 include bilateral vestibular schwannomas or family history of NF2 in first degree relative and unilateral vestibular schwannoma in below 30 years of age and any two of the lesions, i.e. meningioma, schwannoma, glioma, posterior subcapsular lenticular opacities/juvenile cortical cataract.
  - The majority of patients present with hearing loss which is usually unilateral at onset and may be accompanied by tinnitis.
- Somatic mutation in the NF2 gene is implicated in schwannomatosis characterized by the development of multiple schwannomas involving nerves through the body, and additional isolated nervous system benign and malignant tumors including meningiomas, ependymomas and schwannomas, inactivation or loss of NF2 gene is associated with mesothelioma arising from pleural cavity and abdominal cavity.

PTCH 1 Tumor Suppressor Gene

PTCH 1 is a tumor suppressor gene located on chromosome 9q22.32, that encodes patched 1 protein, which functions as a receptor protein, that possesses specific sites to which ligand fits like keys into locks.

Physiologic State

A patched 1 protein encoded by PTCH 1 gene acts as a ligand for patched 1 receptor, which triggers Sonic Hedgehog signaling pathway essential for cell growth, cell differentiation and determining the shape of cells of different body parts during embryonic development. When Sonic Hedgehog signaling is absent, patched 1 protein inhibits cell growth and division.

Pathologic State

Mutations in PTCH 1 gene are implicated in development of Gorlin syndrome, nonsyndromic holoprosencephaly (failure of embryonic forebrain), 9q22.32 microdeletion, coloboma, cancers and other disorders.

- Gorlin syndrome (also known as nevoid basal cell carcinoma syndrome) increases the risk for development of various benign and malignant tumors, microcephaly, skeletal abnormalities and small pits in the skin of palms and soles.
- The chromosomal 9q22.32 microdeletions are characterized by delayed development, intellectual disability, overgrowth of the body due to loss of additional genes in the deleted region of chromosome 9.
- Somatic mutations in PTCH 1 gene are implicated in development of coloboma involving retina, basal cell carcinoma, medulloblastoma, breast carcinoma, colon carcinoma and keratocystic odontogenic tumor.

SMADs for Tumor Suppressor Gene

SMAD4 is a tumor suppressor gene located on chromosome 18q21.2, that encodes a DNA binding protein, which transmits chemical signal from the cell surface to the nucleus. SMAD4 mediates the downstream effect of TGF-β signaling pathway resulting in cell cycle arrest, apoptosis, and inhibition of epithelial–mesenchymal transition (EMT).

Physiologic State

Transforming growth factor-β (TGF-β) binds to transmembrane serine/threonine kinase receptors (TGFR1 and TGFR2) and initiates TGF-β/SMAD4 signaling pathway, which phosphorylates SMAD2/SMAD3. The phosphorylated SMAD2/SMAD3 form a heterodimeric complex with SMAD4 and undergo nuclear accumulation, and then bind to SMAD binding element directly, and regulate transcription of target genes with the help of transcriptional factors. These target genes are involved in cell growth arrest and apoptosis.

Pathologic State

Mutation in SMAD tumor suppressor gene is implicated in hereditary hemorrhagic telangiectasia characterized by arteriovenous malformations, and risk for development of intestinal polyps and malignant transformation, juvenile polyposis syndrome, Myhre syndrome, pancreatic adenocarcinoma, breast carcinoma, prostatic carcinoma, squamous cell carcinoma of head and neck, non-small cell lung carcinoma, and cholangiocarcinoma.

PTEN (Phosphatase and Tensin Homolog) Tumor Suppressor Gene

PTEN (phosphatase and tensin homolog) is a tumor suppressor gene located on chromosome 10q23, that encodes lipid phosphatase, that serves to antagonize cell proliferation and survival generated by the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway.

Physiologic State

Normally, phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway plays an important role in regulation of signal transduction, that plays an important role in cell cycle regulation and apoptosis in response to extracellular signals.

- Normal binding of growth factor to its cell surface receptor leads to phosphorylation of phosphatidylinositol-3,4,5-triphosphate (PIP3). The level of PIP3 is regulated by PTEN dephosphorylation.
- PTEN is a multifunctional protein, that maintains the stability of the genome and chromosomes by regulating proper spindle formation, and accurate chromosome segregation to prevent gross genomic instability via regulation of mitotic arrest deficient 2 (MAD2). PTEN gene expression knockdown induces G2/S checkpoint cell cycle arrest, and abnormal chromosome segregation.

Pathologic State

Frequent inactivation of PTEN tumor suppressor gene has been implicated in development of PTEN hamartoma tumor syndromes, glioblastoma multiforme, endometrial carcinoma, prostatic carcinoma, lung carcinoma, breast carcinoma and lymphomas. Polymerase chain reaction and next generation sequencing have been used to detect PTEN gene mutations.

TUMOR SUPPRESSOR GENES FUNCTION TO INHIBIT CELL CYCLE PROGRESSION

Gene products of CDKN2A and RB tumor suppressor genes inhibit progression of cell cycle. Tumor suppressor gene products restrict cell cycle progression. Their control over cell division is lost due to genetic alterations resulting in their inactivation.

- CDKN2A tumor suppressor gene encodes $p16^{INK4A}$ and $p14^{ARK}$ proteins, which inhibit cell growth and cell division in older cells (senescence). The $p16^{INK4A}$ inhibits CDK4 and CDK6 and restricts cell cycle. The $p14^{ARK}$ inhibits degradation of p53 protein, thus indirectly activates p53 protein.
- Tumor suppressor protein p53 regulates progression of cell cycle through the G1/S checkpoint. In particular, p53 is activated in response to DNA damage and serves to restrict cell cycle progression in G1/S checkpoint and hence allow time for DNA repair. The RB gene product is a tumor suppressor, which plays a pivotal role in the negative control of cell cycle and prevents tumorigenesis. The RB protein is responsible for G1 checkpoint, blockage of S phase entry and cell growth.

CDKN2A Tumor Suppressor Gene

Cyclin-dependent kinase inhibitor 2A (CDKN2A) is a tumor suppressor gene located on chromosome 9p21.3, that encodes several proteins including well studied $p16^{INK4A}$ and $p14^{ARK}$ proteins. Both proteins inhibit cell growth and cell division in older cells (senescence).

Physiologic State

The $p16^{INK4A}$ encoded by CDKN2A tumor suppressor gene inhibits cyclin-dependent kinases (e.g. CDK4 and CDK6) and restricts cell cycle progression. CDK4 and CDK6 normally stimulate cell division through the cell cycle progression.

- The $p14^{ARF}$ protein encoded by CDKN2A tumor suppressor gene protects a different tumor suppressor protein is called p53 protein from its degradation, thus indirectly activates p53 protein. The p53 protein regulates cell division, senescence and apoptosis. By protecting p53 protein, $p14^{ARF}$ also inhibits tumorigenesis. Both $p14^{ARF}$ and p53 proteins are produced in the cells, that are unable to undergo cell division.
- In human papillomavirus (HPV) infection, HPV produces oncoproteins E6 and E7. E6 oncoprotein induces the degradation of the tumor suppressor p53 protein. E7 oncoprotein inactivates RB protein (pRB) and thus resulting in cytoplasmic overexpression of $p16^{INK4A}$. The $p16^{INK4A}$ interaction with other proteins Y2 chain of laminin, β-catenin or vascular endothelial growth factor (VEGF) is thought to be related to new functions of $p16^{INK4A}$ by inhibition of angiogenesis and CSC invasion.

Pathologic State

CDKN2A tumor suppressor gene mutation is associated with familial (germline gene mutation associated melanoma), and sporadic tumors (e.g. somatic mutation associated breast carcinoma, pancreatic carcinoma, urothelial carcinoma, lung carcinoma, esophageal carcinoma, and head and neck squamous cell carcinoma). Promoter hypermethylation of CDKN2A gene turns off the partial or complete production of $p16^{INK4A}$ and $p14^{ARF}$. Without one of these tumor suppressor proteins, resulting in unrestricted CSC proliferation, development of malignant tumor, invasion and metastasis.

RB1 Tumor Suppressor Gene

Retinoblastoma 1 (RB1) is a tumor suppressor caretaker gene mapped on chromosome 13q14.2. Normal people have two alleles of the RB gene, also known as governor of cell cycle encodes RB protein, which acts as master brake on cell cycle.

- Retinoblastoma is the most common primary intraocular malignant tumor caused by inactivation/biallelic loss of tumor suppressor. Genetic testing can determine whether a child has heritable or nonheritable form of retinoblastoma.
- Knudson two-hit hypothesis explains that most tumor suppressor genes require inactivation/biallelic loss, either through mutations or epigenetic silencing to cause a phenotypic change and development of malignant tumor.
  - If a person is born with mutation in one allele of RB tumor suppressor gene, that person already has 'first hit' (germline inherited mutation in one allele), somatic mutation 'second hit' involving remaining functional allele of RB tumor suppressor gene, essential for development of retinoblastoma.
  - The acquisition of the 'second hit' is called loss of heterozygosity (**LOH**). If retinoblastoma is left untreated, tumor can eventually metastasize to distant organs.

Physiologic State

RB protein encoded by RB tumor suppressor gene plays a pivotal role in the negative control of cell cycle and tumor progression. Dephosphorylated RB protein inhibits cell division, whereas phosphorylated RB protein promotes cell division. RB protein represses gene transcription required for transition from G1/S phase of cell cycle, and DNA replication. Additionally,

RB protein interacts with other proteins to influence cell survival, apoptosis and cell differentiation.

Pathologic State

RB1 tumor suppressor caretaker gene germline mutation is implicated in heritable tumors (e.g. retinoblastoma and osteosarcoma) and nonheritable tumors (e.g. retinoblastoma, osteosarcoma, breast carcinoma, colon carcinoma, prostate carcinoma, urothelial carcinoma and lung carcinoma).

- Heritable retinoblastoma develops due to a RB1 germline mutation and subsequent somatic inactivation of the other allele of RB gene. This form of retinoblastoma may be multifocal and/or bilateral or unilateral. Mean age of diagnosis is within first year of life.
- Nonheritable retinoblastoma develops in children with no family history, and accounts for 60% of all cases. Tumor is unilateral with onset after the first year of life in majority of cases. Both alleles of RB gene must mutate to produce the disease. Adult relatives of patients with retinoblastoma may show retinal scars, which are indicative of benign nonprogressive tumors.
- Diagnostic testing to diagnose retinoblastoma includes fundus examination, fundus photography, ultrasonography, MRI scan and genetic testing. Histologic examination of enucleated tumor reveals features of retinoblastoma.
- Significant advancements have been made in retinoblastoma treatment, which include systemic venous or intra-arterial or local intravitreal administration of chemotherapy, laser treatment and enucleation.

TUMOR SUPPRESSOR GENES FUNCTION TO INHIBIT TUMOR ANGIOGENESIS

Angiogenesis is a biological process in which novel capillary blood vessels grow from pre-existing vasculature providing tissues with oxygen and nutrients.

- In normal conditions, angiogenesis only occurs during embryonic development, female reproductive cycle and wound healing.
- Vasculogenesis is the embryonic establishment of the blood supply from mesodermal precursors (i.e. angioblasts and hemangioblasts).
- However, aberrant angiogenesis is an essential step in the growth of malignant tumors. Numerous investigators have established the association between tumor angiogenesis and tumor metastasis.
- Numerous gene products of vHL, SDHB, SDHD and STK11 tumor suppressor genes inhibit tumor angiogenesis.

vHL (von Hippel-Lindau) Tumor Suppressor Gene

The vHL (von Hippel-Lindau) tumor suppressor gene is located on chromosome 3p25.3, that encodes vHL protein involved in regulation of other genes, cell division, and synthesis of extracellular matrix. vHL protein is found in both nuclear and cytoplasmic compartments. Trafficking between these compartments is essential for vHL protein function.

Physiologic State

The vHL protein functions as a subunit of a multiprotein ubiquitin ligase, that inhibit expression of hypoxia-inducible genes controlled by hypoxia inducible factors (HIFs).

- The vHL ubiquitin ligase prevents inappropriate expression of these hypoxia-inducible genes, when cells are grown in abundant supply of oxygen by targeting **HIFs** for rapid ubiquitylation and degradation by the proteosome, when they are no longer needed. Protein degradation is a normal process that removes damaged or unnecessary proteins and helps in the maintenance of normal cellular functions.
- Hypoxia-inducible factor 2α (HIF-2α) is a multiprotein complex called HIF, which plays a pivotal role in the body's ability to adapt to change in oxygen levels. HIF controls several genes involved in cell division, angiogenesis (VEGF synthesis), and erythropoiesis. HIF functions when oxygen levels are lower than normal in the body. However, when adequate oxygen is available, VCL-CUL2 multiprotein complex keeps HIF from building up in approximately in the cells.

Pathologic State

Mutation in vHL tumor suppressor gene encoding defective vHL protein can give rise to heritable von Hippel-Lindau syndrome, familial erythropoiesis, nonsyndromic paraganglioma of nervous system, pheochromocytoma and hemangioblastoma. Somatic nonheritable vHL mutation is implicated in development of clear cell variant of renal cell carcinoma.

- The vHL gene mutation encodes abnormally short vHL protein as a result of change in single amino acids in the vHL protein is found in nonsyndromic paraganglioma and pheochromocytoma. These changes disrupt the function of the protein.
- HIF-2α protein is not broken down and instead accumulates in the cells. Excess of HIF-2α stimulates cell division, and triggers angiogenesis, which can lead to the development of paraganglioma or pheochromocytoma.

SDH Tumor Suppressor Genes

SDH tumor suppressor genes (SDHA, SDHB, SDHC and SDHD) encode the four subunits of succinate dehydrogenase (SDH; mitochondrial complex II), mitochondrial enzyme involved in two essential energy producing metabolic processes: oxidative phosphorylation of the cell, the Krebs cycle (also called TCA—tricarboxylic acid), and the electron transport system.

Physiologic State

SDH tumor suppressor gene encodes succinate dehydrogenase that inhibit genetic and epigenetic alterations and thus prevent carcinogenesis.

Pathologic State

Germline mutations in any of the SDH tumor suppressor genes or assembly factor (SDHAF2) cause heritable paraganglioma/pheochromocytoma syndrome through an unknown mechanism. Tumorigenesis generally follows the Knudson 'two-hit' hypothesis. The first copy of the gene is mutated in all cells, however the second copy functions normally. Mutation in SDH tumor suppressor gene encodes defective succinate dehydrogenases, that can result in genetic and epigenetic changes like hypermethylation, which can lead to succinate-mediated inhibition of α-ketoglutarate-dependent dioxygenases. So, hypoxic conditions can generate subsequent transformation of normal cell to cancer stem cell (CSC).

STK11 Tumor Suppressor Gene

STK11 is a tumor suppressor gene, also called liver kinase B1 (LKB1), located on chromosome 19p13.3. STK11 gene, that encodes an enzyme serine-threonine kinase 11, involved in activation of RAS/RAF/MEK/ERK/MAPK signaling pathway.

Physiologic State

STK11 tumor suppressor gene product plays a pivotal role in correct orientation of cells within the tissues (polarization), determination of amount of energy used by the cells, apoptosis, and suppression of cell growth, when nutrients and energy levels are low.

Pathologic State

Germline mutation of STK11 tumor suppressor gene is linked to **Peutz-Jeghers syndrome**. Patient with Peutz-Jeghers syndrome have a high risk for developing carcinomas at different locations (e.g. stomach, colorectal region, pancreas, lung, cervix, ovary and breast) during their lifetime. Somatic mutation of STK11 gene is demonstrated in non-small cell lung carcinoma, cervical carcinoma, colorectal carcinoma, pancreatic carcinoma, skin melanoma and testicular tumor.

TUMOR SUPPRESSOR GENES: GENOMIC STABILITY ENABLER (MOST IMPORTANT)

Genomic stability is a feature of every organism to preserve and faithfully transmit the error-free genetic material (DNA or RNA) from generation to next generation or from one somatic cell to another. Individual survival depends on genomic stability.

- DNA polymerase and DNA ligase are essential for DNA replication. In addition to maintain the integrity of DNA sequences by DNA repair, an accurate duplication of DNA is a prerequisite for cell division.
- In contrast, genomic instability refers to high frequency of mutations within genome of a cellular lineage caused by external and internal factors. Fortunately, cells have evolved several response systems to tackle DNA damage by accurate DNA repair in order to maintain their genomic integrity. Genomic instability is a defined hallmark of cancer. Example of DNA stabilizer enabler is TP53 tumor suppressor gene.

TP53 Tumor Suppressor Gene

TP53 tumor suppressor gene located on chromosome 17p13.1 encodes p53 protein, that binds directly to DNA and control cell division under check.

Physiologic State

The p53 protein encoded by TP53 tumor suppressor gene is activated as a result of DNA damage caused by toxic chemical agents, ionizing radiation or ultraviolet rays. The p53 protein plays a pivotal role in determining whether damaged has been repaired or not. Failure to repair DNA damage, cell undergoes apoptosis. If the damaged DNA undergoes repair, the p53 protein activates p21, which in turn inhibits the cyclin/CDKs complexes required for cell cycle progression past G1 phase. Cell cycle arrest allows time for DNA repair to occur.

- If DNA damage is beyond repair, p53 protein can induce apoptosis in the mutated cell, an important function, that is crucial for preventing development of malignant tumor growth. Because p53 is essential for regulating DNA repair and cell division, it has been called 'guardian of genome'.
- Without p53 protein, the cell cycle progresses despite DNA damage. The cell eventually accumulates enough mutations through the activation of oncogenes or inactivation/biallelic loss of tumor suppressor genes to transformation of normal cell to CSC to invasion and metastasis. The lack of apoptosis allows multiple cycles of DNA damage and cell proliferation to occur.

Pathologic State

Germline or somatic mutations of TP53 tumor suppressor gene are linked to many human cancers.

- **Germline TP53 tumor suppressor gene mutation and associated cancers:** Germline TP53 tumor suppressor gene mutation is implicated in many human cancers in Li-Fraumeni syndrome (e.g. breast carcinoma, osteosarcoma, rhabdomyosarcoma, brain tumors, leukemias and adrenocortical carcinoma). Li-Fraumeni syndrome is diagnosed based on clinical criteria and/or genetic testing for the mutation in the TP53 gene.
- **Somatic TP53 tumor suppressor gene mutation and associated cancers:** Somatic mutation in TP53 tumor suppressor gene is implicated in breast carcinoma, urothelial carcinoma (50%), cholangiocarcinoma, head and neck squamous cell carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, melanoma, ovarian carcinoma, Wilm's tumor, osteosarcoma, rhabdomyosarcoma, and adrenocortical carcinoma. Most of the mutations involved in these cancers occur due to change single amino acid in TP53 tumor suppressor gene.

TUMOR SUPPRESSOR GENES FUNCTION DNA REPAIR SYSTEM

DNA damage is defined as any modification of DNA in the chemical structure of DNA (i.e. DNA strands and nucleotide base pairs), that changes its coding properties such as DNA replication and transcription.

- DNA damage can be caused by chemical agents, oxidizing agents, ultraviolet (UV) rays, ionizing radiation and chemotherapeutic agents leading to DNA breaks in single/double-strands and missing of nucleotide base pairs from the backbone of DNA.
- DNA replication errors and DNA damage are actually happening in the cells of our bodies all the time. Many DNA replications are minimized or corrected by proofreading. Sometimes mismatched nucleotide base pairs escape proofreading.
- DNA repair systems are essential for the maintenance of genome integrity. Human DNA repair genes function in a diverse set of pathways that involve the recognition and excision of DNA lesions, tolerance to DNA damage, and protection from errors of incorporation made during DNA replication or DNA repair. Additional genes indirectly affect DNA repair by regulating the cell cycle to provide an opportunity for DNA repair or to direct the cell to undergo apoptosis.
- Major DNA repair pathways are direct reversal by cellular enzymes, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), double-stranded break repair (e.g. non-homologous end joining and homologous recombination).
  - DNA repair comes from the double helix structure of DNA, that carries two separate copies of all the genetic information in each of its two strands. When single strand of DNA is damaged, another DNA strand is used as a template to restore the DNA nucleotide base pair sequence to the damaged strand. **PARP-1** encodes a protein, that assists with the repair of single-stranded breaks in the DNA. When both strands of the double helix DNA molecule are broken, however, leaving no template strand for repair, the cells may use one of two distinct alternative mechanisms to address repair.
  - Double-stranded breaks are dangerous because large segments of chromosomes along with their numerous genes may be lost, if DNA break is not repaired. Two pathways are involved in the repair of double-stranded DNA breaks: non-homologous end joining (NHEJ), and homologous recombination (HR) pathways. In NHEJ, chromosome is 'glued back' together, usually a small mutation at the break site. In homologous recombination, the broken chromosome pairs up with its homologue, and the damaged region is replaced via recombination, using sequences copied from the homologue. Homologous recombination is 'cleaner' than NHEJ and it does not usually cause mutations.

Tumor Suppressor Genes Function Mismatch DNA Repair

MLH1 gene (3p21), MSH2 gene (2p15), MSH3 gene (5q11.12), MSH6 gene (2p16), PMS1 gene (2p32) and PMS2 gene (7p22) belong to tumor suppressor caretaker genes. Their gene products ensure the genome integrity by proofreading and fixing mismatched nucleotides during DNA replication.

Physiologic State

A mismatch nucleotide base pair is detected in newly synthesized DNA. The new DNA strand is cut and the mismatch nucleotide base pair and its neighbors are removed. The missing patch of nucleotide base pair is replaced with correct nucleotide base pair by DNA polymerase. DNA ligase seals the gap in the DNA backbone.

Pathologic State

Mutations in one or more mismatch DNA repair genes cause instability regions of repeating nucleotides called 'microsatellite regions'.

- Mutations in one or more mismatch repair genes are linked to hereditary nonpolyposis colon carcinoma (**HNPCC**) also known as **Lynch syndrome**, characterized by increased risk of development

of carcinoma of endometrium, ovary and right colon carcinoma) and sporadic tumors (e.g. colorectal carcinoma, endometrial carcinoma, Muir-Torre syndrome, hepatobiliary carcinoma, genitourinary carcinoma, glioblastoma multiforme).
- Hereditary nenpolyposis colorectal carcinoma with high microsatellite instability confers very poor prognosis. Extensive history of screening of early development of these cancers is required in the management of these patients.

Tumor Suppressor Genes Function Double-stranded Breaks Repair

BRCA1 (17q21), BRCA2 (13q12), ATM (11q22.3), TP53 (17p1.2), and CHEK2 (22q12.1) belong to tumor suppressor genes. Their gene products mediate repair in double-strand breaks caused by ionizing radiation, radiomimetic chemical agents and mechanical stress on chromosomes.

BRCA1 and BRCA2 Tumor Suppressor Genes

BRCA1 and BRCA2 are tumor suppressor genes that encode proteins involved in the repair of DNA double-stranded breaks through homologous recombination repair system pathways. Thus, their gene products are crucial for the activation of cell cycle checkpoints. Both BRCA1 and BRCA2 are high-penetrance caretaker tumor suppressor genes responsible for **breast carcinoma** and **ovarian carcinoma**.

TP53 Tumor Suppressor Gene

TP53 is a tumor suppressor gene located on chromosome 17p13.1, that encodes p53 protein, that maintains genome integrity hence known as 'guardian of genome'.
- **Physiologic state:** TP53 gene products sensors DNA damage and halts cell cycle in G1/S checkpoint for DNA repair by inhibiting nuclear transcription factor, until the DNA is repaired or force the cell to undergo apoptosis via activation of proapoptotic BAX gene.
  - Mutation in TP53 gene is detected in human neoplasia, however its absence does not rule of malignancy.
  - Germline heterogenous mutation in TP53 gene is demonstrated in Li-Fraumeni syndrome (cancers of breast and adrenal cortex, sarcoma, leukemia, brain tumors) and many sporadic tumors.
- **Pathologic state:** TP53 gene mutation promotes carcinogenesis, invasion and metastasis. The p53 immunomarker differentiates malignant tumors, which are most often p53 positive (carcinoma *in situ* and invasive carcinoma) from p53 negative conditions (reactive and metaplastic conditions). The p53 immunomarker can distinguish serous endometrial carcinoma, which is p53 positive from p53 negative endometrioid carcinoma. The p53 immunomarker may be useful as serum tumor marker.

Checkpoint Kinase 2 Tumor Suppressor Gene

Checkpoint kinase 2 (CHEK2) is a tumor suppressor gene located on chromosome 22p12.1, that encodes checkpoint kinase 2 protein that plays key role in activation of DNA repair, cell cycle arrest in G1 phase, and apoptosis in response to the presence of DNA double-stranded breaks.
- **Physiologic state:** CHEK2 tumor suppressor gene encodes checkpoint kinase 2 protein phosphorylates CDC25A, CDC25B and CDC25C, that induces cell cycle arrest in G1 phase, and apoptosis by DNA double-stranded damage. CHEK2 protein also phosphorylates NEK6, which is involved in cell cycle arrest in G2/M phase. CHEK2 protein promotes DNA repair via phosphorylation of BRCA1 and BRCA2 proteins and regulates apoptosis through phosphorylation of TP53 (p53), MDM2 and PML.
- **Pathologic state:** Mutations in CHEK2 gene have been implicated in familial breast carcinoma, sporadic breast carcinoma, ovarian carcinoma, prostatic carcinoma, thyroid carcinoma, and renal cell carcinoma following radiation therapy.

Tumor Suppressor Genes Function Nucleotide Excision Repair

XPA gene (9p22.33), XPB (ERCC3) gene (2q21), XPC gene (3p25.1), XPD gene (19q13.32), XPE gene (11p11.2), XPF gene (16p13.12), XPG gene (2q13.3) and XPV gene (6p21.1) belong to tumor suppressor genes, that encode XP proteins involved in nucleotide excision repair.

Physiologic State

XPA to XPG genes make up the nucleotide excision repair (NER) pathway that detects bulky DNA adducts induced by ultraviolet (UV) radiation and repairs. DNA damage lesion is removed, and replaced by DNA synthesized using the undamaged DNA strand as template by DNA polymerase. XPV gene acts to bypass unrepaired damage.

Pathologic State

Xeroderma pigmentosum is an autosomal recessive disorder caused by alterations in nine different XP genes. XPA and XPC gene mutations are relatively common, XPE gene mutation is rare in XPG. XPG gene mutation results in severe disorder. XPF gene mutation results in mild disorder.
- Xeroderma pigmentosum is characterized by an excessive sensitivity to ultraviolet rays from sunlight affecting exposed parts of body (face, lips and upper limbs).

- There is increased risk for development of skin cancers (e.g. squamous cell carcinoma, basal cell carcinoma and melanoma) in the affected persons.

Tumor Suppressor Genes Function DNA Replication Fork Repair

DNA replication is the process by which DNA makes a copy of itself during cell division. During DNA replication, double helix structure of the DNA is unzipped by **helicase enzyme**, which breaks the hydrogen bonds holding the complementary bases of DNA together (adenine with thymine, cytosine with guanine).

- The separation of the two strands of DNA creates a 'Y' shape called a DNA replication fork. One of the DNA strands is oriented in the 3′ to 5′ direction (towards the replication fork) called 'leading strand'. Other DNA strand is oriented in the 5′ and 3′ direction (away from the replication fork), called 'lagging strand'. As a result of their different orientations, the two strands (i.e. leading and lagging strands) are replicated differently.
- Primase enzyme synthesizes a short piece of RNA called primer, that binds to the end of the leading strand. The primer acts as the starting point for DNA synthesis.
- DNA polymerase binds to leading strand and then walks along it, adding new complementary nucleotide bases (adenine, cytosine, guanine and thymine) to the strand of DNA in 5′ to 3′ direction. This sort of DNA replication is known continuous.
- Chunks of DNA, called '**Okazaki fragments**', are then added to the lagging strand in 5′ to 3′ direction. Once all the nucleotide base pairs are matched (A with T, C with G), exonuclease enzyme strips away the primer(s). These gaps created by stripping away of primer(s) are then filled by more complementary nucleotides.
- Proofreading of new strand ensures no mistake in the new DNA sequence. Finally, DNA ligase enzyme seals up the sequence of DNA into two continuous double-strands. The result of DNA replication is two DNA molecules consisting of one new and one old chain of nucleotide base pairs. That is the reason that DNA is described as semi-conservative, half of the chain is part of this original DNA molecule and half is a brand new. Following DNA replication, the new DNA automatically winds up into double helix.

Werner Syndrome Tumor Suppressor Gene

Werner syndrome (WRN) tumor suppressor gene located on chromosome 8p, that encodes WRN protein involved in replication fork progression, facilitation of repair of stalled forks and DNA double-strand breaks associated with replication fork. WRN protein also blocks nuclease-mediated excessive processing of replication fork.

- Mutation in WRN gene results in an autosomal recessive disorder is called Werner syndrome characterized by premature development of aging features and higher risk for cancer of mesenchymal origin.
- Patient with Werner syndrome shows great predisposition to arteriosclerosis, cataracts, osteoporosis and type 2 diabetes mellitus. Myocardial infarction and development of malignant tumors are most common causes of mortality among patients with Werner syndrome.

RECQL4 Tumor Suppressor Gene

RECQL4 is a tumor suppressor gene located on chromosome 8q24.3, that encodes protein family called RECQ (RECQL4) helicase, which binds to DNA temporarily and unwind the two spiral double helix strands of the DNA molecule, which are essential for the initiation of DNA replication in preparing for cell division, sister chromatids separation, double-strand break (DSB) repair, base excision repair (BER), and telomere replication leading to maintenance of genomic stability. RECQ (RECQL4) helicase protein remains active many cells during embryogenesis and postnatal life. RECQL4 gene mutation is linked to Baller Gerold syndrome, PAPADILNO syndrome and Rothmund-Thompson syndrome. Overall, the prevalence of cancer among RECQL4 heterozygous family members is 2.5%.

TERT Tumor Suppressor Gene

Telomerase reverse transcriptase (TERT) is a tumor suppressor gene located on chromosome 5p15.33, that encodes telomerase enzyme, which maintains structure of chromosomes called telomeres. Telomeres are composed of repeated segments of DNA (tandem repeats of ATTAGGG) found at the ends of the chromosomes.

Physiologic State

Telomeres protect structure of chromosomes from DNA repair pathways initiated by a DNA damage response. In most cells, telomeres become progressively shorten, as the cell divides a certain number of cell divisions, then telomeres become so short, that these trigger the cell to stop dividing and undergoing apoptosis.

- Telomerase enzyme counteracts the shorting of telomeres by adding small repeated segments of DNA to the ends of chromosomes each time the cell divides. In most type of cells, telomerase activity is either undetectable or active at low levels.

- However, telomerase activity is high in lungs, gastrointestinal tract and bone marrow during embryonic development. Telomerase enzyme allows these cells to divide many times without undergoing apoptosis.

Pathologic State

Telomerase enzyme is abnormally active in most CSCs, which grow and proliferate in uncontrolled manner.

- TERT gene can be upregulated by gene amplification, rearrangements and mutations within promoter region in 4% of human cancers.
- TERT promoter mutations are demonstrated in 80–90% of human malignant tumors and can be used as biomarker in diagnosis and outcome prediction.
- TERT promoter mutations cause upregulation of TERT transcription/activity in urothelial carcinoma (68%), central nervous system tumors (28%) and thyroid follicular, papillary and differentiated/anaplastic carcinomas (15%), prostatic carcinoma, endometrial carcinoma, rhabdomyosarcoma (2%), neuroblastoma, lung carcinoma, colorectal carcinoma, ovarian carcinoma, breast carcinoma, adrenocortical carcinoma and acute myelogenous leukemia (AML).
- Rearrangements of TERT promoter have been implicated in neuroblastomas.
- Mutations in TERT gene have been demonstrated in familial idiopathic pulmonary fibrosis and dyskeratosis congenita (e.g. aplastic anemia or cancer).

Fanconi Anemia (FA) DNA Repair Tumor Suppressor Genes

The maintenance of genomic stability is crucial for species survival, and its failure is closely associated with carcinogenesis.

- Fanconi anemia DNA repair pathway, involving 22 identified Fanconi anemia DNA repair genes, plays a central role in repairing DNA inter-strand cross-links (ICLs).
- Recent studies reveal that the Fanconi anemia DNA repair pathway functions in a critical tumor suppressor network to preserve genomic integrity by stabilizing replication forks, migrating replicating stress and regulating cytokinesis.

Physiologic State

The canonical function of the Fanconi anemia (FA) proteins is to collaborate with several other DNA repair proteins to eliminate chromosome-breaking (clastogenic) effects of DNA inter-strand crosslinks.

Pathologic State

Germline mutations in Fanconi anemia DNA repair tumor suppressor genes are linked to Fanconi anemia, inability to repair DNA inter-strand crosslinks and cancer predisposition (e.g. acute myelogenous leukemia, liver tumors, and squamous cell carcinoma of oropharyngeal and anogenital regions). Fanconi anemia significantly increases cancer susceptibility sporadically especially breast carcinoma in the general population.

TUMOR SUPPRESSOR GENES FUNCTION BY VARIOUS MECHANISMS

Numerous tumor suppressor genes function by different mechanisms to inhibit tumorigenesis, which include WT1, MEN1, DPC4 (MADH4/SMAD4), ATM, p16, TGF-β, and CHEK2 genes.

WT1 Tumor Suppressor Gene

WT1 tumor suppressor gene is located on chromosome 11p13, that encodes WT1 protein that is essential for the development of kidneys and gonads (ovaries in females and testes in males) during intrauterine life. WT1 protein activity is limited to glomerulus after birth. WT1 protein plays a key role in cell growth, differentiation and apoptosis.

Physiologic State

WT1 protein encoded by WT1 tumor suppressor gene binds to specific regions of DNA via zinc finger proteins to the promoter regions of >20 putative downstream target genes, and regulates their activity either by repression or activation of the target genes.

Pathologic State

WT1 gene mutation is associated with Wilm's tumor (heritable/nonheritable), Denys-Drash syndrome, WAGR syndrome, Frasier syndrome, congenital nephrotic syndrome, cytogenetically normal acute myelogenous leukemia (AML), aggressive desmoplastic round cell tumors, and prostatic carcinoma. WT1 gene mutations, including deletions, truncations, translocations have been observed in approximately 20% of Wilm's tumors.

- WAGR syndrome is diagnosed on the basis of clinical manifestations (e.g. Wilm's tumor, aniridia, genitourinary abnormalities and intellectual disability) and genetic testing (karyotyping 11p13 deletion). A more specific genetic testing is done by fluorescent *in situ* hybridization (FISH) to detect deletion of specific genes on chromosome number 11.
- Development of Wilm's tumor is unusual in Frasier syndrome. However, gonadoblastoma is more common in Frasier syndrome than in Denys-Drash syndrome.

MEN1 Tumor Suppressor Gene

The MEN1 gene located on chromosome 11p13.1 encodes a tumor suppressor protein called menin present in the nucleus of numerous cells.

Physiologic State

Menin protein encoded by MEN1 gene binds to specific sequences of DNA and control expression of targeted genes. Some of these play a pivotal role in DNA repair, cell growth, cell division and apoptosis.

Pathologic State

Mutation in MEN1 tumor suppressor gene is associated with familial isolated hyperparathyroidism, multiple endocrine neoplasia (tumors of pituitary, parathyroid and pancreas), primary macronodular adrenal hyperplasia and other tumors (e.g. parathyroid adenoma, nonfunctioning neuroendocrine tumor of pancreas, gastrinoma, insulinoma, bronchial carcinoids). Somatic mutation in MEN1 gene is associated with tumors of pituitary, parathyroid and pancreas.

- MEN1 is an autosomal dominant tumor predisposition syndrome characterized by pituitary, parathyroid and pancreatic tumors. MEN1 shows germline heterozygous mutations in one allele of MEN1 gene. Tumor development occurs upon loss of remaining normal copy of the MEN1 gene in MEN1-targeted tissues.
- MEN1 syndrome is diagnosed on the basis of clinical manifestations, family history, physical examination, hormonal analysis, imaging to look for endocrine system tumors.

DPC4 (MADH4/SMAD4) Tumor Suppressor Gene

DPC4 (MADH4/SMAD4) tumor suppressor gene located on chromosome 18q21.1 encodes DPC4 protein.

Physiologic State

DPC4 protein encoded by DPC4 (MADH4/SMAD4) tumor suppressor gene mediates the downstream effect of the TGF-β-stimulated gene transcription through sequence-specific binding to DNA and inhibit cell growth and apoptosis.

Pathologic State

Somatic mutation in DPC4 (MADH4/SMAD4) tumor suppressor gene is specific for pancreatic adenocarcinoma (55%), colorectal carcinoma (20%), breast carcinoma, and ovarian carcinoma. Loss of nuclear immunostaining is considered an abnormal positive result with adjacent pancreatic stroma retaining expression.

ATM Tumor Suppressor Gene

ATM gene is located on chromosome 11p22, that encodes ATM kinase protein, which is recruited and activated by DNA double-strand breaks.

Physiologic State

ATM protein encoded by ATM tumor suppressor gene plays an essential role in the maintenance of **genomic stability** by phosphorylation of downstream targets for activation of the DNA damage-induced cell cycle checkpoints resulting in cell cycle arrest, repair of DNA damage or apoptosis of mutated cell.

Pathologic State

Mutations in both copies of ATM gene result in ataxia-telangiectasia, an autosomal recessive disorder. Depending on the extent of the ATM mutation, the resultant ATM protein expression can result in pleiotropic clinical phenotypes including neurodegeneration, hematologic malignancies (e.g. **leukemia, lymphoma**), immunodeficiency and hypersensitivity to ionizing radiation.

Transforming Growth Factor-β Tumor Suppressor Gene

Transforming growth factor-β (TGF-β) ligands (TGF–β1, TGF-β2 and TGF-β3) transmit through the type 1 and type 2 (TGFR-β1 and TGFR-β2 respectively). Canonical signaling proceeds with phosphorylation of SMAD2 and SMAD3, which then bind with SMAD4 to enter the nucleus to modulate transcription in cooperation with other transcription factors, coactivators and corepressors. In addition, TGF-β ligands bind to its cognate receptors leading to activation of many noncanonical signaling pathways.

- TGF-β can function as tumor suppressor or tumor promoter. Recent studies show that receptors and proteins involved in TGF-β signaling may act as tumor suppressors. Loss of response to TGF-β may be an important step in the tumorigenesis and progression of cancer.
- TGF-β suppresses tumor progression through inhibition of cell cycle (G1/S transition) progression, inducing apoptosis, and suppression of expression of growth factors, cytokines and chemokines.

Physiologic State

One of the significant effects of TGF-β is to inhibit tumor immunosurveillance in the host by directly suppressing the transcription of genes encoding multiple key proteins of the CD8+ cytotoxic T cells, such as perforin and granzymes, and cytotoxins that act through the granule exocytosis pathway.

Pathologic State

TGF-β mediates inflammatory reaction via chemokines and their receptors in the tumor microenvironment. The inflammatory cells provide proangiogenic factors, growth factors, proteases and cell adhesion molecules, that facilitate cancer stem cell proliferation, angiogenesis, epithelial–mesenchymal transition (EMT), invasion and metastasis.

- Alterations in TGF-β signaling have significant effects on tumorigenesis and progression. The mechanisms determining when and how TGF-β switches form a tumor suppressor to a tumor promoter are great challenge in understanding dual role of TGF-β.
- TGF-β is most often produced in large quantity in many human cancers due to its oncogenic activity. Somatic mutation in TGF-β is linked to **pancreatic carcinoma** and **colorectal carcinoma**.

Pathology Pearls: Regulation of TGF-β/SMAD4 Pathway

RAS/RAF/MEK/ERK/MAPK, phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR and Wnt/β-catenin signaling pathways regulate TGF-β/SMAD4 pathway.

- **RAS/RAF/MEK/ERK/MAPK signaling pathway:** RAS/RAF/MEK/ERK/MAPK signaling pathway regulates the TGF-β/SMAD4 pathway by three mechanisms: (a) prevention of phosphorylated SMAD2/SMAD3 translocation into the nucleus, (b) mediating SMAD4 degradation, and (c) promoting activator protein 1 (AP-1) complex formation at the TGF-β1 promoter, thus boost the TGF-β1 transcription and secretion.
  - Binding of substrate of c-Jun N-terminal kinases (JNKs) to the transcriptional corepressor TG-interacting factor (TGIF) inhibits SMAD2-dependent transcription.
  - The p38 can phosphorylate SMAD binding partners, such as activating transcription factor 2 (ATF-2) in the nucleus and therefore enhances TGF-β/SMAD4 induced genes transcription.
- **Phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway:** Phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway suppresses the TGF-βR1 mediated SMAD3 phosphorylation through its downstream mTOR molecule. AKT can directly phosphorylate FOXO and keep in the cytoplasm to prevent its binding to the promoter of p27 and p21, thus blocks the TGF-β1/SMAD-mediated cytostatic signals.
- **Wnt/β-catenin signaling pathway:** Wnt ligand can bind to Frizzled family receptor and initiates Wnt/β-catenin signaling pathway and pass the signal to protein inside nucleus and subsequently to the β-catenin destruction complex consisting of AXIN, APC, GSK3b and CKIa, that causes SMAD7 ubiquitination and degradation. In turn, SMAD7 can disassemble the β-catenin destruction complex by binding AXIN and hence stabilize β-catenin and promotes its translocation in the nucleus.

CHEK2 (Checkpoint Kinase 2) Tumor Suppressor Gene

CHEK2 is a tumor suppressor gene located on chromosome 22p12.1, that encodes CHEK2 protein, CHEK2 protein, which plays key role in activation of DNA repair, cell cycle arrest in G1 phase, and apoptosis in response to the presence of DNA double-stranded breaks.

Physiologic State

CHEK2 protein encoded by CHEK2 tumor suppressor gene phosphorylates numerous effectors (CDC25A, CDC25B and CDC25C) preferentially at the consensus sequence, and inhibit their activities resulting in cell cycle arrest in G1 phase.

- Inhibition of CDC25 phosphatase activity results in increased inhibitory phosphorylation of cyclin/CDK complexes, and inhibits cell cycle progression.
- CHEK2 protein may also phosphorylate NEK6, which is involved in cell cycle arrest in G2/M phase.
- CHEK2 protein interacts with phosphorylates BRCA1 and BRCA2 to restore cell survival after DNA damage, and also protein regulates DNA repair through activation of transcription factor FOXM1, and apoptosis through phosphorylation of p53/TP53, MDM2 and PML. Phosphorylation of MDM2 protein may also reduce degradation of p53.

Pathologic State

Mutation in CHEK2 tumor suppressor gene is implicated in **familial breast carcinoma**, and sporadic tumors such as breast carcinoma, ovarian carcinoma, prostatic carcinoma, thyroid carcinoma, and renal cell carcinoma following radiation therapy.

The $p16^{INK4A}$ Tumor Suppressor Gene

The $p16^{INK4A}$ is the principal members of the INK4 family of cyclin-dependent kinase inhibitor (CDKI). It is encoded by the CDK2NA gene located on chromosome 9p21.3 within the INK4A/ARF locus, which encodes for two different proteins with different promoter activities: $p16^{INK4A}$ and $p14^{ARK}$.

Physiologic State

Both $p16^{INK4A}$ and $p14^{ARK}$ proteins inhibit cyclin-dependent kinases (e.g. CDK4 and CDK6) and restrict cell cycle progression, and are involved in the retinoblastoma (RB) and p53 signaling pathways respectively. The $p16^{INK4A}$ protein inactivates CDKs, that phosphorylate RB, therefore, $p16^{INK4A}$ protein induces cell cycle arrest.

- In human papillomavirus (HPV) infection, the HPV oncoprotein E6 and E7. E6 oncoprotein induces the degradation of the tumor suppressor p53 protein. E7 oncoprotein inactivates RB protein (pRB) and thus resulting in cytoplasmic overexpression of $p16^{INK4A}$.

- The $p16^{INK4A}$ interaction with other proteins Y2 chain of laminin, β-catenin or vascular endothelial growth factor (VEGF) is thought to be related to new functions of $p16^{INK4A}$ such as inhibition of angiogenesis as well as cell division.

Pathologic State

Genetic alterations of the $p16^{INK4A}$ locus may impair both ($p14^{ARK}$ $p16^{INK4A}$)/RB pathways, and provide a selective advantage in carcinogenesis, angiogenesis, invasion and metastasis. Germline mutation in $p16^{INK4A}$ locus is linked to familial melanoma. Somatic mutation in $p16^{INK4A}$ locus is associated with melanoma, mesothelioma, pancreatic carcinoma and astrocytoma.

EPIGENETICS IN CANCER

Epigenetics literally means 'above' or 'on top' of genetics. Epigenetic alterations induce genetic modifications that impact gene expression ('turning on'/'turning off') without changing the nucleotide base sequences of DNA building blocks.

- Epigenetic changes may be caused by environmental chemical agents, cytotoxic drugs/pharmaceutical drugs, dietary factors and advancing age, that alter the physical structure of DNA. Aberrant gene function and altered patterns of gene expression are key features of cancer. Epigenetic machinery governing epigenetic processes is shown in Fig. 6.118.
- Nucleosomes are structural units of the chromosome consisting of DNA wrapped around histone octamer proteins, and play important roles in compaction and regulation of the chromatin structure. When nucleosomes are spaced closely together, transcription factors cannot bind and gene expression is 'turned off'. When the nucleosomes are spaced far apart, the DNA is exposed. Transcription factors can bind to exposed DNA and induce gene expression 'turned on'. Histone octamer proteins also play an important role in the maintenance of gene expression.
- Epigenetic regulation of gene expression is mediated by three main mechanisms: (a) histone octamer modifications, (b) DNA methylation (additional of methyl group to DNA by DNA methyltransferases), and (c) small noncoding RNA (e.g. microRNAs).
  - Histone octamer proteins can be modified by the addition or removal of chemical methyl groups or acetyl groups, which determine how tightly DNA is wrapped around histone octamer proteins and affect whether a gene can be 'turned on'/'turned

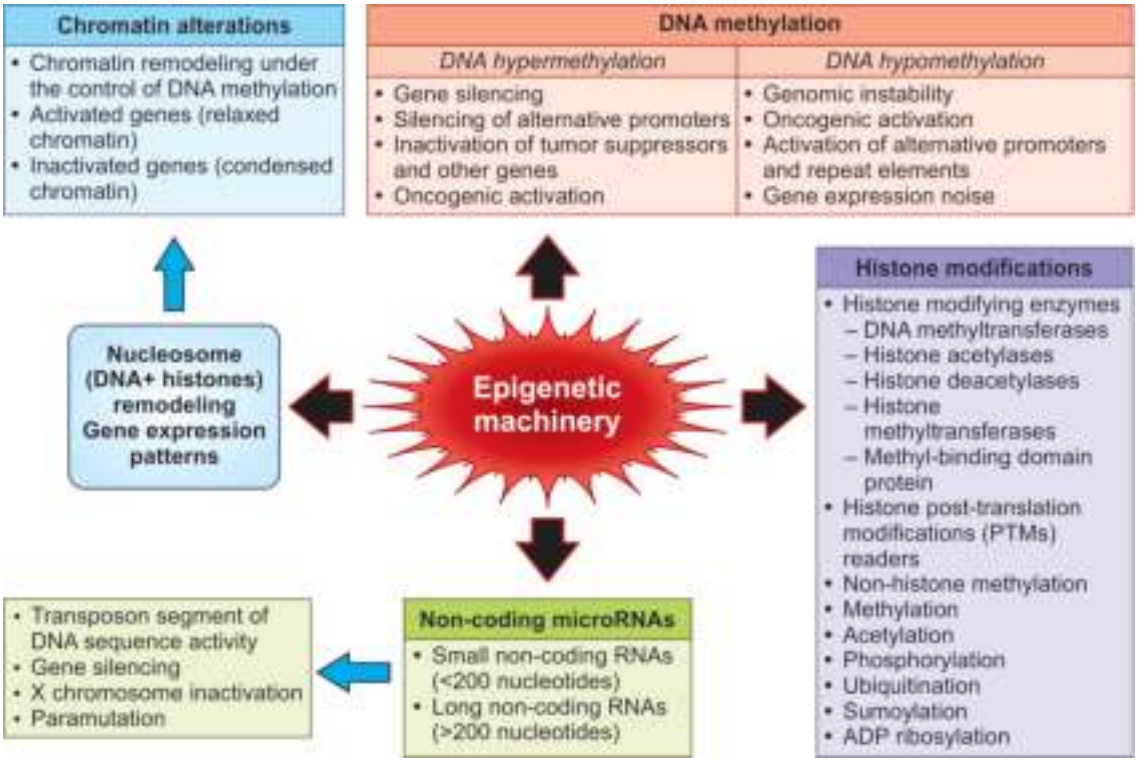


Fig. 6.118: Epigenetic machinery governing epigenetic processes. Three main protein groups control major epigenetic mechanisms targeting DNA, histones, and nucleosome positioning. Genome-wide epigenetic marks introduced by these targeting proteins regulate various biological processes, including gene expression, DNA-protein interactions, chromosomal stability, DNA repair, DNA replication, chromosome structure and recombination events.

off'. An active gene is less bound by histone octamer proteins, whereas inactive gene is highly bound by histone octamer proteins.
  - Transcription-related modifications in histone octamer proteins can occur by methylation (lysine and arginine residues), acetylation (lysine residue), phosphorylation (serine, tyrosine, and threonine residues), ubiquitination (lysine residue) and sumoylation (lysine residue).
  - Proper coordination of temporal activation and silencing of gene expression is the key to the orchestration of normal physiological processes.
  - Deregulation of gene expression results in disruption of normal cellular processes, which in turn, paves the way to the development of cancers.
  - Epigenetic changes can collaborate with genetic changes to cause the evolution of human cancers.
- Recent studies revealed that genome in CSCs in malignant tumor is characterized by global (genome-wide) hypomethylation and site-specific promoter hypermethylation. Many of the epigenetic changes probably contribute in the initiation of malignant tumor growth.
  - Global (genome-wide) hypomethylation increases genomic instability due to chromosomal rearrangements and activates growth-promoting genes (proto-oncogenes), thereby 'turning on' mitotic signals. DNA hypomethylation can also result in loss of imprinting (**LOI**).
  - Methylation-mediated silencing of tumor suppressor genes provides one of the two hits advocated in Knudson's two-hit hypothesis.
  - Silencing of gene expression in carcinogenesis also involves global (genome-wide) loss of transcription-activating histone modifications such as acetylation and activating methylation. Histone tail acetylation enhances transcriptional activity of the gene promoter region.
  - On the contrary, histone octamer methylation can activate either transcription (e.g. H4 lysine 20 methylation) or represses transcription (e.g. histone H3 lysine 9 and lysine 27 methylation). Loss of histone acetylation is carried out by histone deacetylase (HDAC), which is often upregulated in various human cancers.
- The third component of epigenetic regulation is the deregulation of microRNAs demonstrated in many human cancers. Normally, microRNAs are involved in transcriptional regulation, cell proliferation and apoptosis. MicroRNAs regulate interaction of CSCs in tumor microenvironment.
  - In the tumor microenvironment, microRNAs regulate the interaction between CSCs and their tumor microenvironment by targeting several genes involved in this interaction.
  - Several tumor suppressor microRNAs are deregulated in the tumor microenvironment through various mechanisms: (a) amplification, (b) deletion, (c) abnormal transcriptional regulation, (d) epigenetic alteration and gene silencing, and (e) defects in the biogenesis machinery. Amplification of oncogenic microRNAs can downregulate tumor suppressor genes. Onco-RNAs are linked to CSCs growth, proliferation, EMT, invasion, and metastasis to distant organs.
  - Downregulation of tumor suppressor miR-29 and miR-148 in cancer-associated fibroblasts (CAFs) target several genes such as ANGPTL4, PDF, LOX, MMPs and VEGF-A for miR-29 and DNMT1 for miR-148, which stimulate CSCs invasion and metastasis.
  - Hypoxia in the tumor microenvironment has a tumor promoting role by altering the expression of tumor suppressor microRNAs, such as miR-200, miR-34, miR-34, miR-29 and miR-15/16. Inhibition of tumor suppressor miR-200 can significantly increase the expression of ETS-1, resulting in the induction of angiogenesis.
  - Hypoxia-inducible factor 1 (HIF1) gene plays a crucial role in the tumor microenvironment and has been associated with the downregulation of microRNAs. Cancer-associated fibroblasts (CAFs) play an important role in unrestricted proliferation of CSCs during carcinogenesis and metastatic process.
  - Tumor suppressor miR-200 plays role in transformation of normal fibroblasts (NFs) to cancer-associated fibroblasts (CAFs). Tumor suppressor miR-320 targets ETS2, a cancer-specific transcription factor, resulting in increased oncogenic secretome synthesis, and this oncogenic secretome transforms normal fibroblasts (NFs) to cancer-associated fibroblasts (CAFs) in the tumor microenvironment.

TUMOR INVASION AND METASTASIS: MOLECULAR MECHANISMS

Tumor invasion and metastasis are 'hallmarks of cancer' specified by Hanahan and Weinberg. The initial transformation of normal epithelial cell to CSC results in carcinoma *in situ*. With reduced adhesiveness and enhanced migratory behavior, CSCs progress to

invasive stage. After degradation of basement membrane, CSCs invade the surrounding stroma, migrate and intravasate into lymph and blood circulation, surviving CSCs arrest in capillaries, and colonize in the distant organs. CSCs may remain in dormant state without reproliferating for considerable time. **Tumor dormancy** can be induced in malignant epithelial tumor through genetic and epigenetic changes, hypoxia, angiogenic switch, immune evasion, and inflammatory switchover. A change in the tumor microenvironment can facilitate tumor progression/recurrence and thereby permit the tumor to exit from dormancy through interaction with vascular endothelial cells, cancer-associated fibroblasts (CAFs), tumor-associated immune/inflammatory cells, and extracellular matrix (ECM). Subsequent reactivation of metastatic dormancy results in micrometastasis, and then macrometastasis in the distant tissues/organs. In the lymph nodes, communications between the lymphatic channels and venous tributaries allow CSCs access to the systemic circulation. Alternately, CSCs may extravasate the lymph, and blood circulation and lead to formation of secondary tumors resulting in unrestricted CSCs proliferation, angiogenesis and tissue microenvironment activation.

- **Tumor angiogenesis:** Angiogenesis plays a key role in increasing malignant tumor growth, invasion, and metastasis. Vascular endothelial growth factor (VEGF) is the most potent stimulator of endothelial cells within existing vascular networks and near the malignant epithelial tumor.
  - Tumor angiogenesis facilitates dissemination of CSCs from primary malignant epithelial tumor to distant tissues/organs via lymphatic and hematogenous routes.
  - Vascular endothelium of newly malformed blood vessels limits immune CD8+ cytotoxic T cells access to the tumor microenvironment, and expresses FasL, that induces apoptosis of effector CD8+ cytotoxic T cells. FasL on vascular endothelium also poses as a physical barrier, that prevents their extravasation into the tumor microenvironment.
  - Poor perfusion and density-packed glycolytic CSCs create a pocket of diminished oxygen levels, acidic pH, poor nutrition loads, anti-inflammatory signaling factors (e.g. cytokines, chemokines), and accumulated metabolic by-products such as lactate.
- **Tumor invasion in local neighboring tissue:** Tumor invasion is the mechanism by which CSCs in primary malignant epithelial tumor site directly extend and penetrate neighboring tissue.
  - Proliferation of transformed CSCs increases malignant epithelial tumor size eventually leads to breach in the barriers between tissues resulting in CSCs extension into surrounding tissue.
  - Local tissue invasion is also the first stage of dissemination, that leads to the development of secondary tumor in distant tissues/organs.
- **Tumor metastasis:** Metastasis is the multistep process by which CSCs detach from original primary malignant epithelial tumor, invades interstitial tissue and extracellular matrix (ECM), intravasate lymphatic and blood vessels, circulate in the bloodstream as single or clusters, survive, extravasate, establishment of the micrometastasis, and eventually colonize as single or multiple metastases and remain in metastatic dormancy and their subsequent reactivation in the distant tissues/organs.
  - Subsequent reactivation of dormant metastasis through interaction with cancer-associated fibroblasts (CAFs), immune cells, vascular endothelial cells, inflammatory cells, and the ECM involving intracellular signaling, extracellular signaling and induction of signals originating from the bone marrow niche.
  - Metastasis is most often associated with several clinical, pathologic, and anatomic characteristics—tumor size, regional lymph nodes involvement, metastasis in distant organs and resistance to therapy.
  - **Integrins' role in cancer progression:** Integrins are the key cellular adhesion receptors, that are involved in nearly every step of cancer progression from primary malignant epithelial tumor to secondary tumor in distant tissues/organs. Altered integrin expression is demonstrated in human cancers, where integrins support oncogenic growth factor receptor, invasion, and metastasis. Furthermore, integrins regulate the colonization process in the distant tissues/organs by easing anchorage independent survival of CSCs.
- **Metastatic cascade—critical steps:** Critical steps to tumor metastasis include alterations in tumor microenvironment and ECM in the formation of pre-metastatic and metastatic niche. Primary malignant epithelial tumor induces premetastatic niche (PMN) in sequence-wise mechanisms.
  - **Step 1:** At primary malignant epithelial tumor site, synthesis of some molecules (VEGF, TGF-β, MMP2, MMPs, CXCR4, SDF-1) induce inflammation and hypoxia responses, which assist proliferation of primary malignant epithelial tumor and unregulation of molecules, e.g. TNF-α, TGF-β, VEGF-1, and G-CSF.

- **Step 2:** TNF-α, TGF-β, VEGF-1, and G-CSF molecules are transported from primary site to secondary site through hematogenous route.
- **Step 3:** TNF-α. TGF-β, VEGF-1, and G-CSF molecules induce pre-metastatic niche (PMN). These molecules function in similar way at primary and secondary sites. The change of PMN build a unique environment which favor metastatic CSCs colonization in distant tissues/organs.

Pathology Pearls: Liquid Biopsy Significance in Metastatic Cascade

- Liquid biopsy has diagnostic, prognostic, and predictive significance in cancer patients. Liquid biopsy is based on the analysis of cellular or molecular biomarkers such as circulating tumor cells (CTCs), free nucleic acids (DNA or RNA), and exosomes in a biological fluid, usually blood. During metastatic cascade different liquid biopsy analytes are involved. Advantages of liquid biopsy is to monitor tumor progression.
- Liquid biopsy has broad potential applications for cancer diagnosis, study of tumor heterogeneity, clonal evolution, and detection of minimal residual disease.
- During cancer progression, liquid biopsy might help the clinical oncologist to stratify patients for treatment and personalization, monitor the treatment response and the development of resistance to chemotherapy and radiation therapy.
- Liquid biopsy analyte involved during metastatic cascade is given in Table 6.96.

- **Chromosomal instability:** Chromosomal instability initially triggers tissue invasion and metastasis that results from unexpected errors in segregation of chromosomes during mitosis leading to structural and numerical chromosomal abnormalities. The chromosomes are susceptible to damage by ionizing radiation and drugs.
  - Chromosomal instability is associated with poor prognosis as a result of invasion, metastasis, and therapeutic resistance. Chromosomal instability differs from microsatellite instability.
  - Microsatellite instability is defined as increased predisposition to mutation that results from impaired DNA mismatch repair.
  - Microsatellite instability (**MSI**) represents phenotypic evidence that mismatch repair is not functioning normally. Each microsatellite consists of a short motif (1–6 nucleotide base pairs) repeated in tandem to form an array over 6,00,00 unique satellites exist in human genome.
- **Epithelial–mesenchymal transition:** Epithelial–mesenchymal transition (EMT) involves the disruption of cell-cell adhesion and cellular polarity, remodeling of the cytoskeleton and changes in cell-matrix adhesion. It is associated with enhancement in migratory and invasive properties.
  - Epithelial cells are characterized by intact cell-cell interactions through cell adhesion molecules such as E-cadherin and cytokeratin within tight junctions, adherens junctions, desmosomes, and gap junctions.
  - Desmosomes are adhering junctions between the membranes of adjacent cells.
  - Hemidesmosomes are present between epithelial cells and their underlying basement membrane. Desmosomes are composed of desmoplakin and other proteins, which form an electron dense structure immediately beneath the plasma membrane.
  - Cadherins are calcium-dependent adherens proteins associated with desmosomes and penetrate the cell membrane to interact with cognate proteins on adjacent cells. Cadherins function to hold the cells in contact with one another across desmosomes.

Table 6.96 Liquid biopsy analyte involved during metastatic cascade

Metastasis Steps	Liquid Biopsy Analyte Involved
Cancer stem cell proliferation in primary malignant tumor	ctDNA, EVs
Angiogenesis in primary malignant tumor	ctDNA, EVs
Local tissue invasion and detachment and intravasation of CSCs	ctDNA, EVs
Embolization and survival of CSCs	ctDNA, TEPs
Cancer stem cells arrest and extravasation at target organs	ctDNA, EVs
Micrometastasis	ctDNA, EV
Macrometastasis	ctDNA

CtDNA: Circulating DNA; EVs: EV-associated metabolite biomarkers (proteins, DNA and RNA), TEPs are used to diagnose glioblastoma multiforme.

EPITHELIAL–MESENCHYMAL TRANSITION IN DEVELOPMENT OF MALIGNANT TUMOR AND ITS CLINICAL SIGNIFICANCE

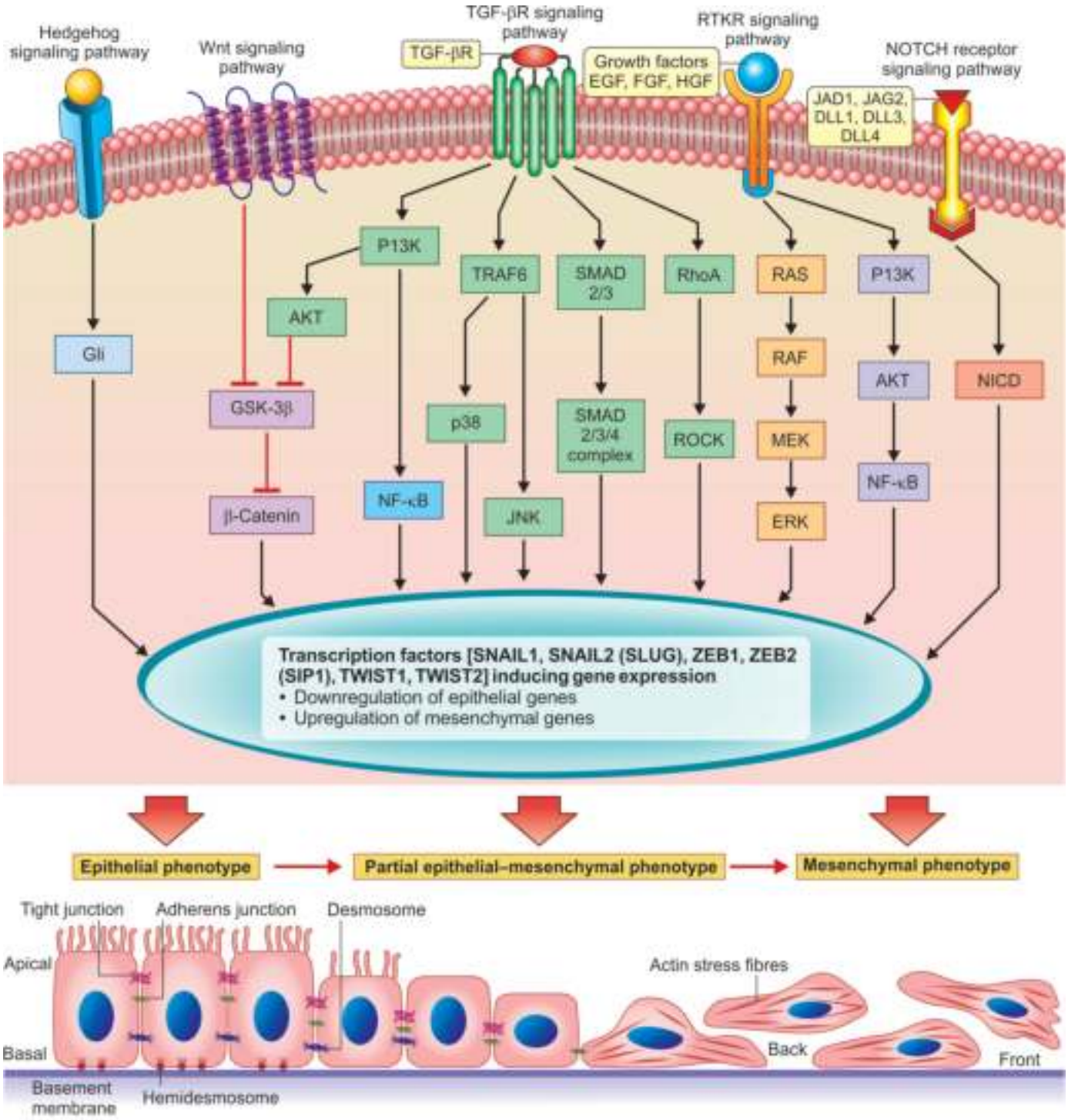
Epithelial–mesenchymal transition (EMT) is biologic process by which epithelial cells lose their cell polarity and cell–cell adhesion and gradually acquire migratory and invasive properties to become mesenchymal stem cells.

- Type I EMT is associated with embryogenesis involved in implantation of embryo, placenta formation and organogenesis. Type II EMT is involved in inflammation, wound healing, and tissue remodeling. Type III EMT plays a critical role in carcinogenesis, invasion, metastasis, recurrence and chemoresistance.
- Cancer stem cells (CSCs) undergoing EMT can acquire invasive properties and enter the surrounding stroma resulting in the creature of favorable microenvironment for invasion and metastasis. EMT signals occur at primary malignant epithelial tumor. Partial EMT state facilitates motility and invasion into basement membrane and extracellular matrix (**ECM**). Mesenchymal phenotype facilitates intravasation and anoikis resistance during lymphatic and blood vessel dissemination and extravasation of CSCs.
- After extravasation, migrating CSCs survive and remain in dormancy in solitary or micrometastasis at distant sites, which later exit from dormancy and undergo mesenchymal–epithelial transition (**MET**), colonization, CSC proliferation, and formation of macrometastasis. Recent studies revealed that CSCs and microRNAs could be involved in EMT process.
- Development of metastasis in distant tissues/organs is the final stage of solid malignant tumor progression and is responsible for cancer-related deaths. The metastasis process consists of multiple steps.
  - First, CSCs escape from the primary malignant tumor site. Then, CSCs invade basement membrane and extracellular matrix (ECM), migrate and invade lymphatic or blood vessels and circulate. Most circulating CSCs undergo apoptosis due to anoikis conditions. If CSCs survive in bloodstream, they may reach more suitable distant sites by attaching to endothelial cells and extravasating from the circulation into the surrounding tissues.
  - Finally, distal colonization requires CSCs to invade and grow in the new microenvironment. The biology of EMT has been clarified in malignant tumor samples through use of EMT-associated markers such as mesenchymal-specific markers (i.e. vimentin and fibronectin), epithelial-specific markers (i.e. E-cadherin and cytokeratin), and transcription factors (i.e. SNAIL and SLUG).

Involvement of Epithelial–Mesenchymal Transition in Cancer Progression

Epithelial–mesenchymal transition is a highly dynamic process by which epithelial CSCs can convert into mesenchymal phenotype, which is involved malignant tumor progression with metastatic expansion and generation of cancer cells with stem cell properties with resistance to cancer treatment.

- Epithelial–mesenchymal transition induces disintegration of cell-to-cell junctions and cell-to-ECM junctions with loss of apical–basal polarization, cytoskeletal modifications (i.e. actin rich membrane projections—invadopodia, lamellipodia, and filopodia; actin stress fiber formation), downregulation of E-cadherin, cytokeratins, claudins and upregulation of mesenchymal markers (i.e. fibroblast-like shape, acquisition of front–rear polarization, N-cadherin, vimentin, fibronectin), multipotency (stem cell traits), motility, invasiveness, anoikis resistance, chemoresistance and radiation resistance, recurrence, immune suppression, metabolic reprogramming, modulation of expression/secretion of invasion-mediated proteolytic enzymes such as matrix metalloproteinases (MMPs).
- Epithelial–mesenchymal transition (EMT) allows CSCs to acquire mobility, invade the surrounding tissue, intravasate into the vasculature, and then extravasate at distant sites, and establish in the tissue stroma, a process that is influenced by cellular plasticity, that requires the ability of CSCs with mesenchymal properties to revert to an epithelial state through mesenchymal–epithelial transition (**MET**). Proliferation of the established CSCs results in clinically relevant macrometastases in distant tissues/organs.
- Signaling pathways in EMT and MET in malignant epithelial tumor progression are given in Fig. 6.119. Signaling pathways in induction of EMT are given in Table 6.97. EMT and MET malignant epithelial tumor progression given in Fig. 6.120. Cellular changes associated with EMT and MET in malignant epithelial tumor progression are given in Table 6.98. EMT-associated biomarkers in clinical tissue samples to predict prognosis of cancer patients are given in Table 6.99.



Epithelial–mesenchymal transition (EMT) transcription factors

- **Epithelial phenotype (downregulation of epithelial markers):** ↓E-cadherin, ↓cytokeratins, ↓claudins, ↓zona occludin 1 (ZO-1), ↓desmoplakin, ↓plakophilin, ↓vitronectin, ↓fibronectin
- **Mesenchymal phenotype (upregulation of mesenchymal markers):** ↑N-cadherin, ↑vimentin, ↑α-SMA, ↑integrins, ↑β1/β3 integrins, ↑MMPs, AKT/GSK3β pathway, ↑neuropilin 2, ↑ETS-1/NF-κB pathway, ↑β-catenin, ↑TGF-β, ↑FGFR2 IIIb/IIIc, ↑rho-kinase, ↑DDR2, ↑ILK, ↑FOXC2

Fig. 6.119: Signaling pathways in epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) in malignant epithelial tumor progression. EMT is essential mechanism in embryonic development and tissue repair. EMT also contributes to the progression of cancer. MET is often implicated in colonization of carcinoma—the last step in the metastatic cascade.

Table 6.97 Signaling pathways in induction of epithelial–mesenchymal transition

Receptor	Ligand	Signaling Molecules	Intermediate Signaling End Point	Effect
TGF-β receptor	TGF-β	▪ SMAD family of signaling pathway ▪ Rho signaling pathway	ZEB	Migration of cancer stem cells
Receptor tyrosine kinase	FGF, HGF, EGF, IGF	▪ Src signaling pathway RAS/RAF/MEK/ERK/MAPK signaling pathway ▪ PI13K/AKT/mTOR signaling pathway	SNAIL2	▪ Cytoskeleton activation ▪ Migration of cancer stem cells ▪ Focal adhesion rearrangement
Integrins	Collagen Fibronectin	▪ FAK signaling pathway ▪ Paxillin signaling pathway ▪ Rac signaling pathway	SNAIL2	Migration of cancer stem cells
Frizzled	Wnt	▪ APC signaling pathway ▪ Axin signaling pathway ▪ GSK3β ▪ β-Catenin	SNAIL2	▪ E-cadherin downregulation ▪ Reduced cell adhesion

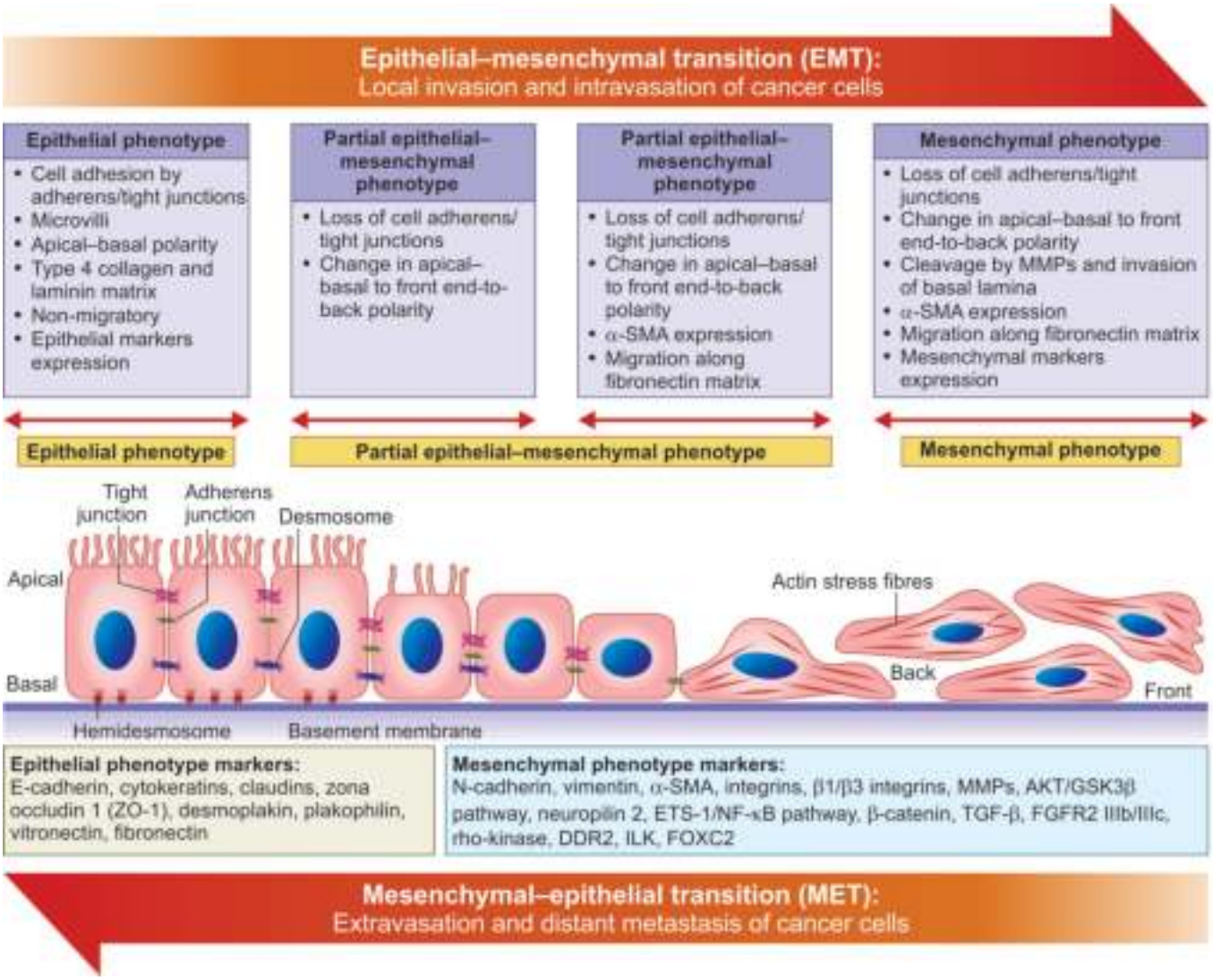


Fig. 6.120: Epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) in malignant epithelial tumor progression. During initial steps of metastatic cascade, epithelial cancer cells undergo an EMT. MET is the reverse process of EMT, and it has been shown to occur in normal development induced pluripotent stem cell reprogramming, cancer metastasis and wound healing.

Table 6.98 Cellular changes associated with epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition in malignant epithelial tumor progression

Downregulation/Loss of Epithelial Cell Phenotype during EMT
Downregulation/loss of E-cadherin (epithelial adherens junction protein)
Noninvasive properties of cancer stem cell
Apical–basal cell polarity
Cytokeratin (intermediate filament) expression
Epithelial gene expression programme
Downregulation/loss of occludin (integral membrane protein), claudin and TJPZO1 (tight-junction protein zona occludin 1)
Acquisition of Mesenchymal Cell Phenotype: Stem Cell-like during EMT
Downregulation/loss of E-cadherin (epithelial adherens junction protein) results in loss of cell-cell interaction and repression of polarity complex proteins
Fibroblast-like shape of CSC
Front-to-back cell polarity
Cytoskeletal changes and motility ▪ Reorganization of actin cytoskeleton to enable cell elongation and directional motility ▪ Formation of actin rich membrane projections: invadopodia—proteolytic, lamellipodia—sheet-like and filopodia—spike-like ▪ Actin stress fiber formation results in increased cell contraction ▪ Actin dynamics are regulated by RHO GTPases: RHO A: actin stress fibers formation, and RAC1 and CDC42—lamellipodia and filopodia ▪ Cytoskeleton changes induce cell shape changes and front–rear polarity essential for migration
Invasiveness and migratory properties (self-renewal, invasion, metastasis, anoikis resistance, evasion of the immune system, resistance to chemotherapy and radiation)
Mesenchymal gene expression programme
N-cadherin (mesenchymal adherens junction protein) expression
Vimentin (intermediate filament) expression
Fibronectin secretion
Protease secretion (MMP2, MMP3, MMP9)
PDGF receptor expression
$\alpha 6\beta 4$ Integrin expression

Transcription factors involved in induction of EMT include SNAIL-1/SNAIL-2, TWIST1, TWIST2, ZEB1, ZEB2, and SMAD2/SMAD3.

Table 6.99 Epithelial–mesenchymal transition (EMT)-associated biomarkers in clinical tissue samples to predict prognosis of cancer patients

EMT-associated Gene	Characteristics	Human Cancers
Downregulation/loss of epithelial biomarkers		
E-cadherin (epithelial cadherin)	Cell-to-cell adhesion glycoprotein	▪ Breast carcinoma ▪ Gastric carcinoma ▪ Colorectal carcinoma
Claudin-1	Cell-to-cell tight junctions restrict lateral diffusion of lipids and membrane proteins	▪ Lung carcinoma ▪ Renal cell carcinoma ▪ Ovarian carcinoma ▪ Colorectal carcinoma ▪ Esophageal carcinoma
Occludins	Cell-to-cell tight junctions	Metastatic breast carcinoma
Desmoplakin	Component of desmosomes present in cytoplasm	Breast carcinoma
ZO-1	Peripheral phosphoprotein	Breast carcinoma

Contd...

Table 6.99 Epithelial–mesenchymal transition (EMT)-associated biomarkers in clinical tissue samples to predict prognosis of cancer patients (*Contd...*)

EMT-associated Gene	Characteristics	Human Cancers
Syndecan-1	Transmembrane proteoglycans	Metastatic breast carcinoma
Nidogen-1 (NID1)	Nidogen-1 (NID1) binds to laminin, collagen IV, fibrinogen, fibronectin; and induces EMT, invasion, metastasis and chemoresistance	Ovarian carcinoma
Upregulation/activators/acquisition of mesenchymal biomarkers		
Vimentin	Intermediate filament represents a class of cytoskeletal elements	▪ Breast carcinoma ▪ Gastric carcinoma ▪ Lung carcinoma
N-cadherin (neutral cadherin)	Cell-to-cell adhesion glycoprotein	▪ Esophageal carcinoma ▪ Lung carcinoma ▪ Urothelial carcinoma
OB-cadherin (osteoblast cadherin)	Calcium-dependent cell adhesion molecule	Prostatic carcinoma
Fibronectin	High molecular weight extracellular matrix glycoprotein	▪ Urinary bladder carcinoma ▪ Colorectal carcinoma ▪ Ovarian carcinoma ▪ Metastatic breast carcinoma
Vimentin	Intermediate filament	Metastatic breast carcinoma
Vitronectin	Glycoprotein	Metastatic ovarian carcinoma
Syndecan-1	Transmembrane heparan sulphate proteoglycans	▪ Metastatic triple negative breast carcinoma ▪ Metastatic endometrial carcinoma
$\beta1\beta3$ Integrin	Cell adhesion molecule	▪ Breast carcinoma ▪ Prostatic carcinoma ▪ Pancreatic carcinoma
$\alpha2\beta2$ Integrin	Cell adhesion molecule	Breast carcinoma
Neuropilin-2	Transmembrane non-receptor tyrosine kinase glycoprotein	▪ Breast carcinoma ▪ Prostatic carcinoma
Transcription factors		
SNAIL-1	CH2H2 type zinc finger transcription factor	▪ Breast carcinoma ▪ Ovarian carcinoma ▪ Skin cancer ▪ Hepatocellular carcinoma ▪ Head and neck cancer
SNAIL-2	CH2H2 type zinc finger transcription factor	▪ Breast carcinoma ▪ Melanoma
SLUG	Zinc finger transcription factor	▪ Lung carcinoma ▪ Colorectal carcinoma ▪ Esophageal carcinoma
TWIST-1	Basic helix-loop-helix (bHLH) transcription factor	▪ Cervical carcinoma ▪ Ovarian carcinoma ▪ Breast carcinoma ▪ High-grade melanoma ▪ Neuroblastoma
TWIST-2	Basic helix-loop-helix (bHLH) transcription factor	Metastatic breast carcinoma

Contd...

Table 6.99 Epithelial–mesenchymal transition (EMT)-associated biomarkers in clinical tissue samples to predict prognosis of cancer patients (*Contd…*)

EMT-associated Gene	Characteristics	Human Cancers
ZEB1 (δ-EF1)	Basic helix-loop-helix (bHLH) transcription factor	▪ Pancreatic carcinoma ▪ Lung carcinoma ▪ Hepatocellular carcinoma ▪ Colon carcinoma ▪ Breast carcinoma
ZEB2 (SIP-1)	Basic helix-loop-helix transcription factor	▪ Ovarian carcinoma ▪ Breast carcinoma ▪ Hepatocellular carcinoma
E12/E-47 (associated with E-cadherin promoter)	Basic helix-loop-helix (bHLH) functions as a subunit of heterodimeric	Gastric carcinoma
ETS (electron transport system)	Helix-turn-helix DNA binding transcription factor	Prostatic carcinoma
LEF1 (lymphoid enhancer binding factor 1)	Transcription factor	▪ Lung adenocarcinoma ▪ Colon carcinoma ▪ Endometrial carcinoma ▪ Prostatic carcinoma ▪ Leukemia
Homeobox protein goosecoid (GSC)	Paired homeodomain	▪ Ovarian carcinoma ▪ Lung carcinoma ▪ Breast carcinoma
FSP1 (fibroblast specific protein 1)	Ferroptosis suppressor protein	Non-small cell lung carcinoma

Pathology Pearls: Epithelial–Mesenchymal Transition and Mesenchymal–Epithelial Transition in Malignant Epithelial Tumor Progression

- Carcinoma arises from epithelial tissues and begins when genetic mutations transform a normal cell into CSC.
  - Loss of E-cadherin, the principal adhesion molecule of epithelial cells is important in the development of carcinoma.
  - Genetic mutations and tumor microenvironment initiate the cancer metastasis via epithelial–mesenchymal (EMT) process.
  - EMT might not be essential for metastasis of lung and pancreatic cancers.
- Epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) are involved in invasion and metastasis of malignant tumor to distant tissue/organ sites.
- Cancer stem cells (CSCs) undergoing EMT detach from primary malignant epithelial tumor, penetrate through the fragmented basement membrane, and degrade extracellular matrix (ECM) and then subsequently intravasate into the blood vessels and lymphatic channels and circulate in the bloodstream as the form of single and clusters.
- Only a small number of CSCs survive and extravasate through basement membrane and surrounding tissues. After CSCs are disseminated, they must reactivate their epithelial properties by means of the reversion of the process of EMT, known as MET. One of the keys to the process of MET is the re-expression of E-cadherin, which enables CSCs to interact the tissue of the recently colonized tissues/organs.
- However, extravasated CSCs may remain dormant over a long period before they activate MET process and proliferate to form micrometastasis and then subsequent formation of macrometastases in distant tissues/organs and become therapy resistance.
- Extravasated CSCs synthesize chemokines and other proteins, which attract immune cells and stimulate angiogenesis, thus promoting the development of highly vascularized and inflammatory niche.
- Cancer-associated fibroblasts (CAFs) drive direct CSCs migration through fibronectin alignment. In addition, hypoxia, metabolic suppressors, and extracellular matrix (ECM) stiffens trigger the EMT programme in CSCs.
- Transcriptional repress epithelial genes and activate mesenchymal genes. Epigenetic and post-transcriptional modulators also play important role in regulation of EMT process.

Molecular Mechanisms of Epithelial–Mesenchymal Transition (EMT) in Cancer Progression

Multiple complexes signaling systems are required for the induction of EMT because epithelial CSCs undergoing EMT must undergo both morphologic and functional changes. Recent studies of the crosstalk among the intracellular signal networks (i.e. receptor tyrosine kinases, Wnt/β-catenin, NOTCH, Hedgehog signaling pathway, and bone morphogenic proteins) and molecules such as E-cadherin inducer of EMT,

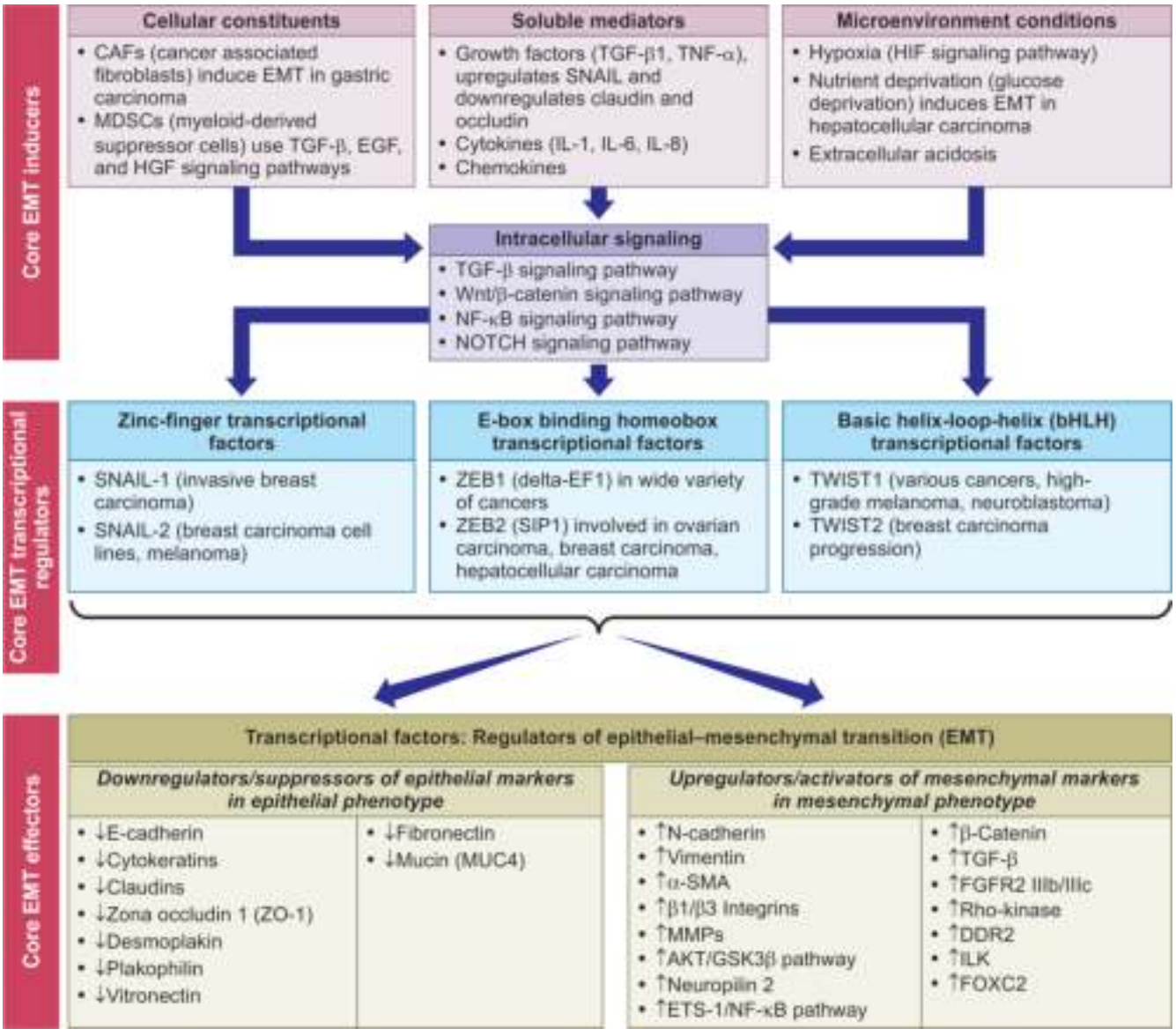


Fig. 6.121: Regulators and biomarkers of EMT in cancer. During the process of EMT, cells undergo changes and molecular alterations representing mesenchymal differentiation. This leads to cancer cells losing epithelial markers and gaining mesenchymal markers. Regulators and biomarkers of EMT include core inducers, transcriptional regulators, and effectors.

transforming growth factor-β (TGF-β) signaling pathway and microRNAs could help to understand the mechanism of regulating EMT. Regulators and biomarkers of EMT in cancer are shown in Fig. 6.121.

E-Cadherin Transmembrane Glycoprotein-associated EMT

The CDH1 tumor suppressor gene provides instructions for making a protein called E-cadherin (i.e. epithelial cadherin) that maintains epithelial tissue structure and its integrity.

- E-cadherin is calcium-dependent transmembrane adherens glycoprotein associated with desmosomes and penetrate the cell membrane, which facilitates normal cell-cell adhesion of most epithelial cells and desmosomes and maintains tissue homeostasis.
- Altered E-cadherin expression occurs due to somatic nonsense or frameshift mutations, hypermethylation of histones or transcriptional repression (SNAIL, SLUG, TWIST), and phosphorylation and degradation.
- Methylation of the E-cadherin promoter region plays a vital role in tumorigenesis (i.e. gastric carcinoma and breast lobular carcinoma), angiogenesis, EMT, invasion, and metastasis in distant tissues/organs. Besides genetic and epigenetic regulation, E-cadherin is regulated by various signaling networks and transcription factors.
- Tumor suppressor gene E-cadherin and its role in normal and cancer stem cells are shown in Fig. 6.122.

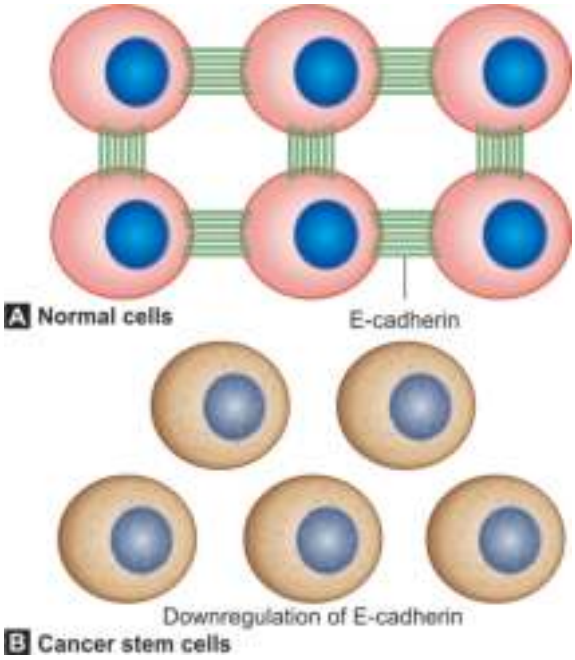


Fig. 6.122: Tumor suppressor gene E-cadherin and its role in normal and cancer stem cells. E-cadherin is a transmembrane glycoprotein, which mediates calcium-dependent cell-cell adhesion, regulates cell growth and differentiation, and maintains tissue homeostasis. Altered E-cadherin expression plays a vital role in tumorigenesis, angiogenesis, EMT invasion, and metastasis.

Transforming Growth Factor-β Signaling-associated Epithelial–Mesenchymal Transition

Transforming growth factor-β (TGF-β) produced by cancer associated fibroblasts (CAFs), and hypoxia-inducible factor in the tumor microenvironment activate EMT in epithelial CSCs, which maintain mesenchymal phenotype through both paracrine and autocrine signaling.

- Canonical TGF-β signaling proceeds with phosphorylation of SMAD2 and SMAD3, which then bind with SMAD4 to enter the nucleus to modulate transcription in cooperation with other transcription factors, coactivators, and corepressors. In addition, TGF-β ligands bind to its cognate receptors leading to activation of many noncanonical signaling pathways.
- TGF-β cytokine plays dichotomous role, i.e. tumor suppressor in normal cells or premalignant cells in initial stage, or tumor promoter in the late-stage by induction of EMT through multiple signaling pathways, including phosphorylation of SMAD2 and SMAD3, invasion, tumor angiogenesis, protection against apoptosis, immune suppression, and metastasis.
- Mutations in TGF-β and SMAD4 are linked to pancreatic carcinoma and colorectal carcinoma. TGF-β activates other EMT-related Wnt/β-catenin, NOTCH, and integrin signal pathways, also trigger EMT programmes.

Transcription Factors Repress E-Cadherin-associated Epithelial–Mesenchymal Transition

Epithelial–mesenchymal transition (EMT) is driven by master transcription factors such as E-box zinc finger proteins (ZEB1, ZEB2), basic helix-loop-helix (bHLH) proteins (TWIST1/TWIST2) and the SNAIL family of zinc finger proteins (SNAIL1, SNAIL2/SLUG), SMADs (SMAD2, SMAD3), SLUG, NF-κB, Gli, E47 via connected TGF-β, Wnt/β-catenin, PI3K (phosphatidylinositol 3-kinase)/AKT/mTOR signaling pathways, that results in the repression of E-cadherin, acquisition of mesenchymal phenotypic traits and activation of extracellular matrix (ECM) degradative enzymes. SNAIL1 and ZEB2 transcription factors would initiate downregulation of E-cadherin. SNAIL2/SLUG and ZEB1 transcription factors would maintain repression of E-cadherin. In the induction of EMT, the activated **SMADs** mediate regulation of transcription through three families of transcription factors leading to repression of epithelial marker gene expression and activation of mesenchymal gene expression.

- **SNAIL family of zinc finger transcription factor:** SNAIL transcription factor blocks the cell cycle and confers resistance to apoptosis. SNAIL causes metabolic reprogramming, confers CSCs with stem cell-like traits resistant to immunosuppression, induces EMT, promotes invasion metastasis, and recurrence.
  - SNAIL-1 overexpression is linked to progression of breast carcinoma, cervical carcinoma, and ovarian carcinoma.
  - SNAIL-2 overexpression is linked to progression of breast carcinoma, and ovarian carcinoma.
- **E-box zinc finger binding transcription factor:** ZEB1 transcription factor represses E-cadherin promoter and induces EMT process by recruiting SMARCA4/BRG1. ZEB2 is involved in chemical signaling pathways that regulate early growth and development of colon carcinoma, breast carcinoma and ovarian carcinoma.
- **Basic helix-loop-helix (bHLH) transcription factor:** TWIST1 transcription factor exerts its multiple biological effects (e.g. angiogenesis, chemoresistance, metastasis, senescence, and stemness) via various downstream signaling pathways, acting as a transcription factor regulating the expression of an array of target genes such as PDGFRA, YB1, MDR1, AKT2, N-cadherin, E-cadherin, ARF, P53 and CD24 in the nucleus or modulating the function of effectors (e.g. Jagged-1, VEGF, mTOR, BCL-2, p53, and Bmi-1) at the protein level in the cytoplasm. Overexpression of TWIST1 induces EMT, a key process in metastasis of breast carcinoma and gastric carcinoma.

- **SLUG, FOXC1/FOXC2, NF-κB and E12/E47 transcription factors:** SLUG transcription factor downregulates E-cadherin during EMT process. FOXC1 partially promotes tumor metastasis by EMT process to support microvascular invasion, thereby increasing angiogenesis. FOXC2 overexpression is linked to breast carcinoma. E12/E-47 basic helix-loop-helix transcription factor represses E-cadherin and induces EMT leading to development of gastric carcinoma.

Regulation of Epithelial–Mesenchymal Transition by Small Non-coding MicroRNAs

Small noncoding microRNAs regulate target gene expression at the post-translation level involved in a wide array of biological processes including carcinogenesis with metastatic potential.

- MicroRNAs are most often dysregulated in human cancers. MicroRNA-10b is overexpressed by EMT transcription factor by TWIST in metastatic breast carcinoma.
- MicroRNA-200 family (miR-200a, miR-200b, miR-200c, miR-141, miR-29) and miR-205 play key roles in regulating of EMT and targeting E-cadherin repressors ZEB1 and ZEB2. MicroRNA-200 family can induce EMT process.

Tumor Microenvironment and Epithelial–Mesenchymal Transition

Tumor microenvironment (TME) is composed of extracellular matrix (ECM), cancer-associated fibroblasts (CAFs), myofibroblasts, immune cells, and soluble factors required for invasion and metastasis. Interaction among CSCs in the tumor microenvironment can induce EMT by autocrine and/or paracrine synthesis of cytokines, growth factors, and ECM proteins.

Cancer Drug Resistance induced by Epithelial–Mesenchymal Transition

Epithelial–mesenchymal transition (EMT) plays a role in development of CSCs properties of invasiveness, metastasis, recurrence and chemoresistance. Cancer drug resistance is still a major persistent challenge for cancer patient management in clinical oncology.

- Cancer stem cells undergoing EMT have invasive and migratory capabilities, and develop resistance to anti-cancer agents. Lung CSCs undergoing EMT express vimentin and/or fibronectin become insensitive to EGFR inhibitors.
- There are several mechanisms of cancer drug resistance induced by EMT, i.e. inactivation of the anti-cancer drug, multidrug resistance, changes in drug targets, alteration in drug metabolism, epigenetic alterations, cell death inhibition (apoptosis evasion), gene amplification, and enhance DNA repair system cause resistance to chemotherapy. Hence, it is important to overcome therapy resistance by using new targeted-therapy strategies.
- The novel cancer treatments by analyzing molecular targets of oncogenes, tumor suppressor genes and RNA interference (RNAi) are expanded, which include: inhibition of receptor tyrosine kinase involved in unrestricted CSC proliferation, enhancement of immune system response in cancer, promotion of drug delivery to CSCs, alteration of drug metabolism and reduction of side effects of anticancer drugs.

Clinical Significance of Epithelial–Mesenchymal Transition

Most EMT-associated markers have been identified in histologic specimens. Clinical significance of the EMT process has been implicated in carcinogenesis, invasion, metastasis, recurrence and chemoresistance.

TUMOR MICROENVIRONMENT

Tumor microenvironment (TME) consists of CSCs, nonmalignant stromal cells such as cancer-associated fibroblasts (CAFs), tumor-associated adipocytes, vascular endothelial cell and extracellular matrix (ECM), activating immune cells such as CD8+ cytotoxic T cells, natural killer cells (NK cells) and dendritic cells (DCs) and immunosuppressive immune cells such as CD4+ regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), tumor associated neutrophils (TANs) and tumor-associated macrophages (TAM-1, TAM-2), signaling molecules and ECM. Malignant epithelial tumor and the surrounding microenvironment are closely related and interact constantly.

- CSCs recruit immune cells and stromal cells from neighboring tissue. CSCs interact with surrounding stromal cells via production of soluble and other mediators, through lymphatic and blood vessels to influence the tumor growth and progression.
  - Stromal cells in the tumor microenvironment play critical roles in all stages of carcinogenesis by stimulating and facilitating uncontrolled cancer cell proliferation.
  - Important inflammatory mechanisms that are corrupted by the malignant epithelial tumor include NF-κB transcription factor, immune checkpoints signaling, and inflammasome signaling.
- Fibroblasts are recruited by CSCs and activated in the local microenvironment via TGF-β, VEGF, PDGF, FGF, EGF, HGF and other signaling molecules.

- Cancer-associated fibroblasts (CAFs) synthesize TGF-β, which provide a positive feedback mechanism encouraging fibroblast activation, as well as guiding the tumor microenvironment (TME).
- CAFs also synthesize paracrine signaling molecules including IL-1, IL-8, NF-κB, IFN-γ, CTGF, CCL5, HGH and PGE_2, as well as extracellular matrix (ECM) molecules such as collagen fibers, MMPs, tenascin C, and periostin.
- Growth factors and cytokines secretion in tissue microenvironment are given in Fig. 6.123.

▪ Immune cells of myeloid and lymphoid lineage such as neutrophils, lymphocytes, macrophages, and natural killer cells respond to the malignant tumor. Both CSCs, via VEGF synthesis and CAFs, are involved in stimulating tumor angiogenesis. Newly formed blood vessels are leaky and dysfunctional, which can limit immune cells to access to the tumor. CAFs regulate the immune response in tumor microenvironment.

▪ Cancer stem cells, under the influence of CAFs signaling, can undergo EMT with downregulation of E-cadherin expression and upregulation of β-catenin and TWIST, and escape through basement membrane, extracellular matrix (ECM), and access to vasculature for dissemination to distant tissues/organs. CSCs can also metastasize to lymph nodes via lymphatic route.

▪ Immunosuppressive tumor microenvironment occurs through several mechanisms: (a) immune

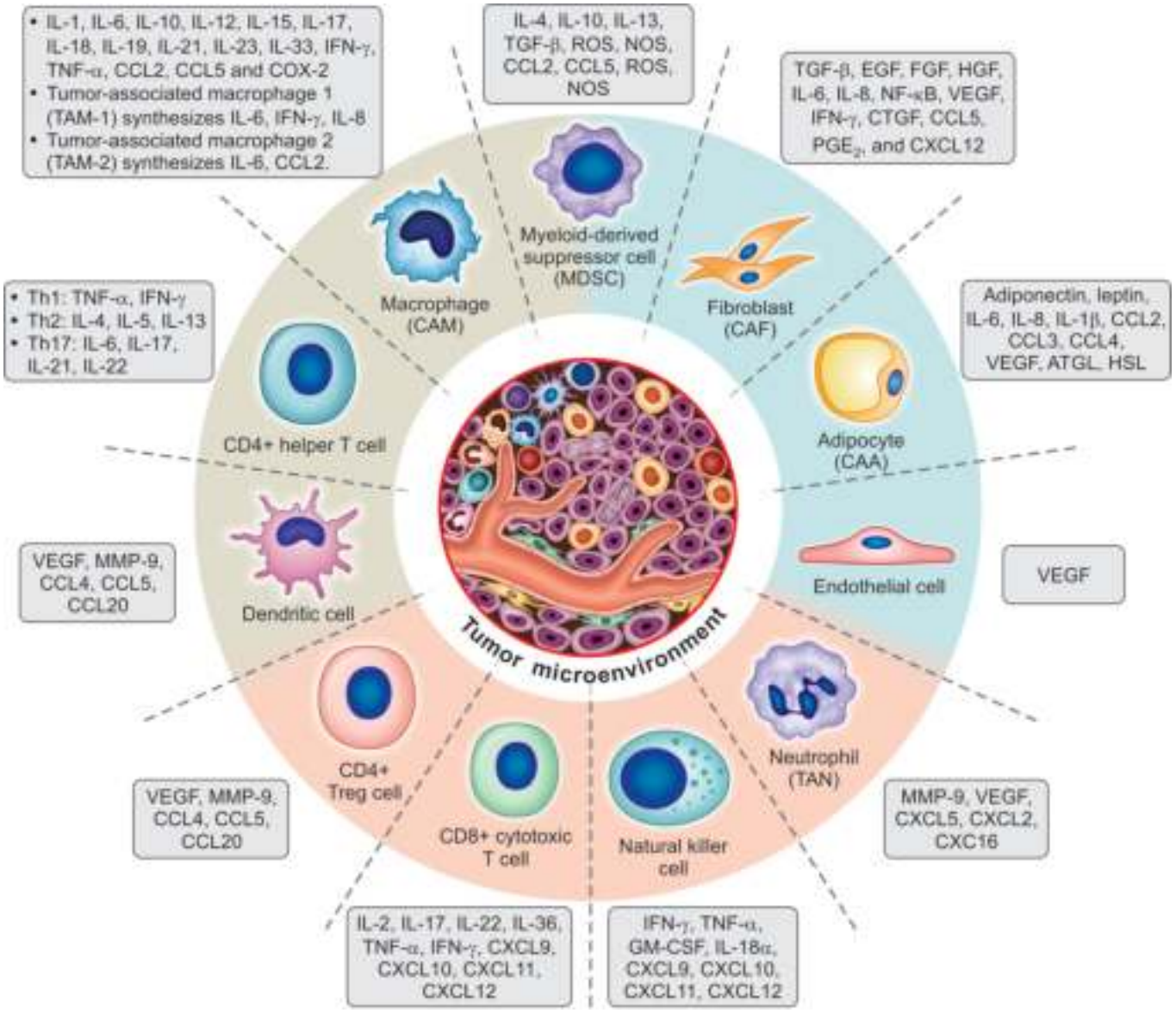


Fig. 6.123: Growth factors and cytokines secretion in tumor microenvironment. The tumor microenvironment is composed of CSCs and surrounding cells, which secrete growth factors and cytokines involved in tumor growth and survival.

checkpoint (PD-1/PD-L1, CTLA-4/B7 and LAG-3, TIM-3), (b) immunosuppressive cells (M2-polarised TAM, CD4+ regulatory T cells and MDSCs), and (c) immunosuppressive tumor microenvironment due to dense extracellular matrix (ECM).

- Molecules expression in tumor microenvironment are given in Table 6.100. Activating immune cells and immunosuppressive cells in tumor microenvironment are given in Table 6.101. Key chemokines involved in recruitment of activating immune cells and immunosuppressive cells into solid malignant epithelial tumors are given in Table 6.102.

Pathology Pearls: Immune Checkpoint Molecules in Cancers

- Immune checkpoint molecules are inhibitory regulators of the immune system, that are crucial in maintaining self-tolerance, preventing autoimmunity, and regulating the duration and extent of immune responses in order to minimize collateral tissue damage.
- Immune checkpoint molecules are expressed on cancer stem cells (CSCs), which play important roles in the maintenance of self-renewal, angiogenesis, EMT, metastasis, chemoresistance, and altered energy metabolism. Immune checkpoint mechanisms are often activated to suppress nascent immune system in cancers.
- Four stimulatory checkpoint molecules belong to tumor necrosis factor receptor superfamily, which include CD27, CD40, OX40, GITR and CD137.
- In addition, two stimulatory immune checkpoint molecules belong to B7-CD28 superfamily, which include CD28 and 40-inducible co-stimulator (ICOS).
- Programmed cell death protein 1 (**PD-1**) receptor and its programmed cell death ligand 1 (**PD-L1**) are checkpoint proteins found on the surface of T cells.

Recruitment of Activating Immune Cells in Tumor Microenvironment

Tumor microenvironment (TME) contains various immune cells: CD8+ cytotoxic T cells, natural killer cells (NK cells), dendritic cells (DCs), B cells, cancer-associated macrophages (CAMs), tumor-associated neutrophils (TANs) and delta-gamma T cells (δ-γ T cells). It is worth mentioning that tumor associated neutrophils and delta-gamma T cells (δ-γ T cells) can have both pro-tumor and anti-tumor activity.

- Activating immune cells secrete cytokines in the tumor microenvironment. Delta-gamma T cells recognize CSCs and release perforin and granzyme leading to CSC destruction.
- Signaling molecules within tumor microenvironment (TME) operate to hijack immune cells to evade CSCs destruction leading to promote CSCs survival.

Table 6.100 Molecules expression in tumor microenvironment

Upregulation of Molecules in Tumor Microenvironment
↑↑**Adenosine**
↑↑**VEGF** (angiogenesis, CSCs proliferation and stromal invasion)
↑↑**TGF-β** (angiogenesis, chemoresistance, EMT, CSCs proliferation)
↑↑**IL-4**
↑EGF (CSCs proliferation and stromal invasion, EMT)
↑FGF (angiogenesis, CSCs proliferation, EMT, chemoresistance)
↑HGF (angiogenesis, CSCs proliferation and stromal invasion, chemoresistance)
↑Hypoxia/acidosis
↑Lactate
↑IL-10
↑IL-1β
↑CXCL10
↑Integrins
Downregulation of Molecules in Tumor Microenvironment
↓IFN-γ
↓TNF-α
↓IL-12
↓Glucose
↓Amino acids
↓Oxygen

- Immunosuppressive molecules within tumor microenvironment and metabolic competition for nutrients have been shown to induce the exhaustion and inactivation of CD8+ cytotoxic T cell infiltrates, thereby limiting the durability of immune checkpoint inhibition leading to survival of CSCs.

T Cells in Tumor Microenvironment

Immune T cells are abundant in most human cancers, which constitute ≤10% of cells of tumor microenvironment within and surrounding the malignant tumor. Phenotypes of pro-tumor or anti-tumor T cells can vary with malignant tumor type and stage. CD8+ cytotoxic T cells, CD4+ helper T cells (Th1) and γ-δ T cells are usually associated with a good prognosis. FOXP3+ T cells, CD4+ helper T cells (Th2 and Th17 cells) are usually associated with poor prognosis.

B Cells in Tumor Microenvironment

Immune B cells are sometimes found at the invasive margin of some solid malignant tumors, but more often in secondary and tertiary structures adjacent to the tumor microenvironment. B cell infiltration in some solid malignant tumors is associated with good prognosis.

Table 6.101 Activating immune cells and immunosuppressive cells in tumor microenvironment

Immune Cells	Upregulation of Molecules
Activating immune cells	
CD4+ helper T cells	▪ Th1 (TNF-α, IFN-γ) ▪ Th2 (IL-4, IL-5, IL-13) ▪ Th17 (IL-6, IL-17, IL-21, IL-22)
CD8+ cytotoxic T cells	IL-2, IL-17, IL-22, IL-36, TNF-α, IFN-γ, CXCL9, CXCL10, CXCL11, CXCL12
Natural killer cells (NK cells)	IFN-γ, TNF-α, GM-CSF, IL-18α, CXCL9, CXCL10, CXCL11, CXCL12
Dendritic cells (DCs)	VEGF, MMP-9, CCL4, CCL5, CCL20
Immunosuppressive immune cells	
CD4+ regulatory T cells (Treg cells)	TGF-β, IL-10, IL-35, IL-37, CCL17, CCL22
Myeloid-derived suppressor cells (MDSCs)	IL-4, IL-10, IL-13, TGF-β, ROS, NOS, CCL2, CCL5
Tumor-associated neutrophils (TANs)	MMP-9, VEGF, CXCL5, CXCL2, CXC16
Tumor-associated macrophages (TAM-1, TAM-2)	▪ IL-1β, IL-6, IL-10, IL-12, IL-15, IL-17, IL-18, IL-19, IL-21, IL-23, IL-33, IFN-γ, TNF-α, CCL2, CCL5 and COX2 ▪ Tumor-associated macrophage 1 (TAM-1) synthesizes IL-6, IFN-γ, IL-8 ▪ Tumor-associated macrophage 2 (TAM-2) synthesizes IL-6, CCL2
Other components in tumor microenvironment	
Cancer-associated fibroblasts (CAFs)	TGF-β, EGF, FGF, HGF, IL-6, IL-8, NF-κB, VEGF, IFN-γ, CTGF, CCL5, and PGE_2, and CXCL12
Tumor-associated adipocytes	Adiponectin, leptin, IL-6, IL-8, IL-1β, CCL2, CCL3, CCL4, VEGF, ATGL, HSL (lipolysis and decreased adipose tissue mass)
Extracellular matrix (ECM)	Matrix metalloproteinases (MMPs)
Vascular endothelial cell	Vascular endothelial growth factor (VEGF)

Immunosurveillance evasion, tumor growth, angiogenesis, invasion, and metastasis.

Table 6.102 Key chemokines involved in recruitment of activating immune cells and immunosuppressive cells into solid malignant epithelial tumors

Cells	Cell Surface Receptors	Chemokines Secretion
Activating immune cells		
CD8+ cytotoxic T cell	CCR3	CXCL12
	CCR4	CXCL9, CXCL10, CXCL11
Natural killer cell (NK cell)	CCR3	CXCL12
	CCR4	CXCL9, CXCL10, CXCL11
Dendritic cell (DCs)	CCR5	CCL4, CCL5, CCL20
	CCR6	CCL20
Immunosuppressive cells		
CD4+ regulatory T cells (Tregs)	CCR4	CCL17, CCL22
Myeloid-derived suppressor cells (MDSCs)	CCR2	CCL2
	CCR5	CCL5
Cancer-associated macrophages (CAMs)	CCR2	CCL2
	CCR5	CCL5
Tumor-associated neutrophils (TANs)	CCR1	CXCL2, CXCL5, CXCL6
	CCR2	CXCL2, CXCL5, CXCL6

Natural Killer Cells in Tumor Microenvironment

Natural killer cells (NK cells) are innate cytotoxic, large granular lymphocytes found outside region of malignant tumor. Unlike lymphocytes, natural killer cells lack antigen-specific receptors. Presence of natural killer cells in tumor microenvironment is associated with good prognosis in some human cancers.

Dendritic Cells in Tumor Microenvironment

Dendritic cells (DCs) are professional antigen-presenting cells (APCs), uniquely able to induce naïve T cells activation, and effector differentiation. Dendritic cells are involved in linking of innate and adaptive immune systems leading to induction of protective immune responses. Dendritic cells stimulate immune response against tumor-associated antigens in the tumor microenvironment.

Recruitment of Immunosuppressive Cells in Tumor Microenvironment

Tumor microenvironment contains many immunosuppressive cells such as cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSCs), tumor-associated neutrophils (TANs: N1 and N2), which evade CSCs destruction by immune system.

- Signaling within the tumor microenvironment (TME) operates to hijack immune cells to promote CSCs survival. Immune cells within the TME secrete various cytokines, that allow growth and metastasis, rather than recognizing and destroying the cancer stem cells (CSCs).
- Important inflammatory mechanisms, that are corrupted by the tumor microenvironment include: NF-κB transcription factor, immune checkpoints signaling, and inflammasome signaling. The inflammasome promotes the cleavage of capase 1 and subsequent cleavage of proinflammatory cytokines IL-1β and IL-18.

Myeloid-derived Suppressor Cells (MDSCs) in Tumor Microenvironment

Myeloid-derived suppressor cells (MDSCs) are heterogenous population of immature myeloid progenitor cells, which fail to differentiate into granulocytes, macrophages, and dendritic cells.

- Immunosuppression is the main functional feature of MDSCs, which inhibit T cell activity in the tumor microenvironment (TME) and promote tumoral immune escape. In the context of malignant epithelial tumors, MDSCs are abnormally produced and recruited to assist in the establishment of an immunosuppressive TME that facilitates CSCs escape. Additionally, MDSCs contribute to non-immunologic aspects of tumor biology, including angiogenesis, invasion, metastasis, and chemoresistance.
- MDSCs are capable to deplete amino acids required for T cell activation and proliferation. A high level of arginase 1 (**ARG1**) expression by MDSCs depletes L-arginine leading to downregulation of the CD3 ζ-chain of the TCR complex, and proliferative arrest of T cells.
- MDSCs synthesize excess IL-10 and TGF-β, which stimulate CD4+ regulatory T cells (Treg) cells. Presence of CD4+ regulatory T cells (Treg cells) in tumor microenvironment (TME) suppresses the immune system by inhibiting functions of CD8+ cytotoxic T cell, and natural killer cells (NK cells), thus paving the way for oncogenesis. MDSCs inhibit CD8+ cytotoxic T cells, and polarize tumor associated macrophages (TAMs) to be a tumor promoting phenotype.

Tumor-associated Neutrophils in Tumor Microenvironment

Neutrophils originating from bone marrow are recruited in the tumor microenvironment by cytokines and chemokines. Tumor associated neutrophils (TANs) are categorized into N1-TANs (anti-tumor activity) and N2-TANs (immunosuppression and tumor progression).

- In cancer patients, either a high number of TANs and neutrophil-to-lymphocyte ratio (NLR) are associated with poor prognosis. TANs count and NLR can be regarded as biomarkers.
  - TANs are exposed to hypoxia as well as stroma cell signals, which can trigger the formation of neutrophil extracellular traps (NETs).
  - NET-associated proteases alter the extracellular matrix (ECM) and NET-derived HMGB1 molecules activate CSCs to jointly promote angiogenesis, unrestricted cancer stem cell proliferation, migration, invasion, and metastasis to distant tissues/organs.
- Owing to the pivotal role of TANs in stimulating angiogenesis, and tumor progression; therapeutic strategies to target TANs have been proposed: (a) targeting the CXCL8/CXCR-1/CXCR-2 axis, which block TANs, and (b) targeting substances synthesized by polymorphonuclear cells, which promote tumor growth. Clinical trials are restrained due to risk of induction of immunosuppression. Ongoing clinical trials aimed to inhibit neutrophil recruitment into the tumor microenvironment are also accurately debated.

Tumor-associated Macrophages in Tumor Microenvironment

Tumor-associated macrophages (TAMs—M1 and M2) are derived from bone marrow stromal cells, that promote tumor development, angiogenesis via vascular endothelial growth factor (VEGF), fibrous stroma deposition, invasion, and metastasis to distant organ(s). TAMs synthesize TGF-β that negatively regulates effector T cells function, and induces CD4+ regulatory T (Treg) cells differentiation, and maintenance.

Defective Terminally-differentiated Myeloid Dendritic Cells in Tumor Microenvironment

Terminally-differentiated myeloid dendritic cells might be defective in the tumor microenvironment, which cannot adequately stimulate an immune response to tumor-associated antigens.

Recruitment of Nonmalignant Stromal Cells in Tumor Microenvironment

Cancer stem cells recruit nonmalignant stromal cells from neighboring tissue in tumor microenvironment. Stromal cells and CSCs are in dynamic relationship promoting the malignant tumor progression. As CSCs enter different locations, they encounter distinct stromal microenvironments in the primary tumor site, locally invasive tumor phenotype, and disseminated tumor phenotype in distant tissues/organs.

- Tumor microenvironment contains another stromal cell component, which is composed of cancer-associated fibroblasts (CAFs), cancer-associated adipocytes (CAAs), and cancer-associated angiogenic vascular and lymphatic endothelial cells. There is downregulation of perivascular stromal cells (i.e. pericytes).
- Tumor microenvironment orchestrates angiogenesis, unrestricted cell cancer stem proliferation, clonal expansion, invasion, and metastasis through the secretion of growth factors, and cytokines, resistance to radiation and chemotherapy and recurrence.

Cancer-associated Fibroblasts in Tumor Microenvironment

Cancer-associated fibroblasts (CAFs) are found in many human cancers especially at the margins. CAFs are recruited by CSCs within tumor microenvironment.

- CAFs secrete tumor-promoting growth factors (TGF-β, PDGF, HGF EGF, FGF, HGF, VEGF), chemokines (CCL5, CXCL12), cytokines (IL-6, IL-8), extracellular-matrix (ECM) components and ECM remodeling enzymes.
- CAFs regulate immune response in the tumor microenvironment. Cancer stem cells via vascular endothelial growth factor (VEGF), and CAFs are involved in stimulating angiogenesis within tumor microenvironment.
- CAFs can also have important immunosuppressive activity by secreting 'fibroblast activating protein-α' (**FAP-α**) protein that restricts T cells to the stroma and prevents them from accumulating in the vicinity of CSCs by two mechanisms: (a) production of dense extracellular matrix (ECM) traps T cells in the stroma access to CSCs, and (b) cancer-associated fibroblasts secrete C-X-C motif chemokine 12 (CXCL12), that alters the behavior of cancer cells and helps in progression of tumor.
- CAFs have been demonstrated to enhance malignant tumor phenotype, notably unrestricted CSC proliferation, epithelial mesenchymal transition (EMT), angiogenesis, invasion, and metastasis to distant tissues/organs.

Cancer-associated Adipocytes in Tumor Microenvironment

In some malignant tumors, adipocytes derived from mesenchymal stem cells from bone marrow play pivotal role in recruitment of CSCs through the secretion of adipokines. They also promote malignant epithelial tumor cell growth by providing fatty acids as fuel for CSCs.

Cancer-associated Vascular Endothelial Cells in Tumor Microenvironment

Proangiogenic factors produced by CSCs, myeloid cells or cancer-associated fibroblasts in the tumor microenvironment stimulate sprouting of tumor associated vascular endothelial cells. The new vessels have chaotic branching and uneven vessel lumina. Newly formed blood vessels are also leaky, raising interstitial pressure and uneven blood flow, oxygenation, nutrients, and chemotherapeutic agent delivery in tumor microenvironment.

Cancer-associated Lymphatic Endothelial Cells

Cancer stem cells (CSCs) can invade existing lymphatics or stimulate lymphatic vessel sprouting with the production of factors such as VEGFC or VEGFD. Lymphatic vessels are important in dissemination of CSCs, but might also promote malignant epithelial tumor development by mechanomodulation of the tumor microenvironment, and altering the host immune response to the tumor.

Cancer-associated Perivascular Stromal Cells

Perivascular stromal cells and pericytes provide structural support to blood vessels. Tumor microenvironment demonstrates low pericyte coverage of blood vessels, which correlate with poor prognosis, and increased metastasis.

Extracellular Matrix in Tumor Microenvironment

Extracellular matrix (ECM) provides critical signals to preserve tissue architecture, polarity, and homeostasis, and regulates cell growth and apoptosis.

- The normal tissue ECM comprises a network of biochemically distinct components, including water, minerals glycoproteins, proteoglycans, polysaccharides, and fibrous proteins, such as collagen, elastin and laminin secreted by the resident cells.
- ECM is the major component of the tumor microenvironment, which interacts with the CSCs, immune cells, stromal cells, and cells of myeloid and lymphoid lineage cells. During malignant epithelial tumor progression, alterations in CSCs and ECM interactions drive angiogenesis invasion, metastasis, and treatment resistance.

Versican in Extracellular Matrix

Versican is an extracellular matrix (ECM) proteoglycan present in most tissues. Versican encoded by VCAN gene interacts with cells by binding to non-integrin receptors and other ECM components to influence the ability of CSCs to proliferate, migrate, adhere, and assemble. Versican present in ECM activates EGFR signaling, which leads to CSCs invasion and metastasis.

Chondroitin Sulfate Proteoglycan 4 in Extracellular Matrix

Chondroitin sulfate proteoglycan 4 (CSPG4) present in ECM stabilizes the interactions between cells in the ECM. CSPG4 interacts with the integrin-αβ upon collagen type VI binding to activate phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway in sarcoma cells. CSPG4 also forms complexes with MMP2 and MMP3 on the surface of melanoma cells to facilitate MMP2 activation, and eventual degradation of the ECM.

Lumican in Extracellular Matrix

Lumican present in ECM organizes fibril organization and growth. Lumican plays key role in corneal transparency, epithelial cell migration and tissue repair. Lumican plays a major role in proliferation, migration, and invasion of breast CSCs. Lumican also modifies cellular junction and promotes mesenchymal–epithelial transition (MET) through direct interactions with other ECM molecules or by the modification of membrane receptors and MMP14.

Glypicans in Extracellular Matrix

Glypicans are proteoglycans that play major role in the developmental morphogenesis. Depending on the context, glypicans may have stimulatory or inhibitory activity on Wnt-β, Hedgehog and FGF signalizing pathways.

- Decreased glypican 3 expression is linked to CSCs invasion and metastasis. Complete loss of glypican 3 is associated with overall poor prognosis. However, elevated expression of glypican 3 is linked to reduced CSC differentiation, and lymph node metastasis in lung carcinoma.
- Glypican 5 overexpression promotes tumor invasion and metastasis in salivary adenoid cystic carcinoma and in rhabdomyosarcoma.

Hyaluronic Acid in Extracellular Matrix

Hyaluronic acid is a glycosaminoglycan present in moist skin, connective tissue, and eyes. Hyaluronic acid retains water in the tissues. It is a principal constituent of the tumor microenvironment stroma and CSC surfaces. Metastatic cancers express increased levels of hyaluronic acid, its receptor, and its synthase in the tumor microenvironment, especially in cancers of breast, prostate, ovarian and oral cancers.

- Hyaluronic acid plays major role in EMT driven by the expression of zinc finger E-box-binding homeobox 1 (ZEB1), and its interaction with CD44, which in turn activates hyaluronic acid synthase 2 (HAS2) expression. HAS2 expression regulates TGF-β induced EMT through the expression of fibronectin, SNAIL1 and ZEB1.
- Hyaluronic acid synthase 2 (HAS2) is essential for interaction between CSCs and tumor-associated macrophages (TAMs). This interaction results in enhanced synthesis of PDGF-BB by tumor-associated macrophages (TAMs), which activates stromal cells and rejuvenates CSCs. Inhibiting HAS2 activity via 4-methylumbelliferone limits hyaluronic acid synthesis and prevents tumor metastasis. The striking effect of hyaluronic acid on tumor progression is highly linked with its low-molecular weight, and interactions with other proteins in the ECM.
- Low-molecular weight of hyaluronic acid has well-established tumorigenic properties. In breast carcinoma, decreased hyaluronic acid synthesis significantly inhibits CSCs migration and invasion.
- Moreover, excess hyaluronic acid in the tumor microenvironment facilitates lymphatic metastasis via disruption of intercellular adhesion among lymphatic endothelial cells.
- In addition, excess hyaluronic acid in the interstitial fluid of colorectal carcinoma patients is associated with lymphatic vessel invasion by CSCs and the development of lymph node metastasis. Although, extracellular matrix (ECM) is a complex and dynamic system, that is composed of a wide spectrum of cells and matrikines that participate in invasion and metastasis.

METASTASIS MULTI-STEP PROCESS

Cancer metastasis is the major cause of cancer-related morbidity and mortality. Metastasis is multi-step process in which CSCs from primary malignant epithelial tumor invade basement membrane, and surrounding tissue, intravasate into lymphatics and blood vessels, transport, survive in the circulation, and arrest on vascular endothelium, extravasate, arrest in the capillaries and colonize and subsequent undergo proliferation, angiogenesis, micrometastasis and macrometastasis in favorable secondary sites/organs. Sequence of events of metastatic cascade is given in Table 6.103. (Kindly refer to tumor invasion and metastasis are shown in Figs 6.53 and 6.81 in this chapter).

Malignant Epithelial Tumor Development at Primary Site

Normal cells are transformed to CSCs, which have same genetic abnormalities in key genes coding for aberrant proteins (e.g. growth factors, growth factor receptors, signal transduction proteins, transcription factor and DNA repair system).

- Carcinoma arising from epithelial tissue usually goes through several stages of development: atypical hyperplasia, carcinoma *in situ*, and then invasive carcinoma, which might further metastasize via lymphatic and hematogenous routes to distant tissues/organs.
- Malignant epithelial tumor results from alterations in the expression of multiple somatic oncogene activation and/or inactivation/biallelic loss of tumor suppressor gene involved in cell adhesion, cell survival through growth factors, proangiogenic factors (angiogenesis), and microRNAs.
- Tumor angiogenesis is the process of developing new dysfunctional and leaky blood vessels from pre-existing blood vessels that has a key role in increase malignant epithelial tumor growth, invasiveness, and metastasis.
- Epithelial–mesenchymal transition (EMT) involves the disruption of cell-to-cell adhesion (down-regulation of E-cadherin), apical–basal cell polarity, remodeling of the cytoskeleton and changes in cell-to-extracellular matrix adhesion associated with mesenchymal phenotype, enhanced motility, and invasive properties of CSCs in primary malignant epithelial tumor.
  - Notably, both epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) are required for the process of invasion and metastasis.

Table 6.103 Sequence of events of metastatic cascade

▪ Primary epithelial malignant tumor (carcinoma development and acquisition of invasive potential) • ↑Tumor metastatic signature genes (e.g. EZRIN, SNAIL and TWIST genes) • ↓Metastatic suppressor genes (e.g. NM23, KAI-1, KiSS and NM23) • MicroRNA-10b dysregulation
▪ Expansive malignant epithelial tumor growth, angiogenesis, erosion of tissue boundaries (basement membrane and ECM) and invasion in surrounding tissue • Downregulation of E-cadherin • Loss of cell-cell interaction • Anchorage-independent autonomous cell growth • Protection against apoptosis • Dynamic actin rearrangement • Epithelial–mesenchymal transition (EMT)—loss of apicobasal polarity, stem cell properties, therapeutic resistance • Basement membrane disruption • ECM degradation (MMPs activity, TIMPs loss and integrin downregulation), and migration • Detachment and CSCs (ECM) and access of CSCs to microvasculature
▪ Intravasation of CSCs (MMPs activity), transport through circulation, interaction with platelets, immune cells, and other cells, avoid immune destruction (downregulation/loss of MHC class I antigen, and shedding of ICAM-1 blocks T cell receptor), arrest on endothelium in microvasculature of various organs
▪ Extravasation of CSCs (regulated by MMPs, integrin and laminin receptor), invasion into tissue microenvironment and metastatic dormancy
▪ After extravasation of CSCs, mesenchymal–epithelial transition (MET) is the reverse process of EMT and involved in colonization of CSCs in distant organs
▪ Colonization of CSCs in favorable secondary organs, interaction, and adaptation to tissue microenvironment
▪ Formation of micrometastasis, angiogenesis and macrometastasis in favorable secondary organs

- While EMT mobilizes CSCs in the primary malignant epithelial tumor. MET terminates the migration process and thereby resulting in the distal colonizing of CSCs.

- Cancer stem cells (CSCs) must acquire certain characteristics for cell migration to occur, such as polarity and dissociation from their point of origin. There can be individual or collective CSC invasion.
- Malignant epithelial tumor has clones with different genetic make-up. Some subclones possess ability to metastasize, while other subclones undergo unrestricted proliferation forming poorly-differentiated malignant epithelial tumor.
- The goal of the chemotherapy is to destroy CSCs in the malignant epithelial tumor. CSCs may develop resistance to chemotherapy. Only resistant CSCs survive after chemotherapy. CSCs in recurrent malignant tumor remain resistant to chemotherapy.

Priming of Premetastatic Niche

Primary malignant epithelial tumor primes the tumor microenvironment, termed the premetastatic niche (PMN) before the initiation of metastasis. Development of PMN is a multistep process involving secretory products and membrane bound extracellular vesicles (exosomes), that induce vascular leakage, extracellular matrix (ECM) remodeling and immunosuppression.

- Moreover, exosomes synthesize EMT inducers that stimulate EMT progression in host epithelial cells, providing them the ability to invade and metastasize to distant organs. Exosome-altered ECM along with chemokines, and growth factors results in the formation of a new host microenvironment for CSCs, immune cells and other stromal constituents that is referred to as the priming PMN, where metastatic cancer stem cells may arrest, extravasate and ultimately colonize in secondary organs.
- Microbiomes selectively targets tumors that have rich vascular networks and chemotactic magnetism, which can cause immunosuppression, cancer progression and resistance to treatment.
- In addition to the PMN, exosomes enhance CSCs metastasis to specific target organs, a process known as 'metastatic organotropism'. This metastatic prejudice towards specific organs occurs due to exosomal proteomic avidity for specific CSCs. For example, bone malignant tumor demonstrates different exosomal integrin patterns. Exosomal integrins are linked to metastasis, i.e. α6-β4, α-β1 (lung metastasis) and avβ5 integrin (liver metastasis).
- Possible synapses in CSCs and neural-related factors, such as neurotrophic factors, neuropilins, axonal guidance molecules, neurotransmitters and their receptors make, it possible to establish direct connections between the nervous system, and primary malignant epithelial tumor. Perineural invasion offers another pathway for CSCs dissemination and colonization in distant organs.

Expansile Primary Malignant Epithelial Tumor Growth, Detachment and Migration of Cancer Stem Cells

As the malignant epithelial tumor grows, it exerts mechanical pressure on the surrounding cells and tissues, which eventually undergo cell death due to blockage of blood supply to tumor.

- Loss of mechanical resistance opens the way for the CSCs to spread along the lines of least resistance and occupy the space once filled with dead cells.
- CSCs detach from primary malignant epithelial tumor (carcinoma) due to loss of diverse epithelial proteins such as E-cadherin and α- and β-catenin, claudin, occludin, cytokeratins; diminished number of cell junctions; and other alterations in surface membrane structures and gain of mesenchymal proteins such as vimentin, fibronectin, matrix metalloproteinases, actin and integrins, become motile, undergo a shape change to acquire a polarized phenotype, migrate, and invade through degradation of basement membrane, interstitial tissue, and then extracellular matrix (ECM) by matrix metalloproteinases (MMPs) synthesized by CSCs and stromal cells.

Acquisition of Invasive Potential of Detached Cancer Stem Cells Through Basement Membrane and Extracellular Matrix

Expansile malignant tumor growth and acquisition of invasive potential of CSCs is regulated by metastatic signature genes (e.g. EZRIN, SNAIL and TWIST genes including microRNA-10b) and metastatic tumor suppressor genes (e.g. NM23, KAI-1, KiSS and NM23).

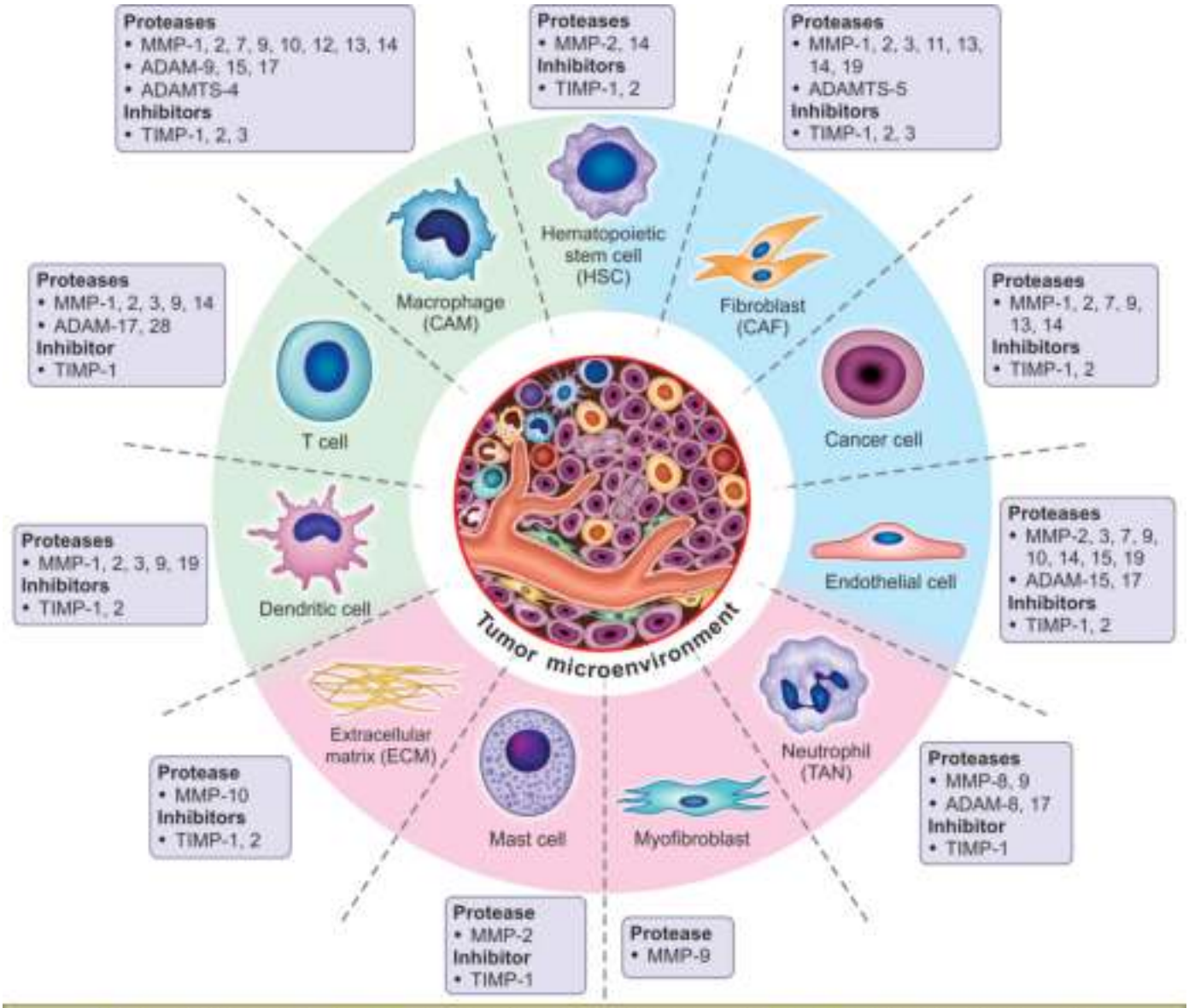
- CSCs possess receptors for laminin, a complex glycoprotein in the basement membrane. Laminin binding to receptors on CSCs permit the CSCs to attach to the basement membrane forming a bridge-like connection.
- Extracellular matrix (ECM) degradation is essential for migration of CSCs detached from primary malignant epithelial tumors. ECM degradation is carried by families of proteases: matrix metalloproteinases (MMP-2 and MMP-9), cathepsin-D and urokinase plasminogen activator. MMP-9 plays most important function in metastatic cascade. MMP-9 induces angiogenesis through VEGF. MMP-9 also acts as gelatinase, that helps in degradation of collagen type IV of epithelial and vascular basement membrane.

- Invading CSCs and surrounding stromal cells in tumor microenvironment synthesize powerful proteolytic matrix metalloproteinases (MMPs) enzymes (e.g. **collagenases** and **proteases**), urokinase-type plasminogen activator (u-PA), elastase, cathepsins. These molecules cause breakdown of collagenous and noncollagenous components of extracellular matrix (ECM). Matrix metalloproteinases (type IV collagenases) cleave (dissolve) basement membrane collagen type IV fibers.
- Plasminogen activator activates elastase and cathepsins, which cleave noncollagenous matrix of basement membrane matrix. Plasminogen activator converts serum plasminogen to plasmin, a serine protease, that degrades laminin. Plasminogen activator also stimulates CSCs to synthesize collagenases in abundance (autocrine action).
- The principal biologic function of MMPs is to degrade ECM proteins and glycoproteins, membrane receptors, growth factors and cytokines. MMPs are strictly regulated by endogenous tissue inhibitor matrix metalloproteinases (TIMPs).
- Localization and interactions between matrix metalloproteinases (MMPs) and tissue inhibitor matrix metalloproteinases (TIMPs) in the tumor microenvironment are shown in Fig. 6.124. Family of matrix metalloproteinases (MMPs) is given in Table 6.104.
- Following extracellular matrix (ECM) breakdown, CSCs become motile and migrate along blood vessels and undergo proliferation to form progeny cells. CSCs are ready to invade lymphatics or vascular channels (capillaries and venules).
- ECM is a macromolecules network, composed of many components: (a) fibers (i.e. collagen, elastin, laminin, and fibronectin), (b) proteoglycans (syndecan 1 and aggrecan), (c) glycoproteins (tenascin, vitronectin and entactin), and (d) polysaccharides (hyaluronic acid), that regulates cell migration, cell growth and differentiation. ECM provides structural and biochemical support. Chondroitin sulphate proteoglycan 4 (CSPG4) present in ECM plays an integral role in stabilizing the interactions between cells in the ECM.
- One important step in tumor invasion is the disassembly of the ECM and its constituents through MMPs.
  - Disassembly of ECM plays a major role in unrestricted CSC proliferation, survival, and angiogenesis, in addition to invasion.
  - **Versican** present in interstitial ECM promotes CSC growth and invasion.
  - **Lumican** in ECM attenuates the CSC proliferation, migration, and invasion of CSCs of breast carcinoma.
  - Glypican 3 in ECM exhibits a tumor suppressor phenotype.
  - Downregulation/loss of **glypican 3** is associated with poor prognosis in cancer patients. **Serglycin** is an intracellular proteoglycan expressed in hematopoietic cells.
  - Serglycin induces EMT, CSCs growth, invasion, metastasis and chemoresistance.
- Autophagy is a highly regulated process through which cells recycle their own constituents by delivering them into lysosomes. In cancer, autophagy has paradoxical roles, acting both as tumor suppressor in early stage and as tumor promoter in more advanced stage (CSC motility, viability, and invasion). Autophagy may exert different functions in response to cancer therapy, causing malignant tumors resistance or increasing sensitivity to chemotherapeutic drugs and radiation.

Resistance to Anoikis of Cancer Stem Cells (CSCs)

Anoikis (Greek word: homelessness) is the name given to induction of apoptosis (programmed cell death) in anchorage-dependent epithelial cells, upon disruption in cell-cell attachment or cell–extracellular matrix (ECM) attachment, due to disruption of integrin ligation. Anoikis is important for maintaining tissue homeostasis.

- Anoikis inhibits detached CSCs from dissemination and colonization in distant tissues/organ(s). Anoikis can engage both extrinsic as well as the intrinsic pathway of apoptosis.
- Resistance to anoikis is an attribute of CSCs with metastatic potential. Numerous studies have revealed that stimulation of pro-survival signals (PI3K/AKT/mTOR, RAS/RAF/MEK/ERK/MAPK and Rho-GTPase), and suppression of death signals including growth factor proteins, cellular acidosis, oxidative stress in CSCs is involved in 'anoikis' resistance, thus enhancing CSC proliferation, invasiveness, and metastasis. This can be achieved through autocrine signaling of growth factors such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF).
  - In addition, overexpression of growth factor receptors such as EGF receptor, TrkB receptor and HGF receptor can suppress anoikis.
  - Similarly, the expression of long noncoding RNA (incRNA) has been linked to anoikis resistance.
- Currently, **anoikis resistance** becomes a hot topic in cancer research. Detailed molecular and functional analyses of 'anoikis' resistant CSCs may provide insight into biology of cancer metastasis and identify novel therapeutic targets for prevention of cancer dissemination to distant organs.



Matrix metalloproteinases (MMPs) and tissue inhibitor metalloproteinases (TIMPs) in tumor microenvironment		
Tissue inhibitor metalloproteinases	*Localization*	*MMPs degradation by TIMPs*
TIMP-1	Soluble and cell surface	• MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9 • ADAM-10
TIMP-2	Soluble and cell surface	• MMP-2, MMP-9, MMP-14, MT1-MMP • ADAM-12
TIMP-3	Extracellular matrix and cell surface	• MMP-2, MMP-9 • ADAM-17, ADAM-10 • ADAMPTS4
TIMP-4	Soluble and cell surface	• MMP-1, MMP-2, MMP-26 • MT1-MMP • ADAM-28

- Metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes that degrade both extracellular matrix (ECM) and non-matrix proteins. MMPs play key roles in morphogenesis, wound healing, tissue repair and remodeling in response to injury and progression of cancer.
- Tissue inhibitors of metalloproteinases (TIMPs) play key role in the homeostasis of extracellular matrix (ECM) by regulating the activity of metalloproteinases (MMPs). TIMPs are well-known for their ability to inhibit metalloproteinase activity thereby inhibiting tumor growth and metastasis.

Fig. 6.124: Localization and interactions between matrix metalloproteinases (MMPs) and tissue inhibitor metalloproteinases (TIMPs) in the tumor microenvironment. MMPs degrade both extracellular matrix (ECM) and non-matrix proteins and play key role in malignant tumor invasion and metastasis. TIMPs inhibit MMPs activity thereby inhibiting tumor growth and metastasis.

Table 6.104 Family of matrix metalloproteinases (MMPs)

MMP	Name of MMP	Substrate Cleaved (Degraded) by MMP	
Collagenases family			
MMP1	Collagenase 1	▪ Collagen fiber types I, II, III, VII and X	▪ HIV Tat protein
MMP8	Collagenase 2	Collagen fiber types I, II, III	
MMP13	Collagenase 3	▪ Collagen fiber types I, II, III, IV, XIV, X	▪ Soluble type II
Stromelysins family			
MMP3	Stromelysin 1	▪ Fibronectin ▪ Laminin ▪ Gelatin types I, II, III, IV	▪ Aggrecan ▪ Collagen types III, IV, X and IX ▪ Activates procollagenase
MMP10	Stromelysin 2	▪ Fibronectin	▪ Gelatin types I, II, III, IV and V
MMP11	Stromelysin type 3	▪ Weakly structures of extracellular matrix	▪ α_1-Proteinase inhibitor
MMP21	Stromelysin type 3	▪ α_1-Anti-trypsin inhibitor	▪ Specific functions in embryogenesis
MMP28	Stromelysin type 3	▪ Casein	▪ Structural proteins of extracellular matrix
MMP19	Stromelysin type 3	▪ Broad range of substrates close to MMP11 and MMP7	
Gelatinases family			
MMP2	Gelatinase A	▪ Gelatin type I ▪ Collagen fiber types IV, V, VII and X	▪ Collagen-like sequence
MMP9	Gelatinase B	▪ Gelatin types I and V ▪ Collagen fiber types IV and V	▪ Fibronectin
Other metalloproteinases (MMPs) family			
MMP12	Not specified	▪ Soluble and insoluble elastin	▪ β-Chain of insulin
MMP20	Not specified	▪ Amelogenin ▪ Aggrecan	▪ Cartilage oligomeric matrix protein (COMP)
MMP27	Not specified	▪ Fibronectin ▪ Laminin	▪ Gelatins ▪ Collagen fibers
Matrilysins family			
MMP 7	Matrilysin 1	▪ Casein ▪ Gelatin types I, III, IV and V ▪ Fibronectin	▪ Activates procollagenase ▪ Specific sequence of β-chain of insulin
MMP 26	Matrilysin 2	▪ Collagen type IV ▪ Fibronectin ▪ Fibrinogen ▪ β-Casein	▪ Gelatin type I ▪ α_1-Proteinase inhibitor ▪ Activates progelatinase B
MT-MMP GPI family			
MMP17	MT4-MMP	▪ Fibrinogen and fibrin ▪ Collagen type I ▪ Gelatin type I ▪ Fibronectin	▪ Laminin ▪ Tenascin ▪ Cartilage oligomeric matrix protein (COMP) ▪ α_2-Microglobulin
MMP25	MT6-MMP	▪ Vimentin	▪ α_1-Proteinase inhibitor
MT-MMP transmembrane type I family			
MMP14	MT1-MMP	▪ Aggrecan ▪ Collage fibers	▪ Activates MMP2 and MMP13
MMP15	MT2-MMP	▪ Broad range of substrates	

Contd...

Table 6.104 Family of matrix metalloproteinases (MMPs) (*Contd...*)

MMP	Name of MMP	Substrate Cleaved (Degraded) by MMP	
MMP16	MT3-MMP	▪ Collagen type 3 ▪ Fibronectin	▪ Activates MMP2
MMP24	MT4-MMP	▪ N-cadherin (CDH2) ▪ Fibronectin partially	▪ Activates MMP2
MT-MMP transmembrane type II family			
MMP23A and MMP23B	Not specified	Regulates the surface expression of some potassium channels	

- Therefore, it is essential to understand molecular and cellular mechanisms underlying anoikis and 'anoikis resistance' in relation to intrinsic and extrinsic death signaling, EMT, growth factors, energy metabolism, acidic environment, reactive oxygen species, oxidative stress, microRNA modulation, membrane microdomains, and lipid domains of plasma membrane.

Cancer Stem Cells Motility

Cancer stem cells (CSCs) secrete chemokines, that stimulate development of finger-like projections (invadopodia) on CSCs involved in motility of CSCs to overcome the dense scaffold of local tissue environment by degradation of extracellular matrix (ECM).

- **Invadopodia:** Invadopodia are actin rich membrane protrusion that facilitates CSCs dissemination on proteolytic activity and clearing paths for migration through physical barriers, such as basement membrane, ECM and endothelial cells.
  - Actin regulatory proteins such as N-WASP, cortactin, and ARP2/ARP3 assist in invadopodia formation. TKS5 regulatory protein plays a key role in invadopodia and maturation of invadopodia because of the delivery of proteins, such as integrins and MT1-MMP.
  - Polarity of CSCs has a leading front and rear regions, which depends on a distinct actin cytoskeleton and generation of various cell junctions.
- **Lamellipodia:** Lamellipodia are flat, sheet-like membrane protrusions formed at the leading edge of migrating CSCs. Lamellipodia contain dendritic arrays of filaments and molecular machinery, that regulates polymerization/depolymerization and organization of actin filaments.

Intravasation, Transport in Circulation and Arrest of Cancer Stem Cells on Vascular Endothelium

Vascular endothelium poses a barrier to CSC intravasation. CSC invasion and transendothelial transmigration is the first step of metastasis to distant tissues/organs.

- Intravasation is the key step in cancer metastasis during which the CSCs invade wall of lymphatic vessel or blood vessel and the lumen of vasculature, thereby becoming circulating CSCs and becoming potential metastatic seeds in distant organs after extravasation to the host tissue environment.
- Intravasation of CSCs depends on the malignant epithelial tumor type, mitosis, tumor microenvironment, microvessel density (MVD) and diameter of vasculature. TGF-β signaling can enhance intravasation of CSCs in part through induction of EMT. In addition to TGF-β signaling, numerous studies demonstrated that activation of EGF receptor family members stimulate intravasation, with downstream signaling through PI3K/AKT/mTOR, N-WASP, RhoA and WASP pathways to induce invadopodia.
- With respect to proteases, there is strong evidence for contributions by urokinase-type plasminogen activator (u-PA)/urokinase-type plasminogen activator receptor (u-PAR), while the roles of matrix metalloproteinases (MMPs) in intravasation may be more tumor specific. Other cells such as macrophages, fibroblasts, neutrophils, and platelets can also play a role in enhancing intravasation of CSCs.
- Furthermore, the architectural constraints of tissue impose some mechanical pressures on invading CSCs during intravasation. Nuclear squeezing of the invading CSC causes genomic rearrangement, which increases the metastatic potential.
- Integrins are the key cell adhesion molecules involved in nearly every step of malignant epithelial tumor progression from primary tumor development to metastasis to distant tissues/organs. Altered integrin expression is most often detected in human cancers.
- A clear understanding of intravasation may provide opportunities for developing new prognostic markers as well as therapeutic options for preventing the spread of malignant tumors.

Cancer Stem Cells Survival in Circulation

The circulatory journey is harsh for most circulating CSCs. Interactions between circulating CSCs and

the microenvironmental components of circulation determine survival, and the ability of circulating CSCs to eventually extravasate in distant tissues/organs via lymphatic and hematogenous routes.

- Majority of circulating stem cells circulate as single cells, whereas others circulate in clusters by forming platelet tumor emboli. However, circulating CSCs in clusters are more likely to form metastases. In addition to intravasating CSCs, clusters contain stromal cells, and immune cells from the original tumor microenvironment that may contribute to the heterogeneity of the cluster of CSCs and enhance their survival in circulation due to evasion of destruction by immune system.
- Neutrophils participate in cluster formation of CSCs and suppress leukocyte activation, which enhance the chances of CSCs survival. Neutrophil clustering with circulating CSCs increases the metastatic potential of CSCs.
- Moreover, circulating CSCs also interact with tumor educated platelets (**TEPs**) result in formation of coating shield of platelets around CSCs that prevent detection of circulating tumor cells by immune cells. Platelets coating cancer stem cells (CSCs) provide protection against physical stresses of circulation.
- Small number of platelets coated CSCs are apparently able to establish metastatic disease. The survival and growth of metastatic CSCs is not a random process but depends upon the selection of CSCs possessing specific properties needed for metastatic growth. Circulating CSCs either arrest, and grow in capillaries or extravasate by transendothelial migration, and colonize premetastatic niche (PMN).

Cancer Stem Cells Margination and Adhesion on the Microvascular Endothelium in Circulation

Margination and adhesion of CSCs on the microvascular endothelium are two critical and closely related steps in tumor metastasis, and generally the former margination step is most often regarded as a prerequisite for the later adhesion step, which may determine the destination of CSCs after extravasation.

- CSCs are more frequently found to adhere at the microvascular bifurcations, and there is a positive correlation between the CSCs adhesion and the microvasculature wall-directed hemodynamic force of the red blood cells (RBCs).
- The larger the wall-directed hemodynamic force is, the closer the CSCs are marginated towards the microvasculature wall, and higher the probability of their adhesion on vascular endothelium. A relatively low or high hematocrit and increasing the shear rate of blood flow can assist to prevent the adhesion of CSCs.
- CSCs may be more likely to extravasate at the microvascular bifurcations, if the shear rate blood flow is slow, and the hematocrit value remains moderate due to high wall directed hemodynamic force from the surrounding red blood cells. These observations suggest the CSCs may be prone to adhere to vascular endothelium at the microvascular bifurcations with low shear rate and moderate hematocrit.
- Hemodynamic forces, cytoskeletal alterations and remolding of microvascular endothelium play important role in the CSCs arrest, adhesion, transendothelial migration, subsequent invasion of surrounding tissue, extracellular matrix (**ECM**) degradation by matrix metalloproteinases (MMPs), and extravasation of CSCs into tissues.

Extravasation of Cancer Stem Cells

Extravasation is a multistep process during which circulating CSCs pass through the blood vessel wall into the tissue.

- Only a small number of CSCs survive in the circulation and extravasate into the tissues, and remain in dormant state, while retaining a stem-like tumor initiating potential.
- Invadopodia are actin rich membrane protrusion that facilitates CSCs dissemination on proteolytic activity (local synthesis of MMPs) and clearing paths for migration of CSCs, local invasion, intravasation and extracellular events through physical barriers, such as basement membrane, extracellular matrix (ECM) and vascular endothelial cells. Invadopodia of CSCs have become therapeutic target for metastasis.

Integrins Role in Extravasation of Cancer Stem Cells

Circulating CSCs express numerous integrins mediating their adhesion via CD44 adhesion molecules on the microvascular endothelial surface, which may either induce retraction of vascular endothelial cells or degradation of vascular basement membrane by matrix metalloproteinases (MMPs) and thereby promoting extravasation, subsequent metastasis in distant organs.

- Secondly, transendothelial migration of CSCs follows subsequent invasion into the vascular basement membrane extracellular matrix (ECM), that requires genetic and molecular mediation to be able to transmigrate and colonize in the new stressful tissue environment.
- In other organs, CSCs extravasation face tight barriers and basement membranes, that require genetic and molecular mediation to be able to migrate. Recent studies revealed that CSCs induce programmed cell death (apoptosis) of vascular endothelial cells, driving metastatic CSCs to extravasate.

Chemokines Role in Extravasation of Cancer Stem Cells

Transendothelial migration of circulating CSCs is dependent on a spectrum of chemokines and complement system components.

- Granulocyte/macrophage colony-stimulating factors (GM/CSFs) and cytokine (IL-5) aid in metastasis of breast carcinoma.
- Circulating CSCs enhance the production of cytokines (IL-6, IL-8), that stimulate biochemical pathways and CSCs migration.
- Dysregulation of interleukin-1β (IL-1β) has been implicated in tumor progression, invasion, and metastasis by inducing the expression of proangiogenic genes and growth factors. In addition, loss of TP53 in CSCs induces the synthesis of Wnt ligands, that stimulate the production of IL-1β, thus driving neutrophilic inflammation.

Cancer Stem Cells Revert to Mesenchymal–Epithelial Transition

After extravasation, CSCs revert to mesenchymal–epithelial transition that involves the transition from mobile, multipolar, or spindle-shaped mesenchymal cells to planar arrays of polarized epithelial cells with cell-to-cell junctions, apical–basal polarity, and epithelial markers. Epithelial–mesenchymal transition (EMT) and its reversal to mesenchymal–epithelial transition (MET) play important roles in metastatic dissemination of CSCs of malignant epithelial tumor subsequent metastasis to distant tissues/organs and therapy resistance.

Cancer Stem Cells Colonization in Secondary Organs at Distant Sites

After extravasation, CSCs undergo adaptation to local tumor microenvironment (TME), successful colonization, and finally establishment of clinically detectable metastatic disease.

- Secondary tumor is defined as metastasis from the primary malignant epithelial tumor at original site. Metastasis suppressors halt metastatic CSCs proliferation at the secondary site without changing the characteristics of primary malignant epithelial tumor. They work through oncogenic signaling pathways to suppress invasion and eventual colonization in tissues/organs.
- Metastasis is a multistep process during which CSCs from primary malignant epithelial tumor disseminate via lymphatic route to lymph nodes and via hematogenous route to lungs, liver, bone marrow, brain, and skin, that represents the main cause of morbidity and mortality. Metastasis is modulated by tumor microenvironment and immunity. Genes play pivotal role in both metastatic dormancy and reactivation of CSCs.

Cancer Stem Cells Evasion of Immune Destruction

Factors which influence the establishment of tumor metastases include: genetic instability of CSCs; enzymatic degradation of basement membrane and extracellular matrix (ECM); ability to withstand rheologic trauma; size of the CSC cluster; interaction with CD8+ cytotoxic T cells, natural killer cells (NK cells), and macrophages; entrapment by fibrin or platelets; and surface glycoproteins. These factors favor the ability of variant CSCs to reach and colonize specific organs.

Cancer Stem Cells Evasion of Immune Surveillance

Cancer stem cells (CSCs) can be recognized by immune system. MHC class I molecules on antigen-presenting cell process and present CSCs to CD8+ cytotoxic T cells leading to CSCs destruction. There is increased risk for cancers in immunocompromised persons. CSCs escape from immune destruction in immunocompetent patients by the following mechanisms: (a) selective outgrowth of antigen negative variant of CSCs, (b) loss of MHC-I molecules, immunosuppression due to synthesis of TGF-β, PD1 ligands and galectins by CSCs, (c) antigen on cancer stem cells masked by enhanced synthesis of glycocalyx on CSCs, and apoptosis of CD8+ cytotoxic T cells.

Metastatic Dormancy of CSCs in Secondary Organs at Distant Sites

After extravasation, CSCs may remain in dormant state over a long period, before they activate the mesenchymal–epithelial transition (**MET**) process and proliferate to form macrometastasis in secondary tissues/organs. Following extravasation, metastatic CSCs cease dividing but survive in a quiescent state of cell cycle in G0–G1 for long-time. Quiescent CSCs possess angiogenic dormancy and immune-mediated dormancy in which the malignant tumor mass is preserved by immune cell cytotoxicity.

- Metastatic dormancy occurs due to the delayed acclimatization of disseminated CSCs to their secondary niches. When the microenvironmental conditions are favorable, these CSCs reinitiate proliferation and colonize sometimes years after treatment of the primary malignant epithelial tumor.
- Metastatic tumor dormancy can be induced in malignant epithelial tumors through several environmental and signaling transcriptional mechanisms involving intracellular signaling, extracellular

signaling and induction signals originating from the bone marrow niche: (a) interaction with vascular endothelial cells, cancer-associated fibroblasts (CAFs), cancer-associated immune cells and the extracellular matrix (ECM), (b) genetic alterations in the tumor microenvironment and induction of EMT, (c) epigenetic alterations in the malignant epithelial tumor, (d) malignant tumor hypoxia, (e) angiogenic switch turning off, (f) immune evasion, and (g) inflammatory switchover.

- Metastatic dormancy occurs due to suppression of RAS-MEK-ERK/MAPK and PI13K/AKT signaling cascades and activation of tumor suppressors. Restricted angiogenesis and/or an active immune cell in microenvironment inhibit expansion of CSC clusters into micrometastasis.
- TGF-β induces chondrocyte 2 (DEC2), which inhibits cyclin-dependent kinase 4 (CDK4), and activates p27 resulting in entry of cell to state of quiescence. Blocking tumor angiogenesis through thrombospondin or inhibiting chaperones such as heat shock protein 27 shifts metastatic CSC clusters into dormant state.
- Immune cells such as CD8+ cytotoxic T cells, natural killer cells (NK cells), and cancer-associated macrophages (CAMs) inhibit CSCs proliferation and clear metastatic CSCs through cytolysis. Metastatic dormant CSCs express weak antigens to escape the immune destruction, which could be the reason behind relapse following immunotherapy.

Metastatic Dormancy and Reactivation of CSCs in Secondary Organs at Distant Sites

Extravasated CSCs may remain in dormant state over a long period with subsequent reactivation to mesenchymal–epithelial transition (**MET**) involving intracellular signaling, extracellular signaling, and induction signals originating from bone marrow niche leading to form macrometastasis.

- Tumor dormancy can be induced through several mechanisms, such as genetic and/or epigenetic changes in the malignant epithelial tumor, tumor hypoxia, angiogenic switch, immune evasion, and inflammatory switch.
- Extravasated CSCs interact and adapt with tissue microenvironment and induce neoangiogenesis in order to ensure sufficient vascularization of malignant epithelial tumor.
- Alteration in the tumor microenvironment can facilitate malignant epithelial tumor growth, recurrence, and metastasis, and thereby permit the CSCs to exit from dormancy through interaction with vascular endothelial cells, cancer-associated fibroblasts (CAFs), tumor-associated immune cells, inflammatory cells and the extracellular matrix (ECM).
- In the novel stromal environment, CSCs form micrometastasis with the ability to generate macrometastasis elsewhere in distant organs such as the lungs, bones, liver, brain, and other tissues.

Metastatic Colonization of CSCs in Secondary Organs at Distant Sites

Metastasis is the main cause of mortality in cancer patients. After extravasation of CSCs leading to dissemination in distant tissues/organs (e.g. liver, lungs, brain, adrenal glands, and bone marrow) is termed 'metastatic colonization'. Cancer stem cells either build a secondary tumor (micrometastasis or macrometastasis) or remain in dormant state.

- The extrinsic traits of CSCs, tumor-secreted factors (e.g. exosomes, cytokines, chemokines) and bone marrow-derived cells signal play a pivotal role in forming the premetastatic niche (**PMN**) promoting cancer metastasis.
- Tumor-derived exosomes play important role in educating bone marrow progenitor cells to become metastatic. In patients with pancreatic carcinoma, exosomes also play important role in the initiation of **PMN** in the liver. Recent study revealed that the local microenvironment and cancer cell–host cell interactions of some organs is favorable for colonization of CSCs.
- Disseminated CSCs can create an adequate stromal microenvironment to allow metastasis in the preparation of an adequate **PMN**.
- Primary malignant epithelial tumor synthesizes proangiogenic VEGFA factor to mobilize progenitor hematopoietic stem cells from bone marrow toward the bloodstream and from there to the site of metastasis. In addition, primary malignant epithelial tumor secretes matrix metalloproteinases, fibronectin and growth factors into bloodstream and later accumulate in the target organ to **PMN**.
- Chemokines synthesized by cells attract CSCs and macrophages toward the premetastatic niche to colonize in target organs. Receptor of chemokine CXCR4 plays an important role in the extravasation of breast cancer stem cells toward their target organs.
- The anatomy of some organs provides a barrier to metastasis in lungs and brain. The vascular endothelium of capillaries surrounded by a basal membrane act as barrier to metastasis in lungs. Capillaries in the blood–brain barrier having tight junctions and astrocyte foot processes act as barrier to metastasis in brain. Presence of fenestrated capillaries

in the liver and bone makes these organs susceptible to metastasis.

- Metastasis in the mechanism by which CSCs break from primary malignant epithelial tumor and disseminate to distant organs.
  - Breast carcinoma, prostatic carcinoma and melanoma disseminate to distant organs years before detection of primary tumor.
  - Pancreatic carcinoma and lung carcinoma rapidly acquire the capacity to infiltrate and colonize without requiring a process of quiescence to generate a macrometastasis.
- Establishing a vascular network is crucial for proper metastatic colonization. Vascular mimicry supports malignant tumor perfusion, enhances intravasation and promotes breast carcinoma metastasis through serpin peptidase inhibitor, clade E member 2 (SERPINE2) and secondary leukocyte peptidase inhibitor (SLPI), which drive distant metastasis to lung and brain by enabling breast CSCs to form vascular channels.
- Breast carcinoma can colonize brain by utilizing neuronal signaling pathways for growth and adaptation. The proximity of the breast carcinoma CSCs to neuronal synapses allows CSCs to hijack N-methyl-D-aspartate receptor signaling to promote brain metastasis. Protocadherin 7 is a protein that promotes the assembly of cancer stem cell-astrocyte gap junctions composed of connexin protein. Metastatic breast cancer cells use these astrocyte gap junctions.
- Clinical observations suggest that most primary malignant epithelial tumors metastasize to specific organs, a process known as '**organotropism**'.
  - Breast carcinoma most frequently metastasize to the bone, often after long latency.
  - Bone metastatic breast disease becomes resistant to therapy. Calcium influx has been detected as a mechanism between the osteogenic niche and CSCs, which promotes the progression of bone metastatic breast disease.
  - Patients with postpartum breast carcinoma develop liver metastasis associated with poor prognosis.
- Metastatic organotropism in females and males is shown in Fig. 6.125.

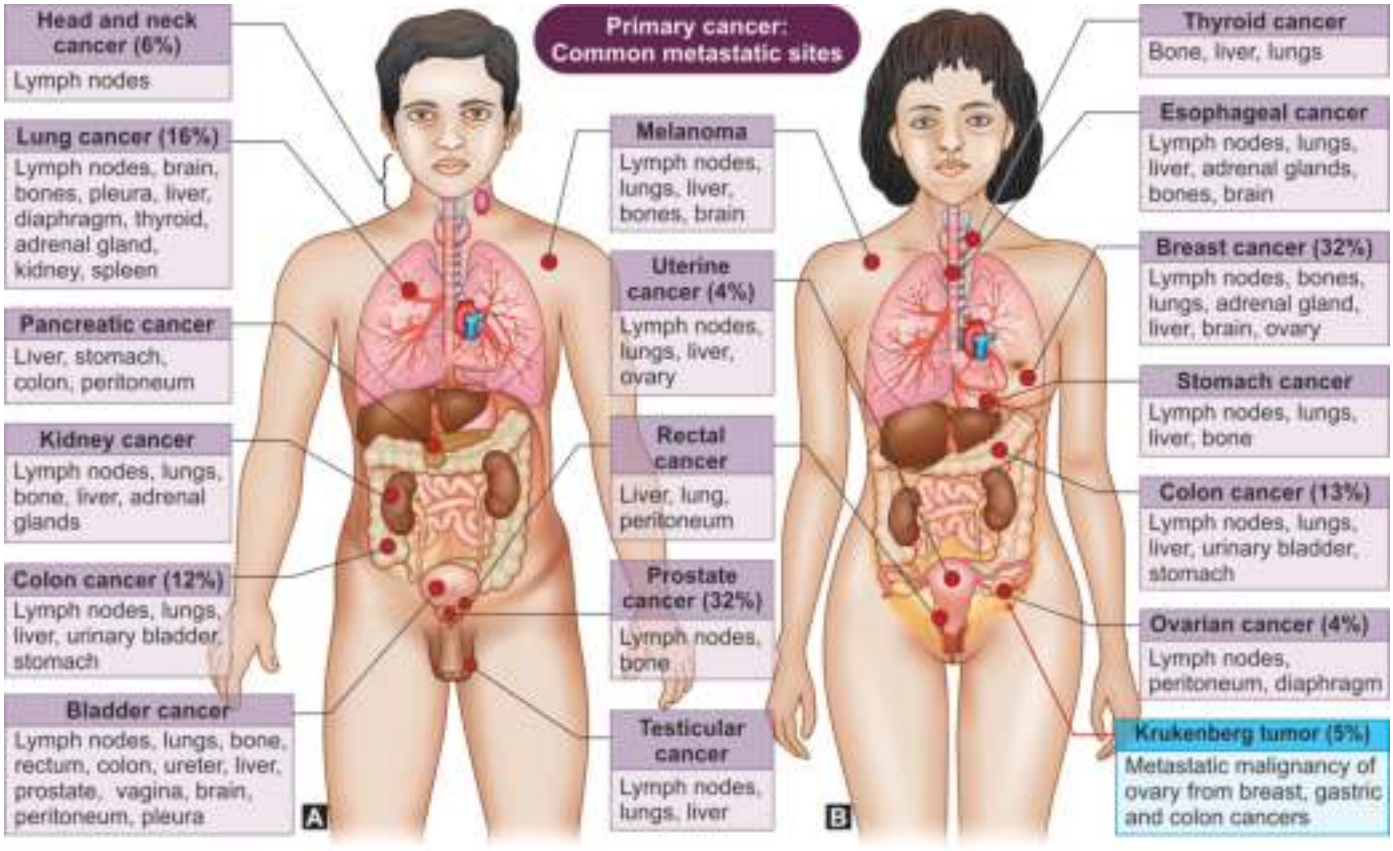


Fig. 6.125: Metastatic organotropism in females and males. Most cancers metastasize to specific target organs, a process known as 'metastatic organotropism.

CLINICAL ONCOLOGY

Cancer develops due to mutations in several key genes, e.g. proto-oncogenes, tumor suppressor genes, apoptosis regulatory genes, and DNA repair genes, that control cell growth, proliferation, and survival.

- **Oncology:** Oncology is a branch of medicine that deals with the prevention, diagnosis, and treatment of cancer. Field of oncology has major areas based on treatments: clinical oncology, medical oncology, radiation oncology, and surgical oncology.
  - **Clinical oncology:** Clinical oncology relates to treatment of diagnosed cancer patients by radiotherapy, chemotherapy, hormonal therapy, biological therapy, and targeted therapy without surgical intervention.
  - **Medical oncology:** Medical oncology focuses on the diagnosis, treatment, and prevention of cancer. Medical oncologist is the main health provider for cancer patients, and also provides supportive care and may coordinate treatment given by other specialists.
  - **Radiation oncology:** Radiation oncology is a type of medicine that uses high-energy radiation to destroy the DNA of CSCs and prevent them from growing and dividing. Radiation oncologist treats cancer using radiation therapy, which is the use of high-energy X-rays or other α and β particles to destroy CSCs.
  - **Surgical oncology:** Surgical oncology is a branch of surgery applied to oncology, that focuses on the surgical management of tumors, especially malignant tumors. Surgical oncologist treats cancer using surgical removal of tumors nearby tissue during operation. Surgical oncologist can also take biopsy to diagnose cancer.
- **Clinical history:** Clinical history of cancer patient is the most important diagnostic tool. Some cancers may be diagnosed by thorough clinical history, and physical examination, even before the person develops signs and symptoms and the suspected body system involved. Other persons may display '**cancer warning signs**': change in bowel or urinary bladder habits, non-healing sore, lump, indigestion/dysphagia, obvious change in a wart/mole, and cough or hoarseness.
  - Common symptoms that point towards cancer include fatigue, weight loss, unexplained anemia, and fever of unknown origin from *Escherichia coli* or *Pseudomonas aeruginosa* infections. Cancers of colon, stomach and kidney over mucosal surface can lead to ulceration, bleeding, and superadded infections.
  - Clinical manifestations of cancer patients are directly attributed to the local effects of the primary malignant tumor or metastases in majority of cases. In 5–10% of cases, systemic effects of cancer on the host independent of direct invasion and metastases occur due to paraneoplastic syndromes mediated by a variety of mechanisms: either 'ectopic' or 'inappropriate' hormone secretion or the production of chemical mediators such as cytokines, interleukins, and growth factors. Immune system effects mediated by antibodies or T cells that cross-react with normal host tissue are particularly observed with neurologic syndromes.
- **Tumor effects on the host:** The effects of tumors on the host depend on the location of tumor. The localized tumor mass may compress neighboring vital structures causing loss of function. Benign endocrine tumors may produce excess hormones. Non-metastatic systemic effects of cancer on the host include cachexia, hormone- and autoantibodies-related paraneoplastic syndromes.
- **Diagnostic modalities:** Cancer is diagnosed by various diagnostic tools such as fine needle spiration cytology, surgical biopsy of the tumor and examining it under light microscope including immunohistochemistry.
  - Other diagnostic tools include endoscopy for gastrointestinal tract, cystoscopy for urinary tract, bronchoscopy for respiratory tract; imaging studies like ultrasonography, X-rays, CT scanning, MRI scanning, scintigraphy, single photon emission computed tomography, positron emission tomography and nuclear medicine.
  - Common diagnostic tests include blood tests for biological or tumor markers. Increased level of tumor markers in blood may be indicative of the cancer.
- **Treatment:** The treatment modalities for cancers include surgery, chemotherapy, and radiation therapy. Cancer therapy varies by the patient's tumor type, and location.
  - **Targeted drug therapy:** Targeted drug therapy uses medicines that targets specific genes and proteins that help CSCs to grow, spread, and survive longer. Two main groups of targeted drug therapy include monoclonal antibodies and small molecule inhibitors. They have side-effects different from chemotherapy.
  - **Bone marrow transplant:** Bone marrow transplant is also called **hemopoietic stem cell transplant**. Bone marrow transplants may use stem cells from

own body (autologous transplant) or from a donor (allogenic transplant). Bone marrow transplant can benefit cancer patients (leukemias, multiple myeloma, and lymphoid neoplasms) and other patients with aplastic anemia, bone marrow failure syndromes, hemoglobinopathies and amyloidosis.

- **Clinical trials:** Clinical trials are studies to discover new methods of treating cancers. Each type of clinical trial is designed to answer different research questions and will help researchers learn things that can help patients in the future.

LOCAL EFFECTS OF TUMORS ON THE HOST

The effects of tumors on the host depend on the location of tumor. The localized tumor mass may compress neighboring vital structures causing loss of function. Benign endocrine tumors may produce excess hormones.

- Pituitary adenoma can compress optic nerve, and cause bilateral temporal hemianopia, and produce excess hormones, e.g. growth hormone causing acromegaly.
- Malignant tumors can impinge on airways, brainstem, nerves, blood vessels, and hollow viscus and cavity.
- Cancers of colon, ovary and urinary bladder may rupture. Colon carcinoma can narrow a hollow viscus, e.g. causing intestinal obstruction, ulceration and bleeding leading to intestinal obstruction, and anemia.
- Pancreatic carcinoma may obstruct common bile duct resulting in obstructive jaundice. A space-occupying tumor can block a cardiac valve orifice. Direct spread of lung carcinoma may compress neighboring viscera, blood vessels and nerves.
- Metastasis to bone causes bone pain and pathologic fracture. Metastasis to the brain causes epilepsy, cerebral stroke and raised intracranial pressure.
- Malignant tumors and their products produce systemic effects such as cachexia, and paraneoplastic syndrome. Initially, cancer patients are asymptomatic, but become symptomatic in advanced disease. Local effects of cancer on the host are shown in Fig. 6.126.
  - **Central nervous system manifestation:** About 2 cm tumor in the brainstem may kill a patient. Central nervous system tumors can compress surrounding normal structures and impair their functions. Intracranial tumors cause compression on brain parenchyma due to inelasticity of the skull vault, that produce headache, nausea, vomiting, confusion, seizure, and localized neurological signs. Invasion of nerve can result in neurologic deficits and pain.

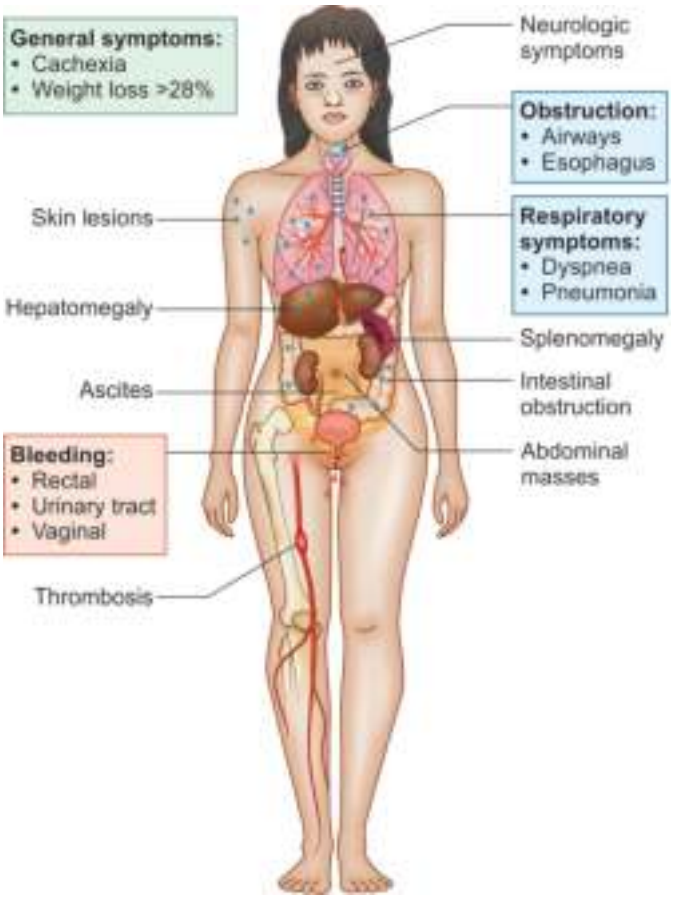


Fig. 6.126: Local effects of cancer on the host. Primary and metastatic tumors can produce same consequences. Malignant tumor can affect normal bodily functions by compressing, invading, and destroying normal tissues.

- **Respiratory system manifestations:** Lung carcinoma can obstruct airways cause pneumonia or bronchiectasis and breathlessness. As the disease progresses, patient develops weight loss, fever, and tiredness. Lung carcinoma metastasizing to lymph nodes and distant organs can develop additional symptoms.
- **Gastrointestinal tract manifestations:** Symptoms and signs of advanced gastric carcinoma include fatigue, weight loss, anemia, black tar stools, severe nausea, and vomiting, some frank blood in stool sometimes. Advanced intestinal and gynecological cancers can cause partial complete bowel obstruction at multiple levels. Peritoneal carcinomatosis has been demonstrated in majority of cases. Patients with malignant tumor developing bowel obstruction present with continuous colic pain, nausea, vomiting, limitation in inferior vena cava venous return, water and electrolytes imbalance, deterioration in general and hemodynamic status, diaphragmatic elevation and ventilatory restriction.

- **Vascular manifestations:** Tumor mass can impinge upon blood vessels leading to ischemia and infarction of tissue/organ. Tumor invasion in blood vessel can lead to hemorrhage within pleural or peritoneal cavity.
- **Trousseau sign:** Trousseau sign is migratory venous thrombosis most often associated with pancreatic adenocarcinoma and bronchogenic carcinoma.
- **Hematologic manifestations:** Space-occupying lesion in the bone marrow causes pancytopenia. Patient develops microcytic hypochromic anemia due to blood loss in colorectal cancer. Macrocytic anemia occurs due to folate deficiency from rapid malignant tumor growth; and bone marrow metastases. Leukopenia and thrombocytopenia occur due to tumor metastasis within bone marrow, chemotherapy, and radiation therapy.
- **Skeletal manifestations:** Bone destruction can lead to pathologic bone fracture in the settings of metastatic bone disease, and multiple myeloma.

NON-METASTATIC SYSTEMIC EFFECTS OF CANCER ON THE HOST

Five non-metastatic systemic cellular and tissue level hallmarks of cancer include: global inflammation, immune system inhibition, metabolic changes-associated cachexia, the propensity to thrombosis and neuroendocrine changes. Other clinical manifestations of cancer are mediated by various mechanisms, other than invasion and metastasis. Systemic effects of cancer on the host include cachexia, hormone- and autoantibodies-related paraneoplastic syndromes.

- **Cancer cachexia:** Cancer cachexia is a wasting disorder that causes extreme loss of adipose tissue and skeletal muscle, anorexia associated with cancer. It occurs either low-caloric intake or altered normal metabolism and switching of CSCs to ineffective aerobic glycolysis. Cancer cachexia is caused by cytokines produced by the malignant tumor and host response to the malignant tumor.
- **Hormone-related paraneoplastic syndromes in cancers:** In minority of patients, CSCs can synthesize hormone-like proteins or substances and produce remote effects, that are not attributable to malignant tumor invasion or to metastasis, and are collectively called paraneoplastic syndromes. Such effects are rarely lethal, but in some cancer patients side effects dominate the clinical course.
  - **Parathormone (PTH)**-related peptide produced by squamous cell lung carcinoma, breast carcinoma and renal cell carcinoma results in osteolysis of bone and thus hypercalcemia.
  - **Adrenocorticotropic hormone (ACTH)**-like protein is produced by small cell lung carcinoma and pancreatic carcinoma results in Cushing syndrome-like manifestations. Small cell lung carcinoma and cerebral tumors are associated with syndrome of inappropriate antidiuretic hormone (SAIDH) leading to retention of water in the body.
  - **Erythropoietin** produced by renal cell carcinoma, cerebellar hemangioblastoma and hepatocellular carcinoma results in polycythemia.
- **Autoantibodies-related paraneoplastic syndromes in cancers:** Autoantibodies-related paraneoplastic syndromes are related to involvement of nerves and skeletal muscles. Lambert-Eaton syndrome is a myasthenia gravis-like syndrome produced by small cell lung carcinoma, in which IgG autoantibodies are formed against presynaptic calcium channels at the neuromuscular junction leading to weakness of pelvic girdles, proximal limbs and trunk due to non-release of acetylcholine. IgG autoantibodies produced by small cell lung carcinoma can cause peripheral neuropathy and cerebellar degeneration.

CANCER-ASSOCIATED CACHEXIA

Cancer-associated cachexia is a syndrome characterized by marked body weight loss (>20%) due to ubiquitin proteasome pathways with specific irreversible loss of skeletal muscle and adipose tissue, weakness, anorexia, anemia, recurrent infections, increased cellular metabolism, high-energy expenditure, and decreased secretion of anabolic hormones in patients with cancers of esophagus, pancreas, gastric region, colorectal region, and lung.

- **Molecular pathogenesis:** Cancer-associated cachexia process involves numerous chemical mediators, e.g. tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interferon-γ (IFN-γ) and C-reactive protein (CRP) derived from the CSCs and inflammatory and immune cells such as macrophages within tumor microenvironment, that promote cellular catabolism of skeletal muscles and adipose tissue. In addition, metabolic, endocrine, and central nervous system perturbations combine with these chemical mediators to induce catabolic changes in skeletal muscle and adipose tissue. At the tissue level, mechanisms of cancer-associated cachexia include activation of inflammation, proteolysis, and lipolysis. Nutritional status of patient is compromised by direct response to malignancy-induced alterations in the cellular metabolism. Cytokine milieu and effects in cancer-associated cachexia are given in Table 6.105. Cancer-associated cachexia is shown in Fig. 6.127.

Table 6.105 Cytokine milieu and effects in cancer-associated cachexia

Cytokine	Cytokine Milieu and Effects
Tumor necrosis factor-α (TNF-α)	Lipolysis, skeletal muscle degradation, increased glucose turnover
Interferon-γ (IFN-γ)	Potentiates lipolysis, decreased protein synthesis
Interleukin-1 (IL-1)	Induction of anorexia, early satiety, peripheral proteolysis, potential release of IL-6
Interleukin-6 (IL-6)	Severe skeletal muscle wasting
Proteolysis	Skeletal muscle degradation
Lipid-mobilizing factor	Lipolysis

- **Tumor necrosis factor-α (TNF-α):** TNF-α increases glucogenesis, proteolysis, and lipolysis. It also decreases synthesis of glycogen, proteins, and lipids. TNF-α activates IL-1 production, that stimulates the expression of uncoupling proteins (e.g. UCP2 and UCP3) in cachectic skeletal muscles.
- **Interferon-γ (IFN-γ):** IFN-γ potentiates lipolysis, and decreased protein synthesis.
- **Interleukin-1 and 6 (IL-1 and 6):** IL-1 induces anorexia, early satiety, peripheral proteolysis, potential release of IL-6. **IL-1** induces anorexia in cancer cachectic patients by increasing plasma concentration of tryptophan, which in turn enhancing serotonin production and causing early satiety and suppressing hunger. Excess plasma tryptophan concentration resulting in increased serotonin production from the hypothalamus has been linked to anorexia. **IL-6** is an important mediator in the defense through its regulation of immune system. IL-6 may play a pivotal role in the development of cachexia.
- **Signal transducers and activation of transcription 3 (STAT3):** STAT3 is a member of STAT family of proteins. STAT3 plays an important role in cytokine-induced pathways, that regulate the embryonic development, cell proliferation, differentiation, and homeostasis of many tissues. STAT3 activation is a common feature of skeletal

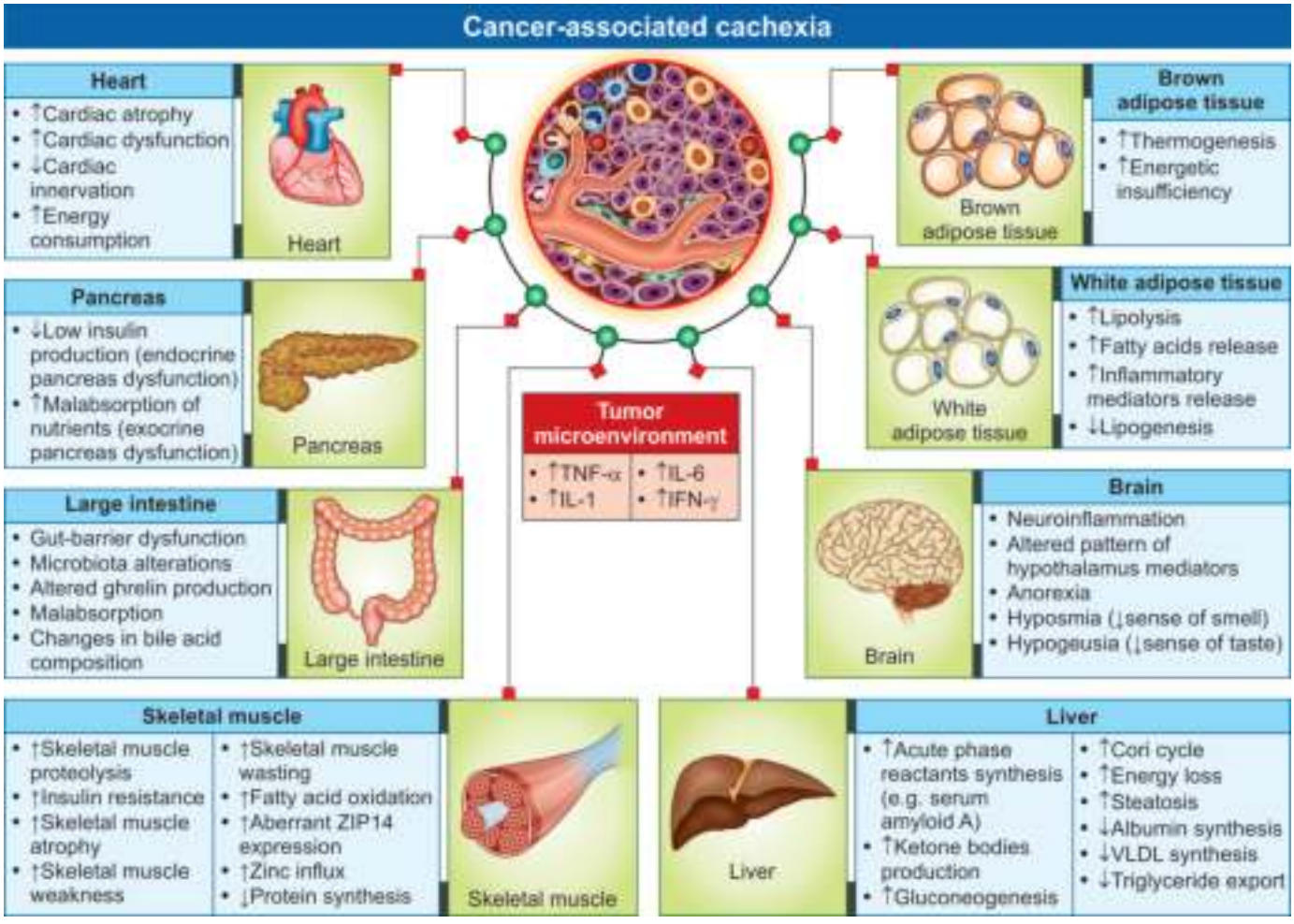


Fig. 6.127: Cancer-associated cachexia. It is host-phagocytic syndrome characterized by a continuous decline in skeletal muscle mass, with or without fat loss, which leads to progressive functional impairment, weakens the effects of chemotherapy, and increases mortality in patients with cancer.

muscle wasting. STAT3 is activated in skeletal muscle wasting by IL-6 in various malignant tumors, and sterile sepsis.

- **Management:** Patients experience impaired quality of life, reduced physical, emotional, and social well-being, and increased use of healthcare resources. The best management strategy of cancer-associated cachexia is to treat the cancer disease as this would completely reverse the cachexia syndrome.

Skeletal Muscle Wasting in Cancer-associated Cachexia

The maintenance of skeletal muscle mass is governed by dynamic processes that regulate protein synthesis and proteolysis. In cancer-associated cachexia, there is a chronic skeletal muscle protein degradation. Changes in the skeletal muscle protein as well as cell organelles, and cytoplasm affect the size of skeletal muscle fibers.

- Mechanical overload, anabolic hormone, insulin, insulin-like growth factor 1 (IGF-1) testosterone and β_2-adrenergic agonists stimulate protein synthesis in skeletal muscle leading to increase in size of skeletal muscle fiber. Binding of insulin and insulin-like growth factor 1 (IGF-1) to the receptors of skeletal muscle fiber activates the phosphatidylinositol 3-kinase (P13K)/AKT/mTOR-mediated signaling propagation system and promotes protein synthesis in skeletal muscle and inhibits its proteolysis.
- **Ubiquitin proteosome pathway (UPP)** is highly-selective proteolytic system, that plays crucial role in protein quality control, cell proliferation, embryonic development, signal transduction, transcriptional regulation, receptor downregulation, and synaptic plasticity. In fact, marked uncontrolled upregulation of UPP pathway underlies the pathogenesis of skeletal muscle wasting in many myopathies and cancer cachexia. These proteolytic systems are activated by various stimuli such as oxidative stress, inflammatory cytokines, growth factors (myostatin/activin A) and glucocorticoids.
- **Myostatin and activin A growth factors** play a central role in the striated muscle growth and skeletal muscle loss in various diseases. Uncontrolled activation of myostatin and activin A cause skeletal muscle wasting in cancer patients. An increase in the extracellular proteases and reactive oxygen species (ROS), severe decrease in pH and follistatin glycoprotein activate myostatin and activin A growth factors.
  - In skeletal muscle fibers, binding of myostatin and actin A to their receptors activate downstream processes, that inhibit IGF-1 P14K/AKT hypertrophy signaling pathway through phosphorylation of AKT resulting in the translocation of SMAD2/SMAD3, and FOXO1 transcription factors into the nucleus and the upregulation of proteolytic genes, and thus inhibits myoblast growth and myogenic differentiation through the suppression of PAX3/PAX7 and myoD.
  - The cycle of events initiates skeletal muscle wasting by upregulating transcription of **atrogin 1**, **MURF1** and **E2 ligases**, which always remain active in the ubiquitin proteasome pathway.
- Recent studies have revealed several emerging mechanisms that may contribute to skeletal muscle wasting including malnutrition, excess cytokines that downregulate genes involved in protein synthesis and upregulation of the ubiquitin proteasome pathway (UPP).

Weight Loss in Cancer-associated Cachexia

Weight loss in cancer-associated cachexia and starvation occurs due to loss of adipose tissue and skeletal muscle mass. In starvation, ketone bodies are produced from the lipid metabolism in the liver, and fat replaces glucose as an energy source and prevents loss of skeletal muscle mass. Depending on the malignant tumor type, weight loss occurs in 30–80% of cancer patients.

- Patients with gastric carcinoma and pancreatic carcinoma have the highest frequency of weight loss. Weight loss frequency is much less in the patients with breast carcinoma, acute myeloid leukemia, non-Hodgkin's lymphoma and sarcoma. Head and neck cancers can cause dysphagia and weight loss.
- Weight loss is an important prognostic factor in cancer; higher the extent of weight loss, the shorter the survival span. Patients with cancer cachexia have fatal outcome, when there is 25–30% of total body weight loss. Proteolysis-inducing factors have been detected, which cause skeletal muscle wasting. Increased energy expenditure is also considered as a factor that contribute to the wasting process.

Increased Resting Energy Expenditure in Cancer-associated Cachexia

Energy expenditure is a metabolic calculator determined by three factors: basal metabolic rate, diet-induced thermogenesis, and physical activity. The best predictor for 24-hour energy expenditure refers to resting energy calculated by indirect calorimetry. Resting energy expenditure (REE) in cancer patients varies with the type of malignant disease.

- **Resting energy expenditure (REE)** is high in lung carcinoma and pancreatic carcinoma. However, REE is not increased in patients with gastric carcinoma and colorectal carcinoma. Reasons for REE alterations depend on acute phase response protein and thermogenesis.

- Acute phase response alterations occur in the liver, where there is a shift from production of albumin to acute phase proteins like C-reactive protein (CRP), fibrinogen, serum amyloid associated (AA) proteins, α_2-macroglobulin and α_1-antitrypsin. There is an increase in the level of C-reactive protein in cachexia patients with head and neck squamous cell carcinoma. Acute phase response induces the rate of loss of body mass. Increased REE occurs due to enhanced thermogenesis in brown adipose tissue (BAT) and skeletal muscle.
- White adipose tissue acts as a fat store, whereas function of brown adipose tissue is thermogenesis related proportional to the oxidative phosphorylation in the mitochondria essential for cellular respiration, and presence of uncoupling proteins in the mitochondria.

- **Uncoupling proteins (UCPs)** mediate protein leakage across the inner mitochondrial membrane decreasing the level in the coupling of cellular respiration to ADP phosphorylation.
  - UCP1 is detected in brown tissue. UCP3 is present in brown tissue and skeletal muscle.
  - Increased levels of UCP3 mRNA in cancer are responsible for increased energy expenditure and tissue catabolism.
  - The increase in UCPs is also associated an increase in circulatory fatty acids. It appears that some cytokines and tumor lipid mobilizing factors (LMFs) can increase the levels of UCP in both brown adipose tissue and skeletal muscle.

Anorexia in Cancer-associated Cachexia

Anorexia is an important cause of weight loss in cancer-associated cachexia. The cancer is associated with activation of proinflammatory cytokines and neuroendocrine responses. These pro-inflammatory cytokines result in reduced food intake and metabolic change. Adrenergic activation and malignant tumor-related lipolytic factors lead to enhanced lipolysis.

- The effects of hypogonadism, insulin resistance, adrenergic activation, and systemic inflammation coupled with semistarvation lead to skeletal muscle atrophy. Protein synthesis in liver and its export is stimulated as part of the acute phase response. In addition, futile substrate of Cori cycle contributes to hypermetabolism. Recent studies also suggest that inflammation plays a role in the increased lipolysis in adipose tissue, and proteolysis in skeletal muscle.
- Primary cause of anorexia is due to increase in proinflammatory cytokines and lactate. Cytokines and lactate modulate the central nervous system neurotransmitter cascades. Numerous causes of anorexia in cancer cachexia can be categorized as being due to central or peripheral mechanisms.
  - Central causes of anorexia in cancer cachexia can be due to alterations in neurotransmitters (e.g. serotonin and corticotropin-releasing factor) leading to depression. Infusion of interferon in cancer patients increases kyreunine/keurinic acid.
  - Peripheral causes of anorexia in cancer cachexia occur in various settings: (a) malignant tumors directly impinging on gastrointestinal tract function, (b) malignant tumors producing substances (e.g. lactate, tryptophan, or parathormone-related peptide) that alter food intake, (c) malignant tumors causing alterations in nutrients such as zinc, or (d) malignant tumor inducing inflammation and cytokine synthesis. Chemotherapy can alter taste perception (dysgeusia) and cause nausea, vomiting, abdominal cramps, bleeding, and paralytic ileus.

PARANEOPLASTIC SYNDROMES

Paraneoplastic syndromes are defined as signs and symptoms, that present distant from the site of malignancy. It is important to recognize paraneoplastic syndromes for several reasons: (a) signs and symptoms of the paraneoplastic syndromes may be the clinical symptoms of a primary malignant tumor, (b) paraneoplastic syndromes may be mistaken for advanced metastatic disease, that need appropriate therapy, and (c) paraneoplastic syndromes can be disabling and treated to relieve palliative effects.

- Recent medical advances have improved the understanding of clinical manifestations, diagnosis, and treatment of paraneoplastic syndromes, which arise from malignant tumor secretion of hormones, peptides or cytokines or immune cross-reactivity between malignant tumor and normal tissues. Paraneoplastic syndrome in small cell lung carcinoma develops due to excessive production of ectopic hormones such as ACTH and ADH.
- Paraneoplastic syndromes may adversely affect diverse organ systems (e.g. brain and skeletal muscle), most notably the endocrine, neurologic, dermatologic, hematologic, and rheumatologic systems associated with the presence of malignancies without direct association with primary malignant tumor invasion and metastasis.
- Paraneoplastic syndromes can occur in the patients with breast carcinoma, gastric carcinoma, small cell lung carcinoma, ovarian carcinoma, pancreatic carcinoma, renal cell carcinoma and hematologic malignancies (i.e. leukemias/lymphomas).

- Symptoms of paraneoplastic syndromes vary depending on the organ systems affected. In >50% of cases, the signs and symptoms appear before an individual is diagnosed with a malignant tumor.
- In addition, patients present with fever, loss of anorexia and weight and night sweats.
- Cancer-associated paraneoplastic syndromes are given in Table 6.106.

Table 6.106 Cancer-associated paraneoplastic syndromes

Syndrome	Underlying Cancers	Mechanism by Synthesis of Molecules
Endocrinopathies manifestations		
ACTH-Cushing's syndrome	▪ Small cell lung carcinoma ▪ Pancreatic carcinoma ▪ Neural tumors	Production of ACTH or ACTH-like hormone
Hypercalcemia	▪ Squamous cell lung carcinoma ▪ Breast carcinoma ▪ Renal cell carcinoma ▪ Ovarian carcinoma ▪ Head and neck cancer ▪ Adult T cell leukemia/lymphoma	Production and release of polypeptide factor with close relationship to parathormone (PTH)
Hypocalcemia	Medullary thyroid carcinoma	Production of calcitonin
Syndrome of inappropriate antidiuretic hormone (SIADH) resulting in hyponatremia	▪ Small cell lung carcinoma ▪ Prostatic carcinoma ▪ Thymoma ▪ Gastric carcinoma ▪ Colon carcinoma ▪ Hodgkin's disease ▪ Non-Hodgkin's lymphoma ▪ Intracranial neoplasms	Production of ADH
Polycythemia	▪ Renal cell carcinoma ▪ Hepatocellular carcinoma ▪ Cerebellar hemangioma	Production of erythropoietin
Hypoglycemia	▪ Fibrosarcoma ▪ Ovarian carcinoma ▪ Medullary thyroid carcinoma ▪ Other mesenchymal sarcomas	Production of insulin-like hormone
Hypocalcemia	▪ Fibrosarcoma ▪ Ovarian carcinoma ▪ Medullary thyroid carcinoma	Production of calcitonin
Bone changes		
Hypertrophic osteoarthropathy and clubbing of fingers	▪ Bronchogenic carcinoma ▪ Thymoma	Bone changes due to periosteal reaction of distal phalanx, often associated with clubbing of nails
Antibody-mediated disorders		
Lambert-Eaton syndrome (myasthenia gravis-like weakness)	▪ Small cell lung carcinoma ▪ Thymoma	Immunologic-mediated disorder due to autoantibodies formed against presynaptic voltage-gated channels in the neuromuscular junction
Peripheral neuropathy	Small cell lung carcinoma	Immunologic-mediated disorder due to IgG autoantibodies against anterior and lateral horn cells of nervous system

Contd...

Table 6.106 Cancer-associated paraneoplastic syndromes (*Contd...*)

Syndrome	Underlying Cancers	Mechanism by Synthesis of Molecules
Stiff-Person syndrome (brain and spinal cord involvement)	▪ Breast carcinoma ▪ Small cell lung carcinoma	Immunologic-mediated disorder due to anti-amphiphysin and anti-GAD antibodies formed
Opsoclonus-myoclonus-ataxia syndrome (uncontrolled eye movements)	▪ Neuroblastoma ▪ Small cell lung carcinoma	Immunologic-mediated disorder due to anti-Ri and anti-Hu antibodies
Cerebellar degeneration	Small cell lung carcinoma	Immunologic-mediated disorder due to IgG autoantibodies against cerebellum resulting in ataxia, vertigo, and dysarthria
Dermatologic manifestations		
Seborrheic keratosis	Gastric carcinoma	Sudden appearance of numerous pigmented seborrheic keratosis (Leser-Trélat sign)
Dermatomyositis	▪ Bronchogenic carcinoma ▪ Breast carcinoma	Immunologic-mediated disorder
Acanthosis nigricans maligna (black verrucoid-appearing lesions)	▪ Lung carcinoma ▪ Gastric carcinoma ▪ Endometrial carcinoma	Acanthosis nigricans occurs by immunologic mechanism due to synthesis of epidermal growth by these cancers
Carcinoid syndrome	▪ Gastric carcinoma ▪ Pancreatic carcinoma ▪ Bronchial adenoma	Production of serotonin
Hematologic and vascular manifestations		
Disseminated intravascular coagulation	▪ Acute promyelocytic leukemia ▪ Prostatic carcinoma	Production of procoagulant factors, which activate coagulation cascade
Trousseau syndrome (superficial migratory thrombophlebitis results in venous thrombosis)	▪ Bronchogenic carcinoma ▪ Pancreatic carcinoma ▪ Solid metastatic tumors	Production of tumor products like mucins that activate clotting
Nonbacterial thrombotic endocarditis	Advanced malignancies	Hypercoagulable state
Renal manifestations		
Nephrotic syndrome	▪ Renal cell carcinoma ▪ Lung carcinoma ▪ Colon carcinoma ▪ Lymphomas	Tumor-derived antigen combined with antibody to form immune complexes deposited in glomeruli leading to glomerular injury
Male breast manifestation		
Gynecomastia	Choriocarcinoma of testis	β-hCG

- In some instances, the timely diagnosis of paraneoplastic syndromes may lead to detection of an otherwise clinically occult malignant tumors at an early and highly treatable stage in 20% of middle-aged persons.
- Treatments include addressing the underlying malignancy, immunosuppression for endocrine, neurologic, dermatologic, hematologic, and rheumatologic systems involved in paraneoplastic syndromes, and correction of electrolyte, and hormonal derangements for endocrinal paraneoplastic syndromes.

Endocrinal Manifestations of Paraneoplastic Syndrome

Endocrinal manifestations of paraneoplastic syndrome are caused by ectopic production of hormones or chemically unrelated substances inducing effects like those of a given hormone.

- The most common endocrinal manifestations include hypercalcemia, syndrome of inappropriate antidiuretic hormone secretion, Cushing's syndrome, and polycythemia. These endocrinal manifestations may be the presenting feature of underlying malignancy.

- Rarely endocrinal manifestations of paraneoplastic syndromes result from ectopic production of less frequently observed bioactive proteins such as growth hormone, human chorionic gonadotropin, insulin-like growth factors, renin and intestinal peptides.

Hypercalcemia

Malignancy-associated hypercalcemia occurs in about ≤10% of all patients with advanced cancer and generally associated with poor prognosis. Biochemical alterations include hypercalcemia (serum calcium >14 mg%), increased excretion of calcium and hydroxyproline containing peptide in the urine indicative of bone matrix destruction.

- Patient with cancer-related hypercalcemia presents with thirst, nocturia, and polyuria due to impaired renal concentrating ability, lethargy, hypotonia, muscular weakness, myoclonus, anorexia, vomiting, abdominal cramps, constipation, psychosis, and coma.
- The pathophysiology of hypercalcemia of malignancy is mainly through four principal mechanisms: (a) excessive secretion of parathyroid-related protein (PTHrP) by CSCs—known as humoral hypercalcemia of malignancy accounts for 80% of cases with squamous cell carcinomas, (b) binding of PTHrP receptor in bone and kidneys regulates bone resorption and renal handling of calcium and phosphate, (c) another 20% of cancer cases arise from osteolytic activity at the sites of skeletal metastases in the settings of breast carcinoma, squamous cell lung carcinoma, head and neck cancers, urinary bladder carcinoma, ovarian carcinoma and non-Hodgkin's lymphoma, and (d) rarely, hypercalcemia may result from secretion of 1,25-dihydroxyvitamin D (calcitriol) in the setting of certain lymphomas or other malignant tumors, which secreting ectopic PTH.

Cushing Syndrome

Cushing syndrome is caused by production of ACTH-like substances by small cell lung carcinoma, bronchial carcinoids, islets cell tumor of pancreas, and thyroid medullary carcinoma. Production of ACTH-like substance causes bilateral adrenal gland hyperplasia,that leads to high plasma concentration of cortisol.

- Biochemical abnormalities produced by continuous exposure to high plasma levels of ACTH-like substance and cortisol are manifested by potassium depletion leading to skeletal muscle weakness and wasting, polyuria and carbohydrate intolerance, sodium retention resulting in edema and hypertension.
- Patient presents with exaggerated facial roundness, weight gain around midsection and upper back, thinning of arms and legs, easy bruising and stretch marks. Pigmentation is a feature in some patients due to secretion of melanocyte-stimulating hormone as well as corticotropin.
- Confirmation of cancer-related Cushing syndrome is obtained by high concentration of 11-hydroxy-corticosteroids and the loss of suppression by exogenous glucocorticoids such as dexamethasone.
- Antihypertensive agents and diuretics, with careful monitoring of serum potassium, may also be used to control symptoms. Mifepristone, which binds competitively to the glucocorticoid receptor, has recently been shown to improve clinical and biochemical parameters of Cushing's syndrome.

Syndrome of Inappropriate Antidiuretic Hormone Secretion

Syndrome of inappropriate antidiuretic hormone (SIADH) secretion results in hyponatremia (decreased serum sodium) in the settings of small cell lung carcinoma (most common), prostatic carcinoma, thymoma, gastric carcinoma, colon carcinoma, Hodgkin's disease, and non-Hodgkin's lymphoma.

- Continued secretion of antidiuretic hormone (ADH) inappropriate to the body needs leads to overhydration in both the intracellular and extracellular compartments. The resulting cerebral edema leads to drowsiness, mental confusion, irritability, disorientation and ultimately to convulsions and coma. The clue to the diagnosis of SIADH is the presence of dilutional hyponatremia rather than depletion hyponatremia type.
- All other plasma constituents will be diluted by the increased plasma content of water (e.g. low blood urea, packed cell volume). Plasma osmolality, which depends on the plasma content of sodium and potassium will also be low.

Spontaneous Hypoglycemia

Spontaneous hypoglycemia occurs due to ectopic secretion of insulin-like hormone in the settings of hepatocellular carcinoma, mesothelioma and leiomyosarcoma that leads to hypoglycemia (blood glucose as low as 20 mg/dl). Non-islet cell tumor hypoglycemia due to increased synthesis of insulin-like hormone, inhibition of insulinase, and impairment of hepatic glucose-6-phosphatase activity are responsible for glucose hunger. Patient presents with sweating, flushing, hunger pain, tachycardia, and drowsiness terminating in convulsions and coma.

Hyperthyroidism

Choriocarcinoma and hydatidiform mole synthesize thyroid stimulating hormone-like substance that

leads to hyperthyroidism. Both serum protein-bound iodine level, and radioactive iodine uptake by thyroid gland are raised. Iodine uptake by thyroid gland is not suppressed by the administration of tri-iodothyronine or thyroxine. Patient presents with tachycardia, increased appetite, heat-tolerance, polydipsia, and muscle weakness.

Hypocalcemia

Fibrosarcoma, ovarian carcinoma and medullary thyroid carcinoma synthesize calcitonin-like protein, that causes hypocalcemia.

Precocious Puberty

Precocious puberty occurs due to increased concentrations of human chorionic gonadotropin in patients with testicular germ cell and Leydig cell tumors. Patient presents with spurt in both weight and height, deepening of voice, virilism, enlargement of penis, and testes and growth of pubic hair. Plasma testosterone and urinary excretion of 17-keto(oxo)steroids are elevated but not suppressed by the administration of exogenous glucocorticoids.

Gynecomastia in Men

Gynecomastia is found in association with tumor producing a luteinizing hormone, which stimulates production of estrogens. Testicular choriocarcinoma secretes β-hCG, that can cause gynecomastia. Males develop gynecomastia in the settings of testicular choriocarcinoma, breast carcinoma, lung carcinoma, choriocarcinoma, metastatic hepatocellular carcinoma, estrogen therapy in prostatic carcinoma, and malignancies treated by cytostatic chemotherapeutic agents.

Gastrin Producing Tumors

Zollinger-Ellison syndrome (**ZES**) is caused by secretion of gastrin by duodenal or pancreatic functional neuroendocrine tumors (e.g. gastrinomas).

- Zollinger-Ellison syndrome is characterized by severe peptic ulcer disease, gastroesophageal reflux disease (GERD, and chronic diarrhea caused by gastrinomas that results in excessive production of hydrochloric acid in the stomach.
- Zollinger-Ellison syndrome also occurs in association with multiple endocrine neoplasia 1 (MEN1). If Zollinger-Ellison syndrome is suspicious, screening for MEN1 needs to investigated by analyzing serum calcium, parathormone level, prolactin, and pancreatic polypeptide.
- Imaging studies are recommended to localize gastrinoma or to evaluate any metastases.

Polycythemia (Erythrocytosis)

Renal cell carcinoma, hepatocellular carcinoma, cerebellar hemangioma, and uterine leiomyoma synthesize excessive **ectopic erythropoietin (EPO)** that interacts with receptor-bearing cells in the bone marrow leading to erythroid hyperplasia, increased red blood cell production, hemoglobin concentration, and packed cell volume. Resection of the tumor reverse the hematological abnormality.

Neurologic Manifestations of Paraneoplastic Syndromes

Paraneoplastic syndromes result from immune cross-reactivity between cancer stem cells (CSCs) and components of the nervous system. In response to developing malignant tumor, patient produces cancer-directed antibodies known as onconeural autoantibodies.

- Because of antigenic similarity, these onconeural autoantibodies and associated onconeural antigen-specific T cells attack components of the nervous system. Immunosuppressive therapy is administered in paraneoplastic neurologic syndromes.
- Depending on the affected nervous system compartment, patient presents with cognitive and personality changes, ataxia, cranial nerve deficits, weakness, or numbness.
- Paraneoplastic neurologic syndromes can affect neuromuscular junction (e.g. Lambert-Eaton myasthenia syndrome), central nervous system (e.g. limbic encephalitis and cerebellar degeneration) or the peripheral nervous system (e.g. autonomic neuropathy and subacute sensory neuropathy).
- Paraneoplastic neurologic syndromes are diagnosed by imaging, serologies, electroencephalography, nerve conduction studies, electromyography, and cerebrospinal fluid analysis for signs of inflammation.
- Onconeural antibodies are classified according to three main categories: (a) cancer-associated onconeural antibodies include anti-amphiphysin, anti-Hu, anti-Yo anti-Ma2, anti-Ri and anti-recoverin, (b) partially characterized onconeural antibodies include ANNA-3, anti-mGluR1, anti-Tr, anti-Zic4, PCA-2, and (c) onconeural antibodies occurring in both cancer and non-cancer-associated syndromes include anti-acetylcholine receptor (AchR), anti-nicotinic AchR, anti-VGCC and anti-VGKC.

Lambert-Eaton Myasthenic Syndrome

Lambert-Eaton myasthenic syndrome (LEMS) is characterized by myasthenia gravis like skeletal muscle weakness as a result of autoantibodies formed against presynaptic voltage-gated channels in the neuromuscular junction in the settings of small cell lung carcinoma and thymoma.

- Clinical manifestations of Lambert-Eaton myasthenic syndrome (LEMS) are like myasthenia gravis, which include insidious and progressive skeletal muscle weakness, difficulty in walking, fatigue, tingling sensation in the hands or feet, eyelid dropping, dry mouth, difficulty in speaking and swallowing, urinary bladder and bowel changes and erectile dysfunction.
- The target of the attack of autoantibodies is different in myasthenia gravis as the acetylcholine receptor on the nerve is affected, whereas in LEMS, target of the attack of autoantibodies is the voltage-gated calcium channel on the nerve.

Peripheral Neuropathy

Peripheral neuropathy occurs due to IgG autoantibodies against anterior and lateral horn cells of nervous system in patients with small cell lung carcinoma. Symptoms of peripheral neuropathy depend on the type(s) and location(s) of the damaged nerves. The most common peripheral neuropathy symptoms include numbness, tingling sensations and shooting pain or burning in fingers or toes.

Stiff-Person Syndrome

Stiff-Person syndrome (SPS) is a neurological disorder, that occurs due to formation of anti-amphiphysin and anti-GAD antibodies in patients with breast carcinoma and small cell lung carcinoma.

- Patient presents with progressive muscle stiffness (rigidity) and repeated episodes of painful muscle spasms in the trunk and limbs, severely impaired disability, and heightened sensitivity to stimuli such as noise, touch and emotional distress.
- Stiff-Person syndrome is treated by γ-aminobutyric acid and immunotherapy.

Opsoclonus-Myoclonus-Ataxia Syndrome

Opsoclonus-myoclonus-ataxia (OMA) syndrome is an immune-mediated disorder of the nervous system, that occurs due to formation of anti-Ri and anti-Hu antibodies in patients suffering from neuroblastoma and small cell lung carcinoma. Patient presents with rapid involuntary conjugate fast eye movements, twitching of a skeletal muscle or group of skeletal muscles, cerebellar ataxia, aphasia, mutism, abnormal behavior, sleep dysregulation and difficulty in talking.

Cerebellar Degeneration

Cerebellar degeneration is a rare acquired neurological disorder characterized by ataxia, vertigo, and dysarthria. It occurs due to formation of **IgG autoantibodies** against cerebellum in patient suffering from small cell lung carcinoma.

Dermatologic and Rheumatologic Manifestations of Internal Malignancies

Dermatologic and rheumatologic manifestations of internal malignancies include a wide variety of non-malignant skin disorders in association with malignancy.

- Dermatologic manifestations strongly associated with internal malignancies include acanthosis nigricans maligna, triple palms, Basex's acrokeratosis paraneoplastica, erythema gyratum repens, necrolytic migratory erythema, paraneoplastic pemphigus, florid cutaneous papillomatosis (Leser-Trélat sign), paraneoplastic dermatomyositis, hypertrophic osteoarthropathy, necrolytic migratory erythema, erythema gyratum repens, acquired hypertrichosis lanuginosa and paraneoplastic pemphigus.
- When paraneoplastic dermatoses develop before an internal malignancy is diagnosed, recognition of these cutaneous disorders can aid in the diagnosis of the internal malignancy.

Carcinoid Syndrome: Cyanotic Episodic Flushes over Face and Chest

Carcinoid syndrome is associated with malignant argentaffin tumors of the midgut (i.e. small intestine, cecum and appendix constitute 80% of cases) metastasizing to liver.

- Carcinoid syndrome is associated with argentaffin tumors of bronchus, larynx, pancreas, gallbladder, and ovary. Bronchial carcinoid tumor secretes histamine and 5-hydroxytryptophan in systemic circulation, which lacks decarboxylase to convert the latter into 5-hydroxytryptamine (serotonin).
- Patient presents with watery diarrhea, abdominal cramps, bronchospasm and cyanotic episodic flushes over face and chest, with right-sided valvular stenosis of the heart caused by the secretion of histamine, 5-hydroxytryptophan, and 5-hydroxytryptamine (serotonin) into the portal circulation, and 5-hydroxyindoleacetic acid (**5-HIAA**) found in the urine.

Acanthosis Nigricans Maligna

Acanthosis nigricans maligna differs from a typical acanthosis nigricans, which is characterized by sudden development of extensive and severe skin lesions over mucosa, palms and soles. Papillomatous thickenings around the lips and eyes may be the presenting feature.

- The vast majority of acanthosis nigricans maligna are secondary to **gastric adenocarcinoma**. But, acanthosis nigricans maligna can also be found in cancers of colon, lung, uterus, ovaries, and urinary tract.
- Patient with gastric adenocarcinoma synthesizes epidermal growth factor (EGF) results in hyperkeratosis, and pigmentation of the axilla, neck, intertriginous areas, flexures, and anogenital region

known as acanthosis nigricans. Approximately 50% patients with gastric carcinoma develop acanthosis nigricans maligna.

Tripe Palms: Unusual Cutaneous Paraneoplastic Syndrome

Tripe palms are cutaneous manifestation of paraneoplastic syndrome in internal malignancy and characterized by a curious thickening of the palms with an accentuation of the normal dermatographia ridges and sulci.

- Patient presents with yellowish, velvety, diffuse palmar hyperkeratosis with accentuated dermatoglyphic patterns leading to rough appearance in the settings of gastric carcinoma and lung carcinoma in 50% of cases.
- Less commonly associated malignancies include cancers of head and neck, breast, and genitourinary tract.

Basex's Acrokeratosis Paraneoplastica

Basex's acrokeratosis paraneoplastica is associated with squamous cell carcinoma of the oral cavity, pharynx, larynx, and esophagus. Clinical features are similar to psoriasis. Patient initially develops poorly defined psoriasiform plaques involving ears (helices), nose, fingers and toes including nail changes. Later, larger cutaneous palmoplantar keratoderma with central clearing appears on cheeks followed by involvement of the legs, knees, thighs, and arms.

Erythema Gyratum Repens

Erythema gyratum repens develops in internal malignancy in 80% of cases and lung carcinoma in 20% of cases. Patient presents with widespread serpiginous, polycyclic, and pruriginous erythema, which is fast growing desquamative lesion around the edges.

Necrolytic Migratory Erythema

Necrolytic migratory erythema is associated with slow-growing malignant tumor **glucagonoma** derived from α cells of pancreas. Patient presents with unusual dermatosis, recent onset of diabetes mellitus and weight loss. Although cutaneous manifestations may precede the diagnosis of glucagonoma by several years. Cutaneous lesions develop within 10 days, which begin with erythematous patch that blisters centrally, erodes, and then crusts over and heals by hyperpigmentation.

Paraneoplastic Pemphigus

Paraneoplastic pemphigus is associated with B cell lymphoproliferative disorders, thymoma, and various sarcomas and carcinomas. Patient presents with painful blisters and denuded areas of the mouth, lips, esophagus, and skin.

Florid Cutaneous Papillomatosis

Florid cutaneous papillomatosis is associated with gastric carcinoma. Patient presents with rapid onset of numerous warty papules on the trunk and the extremities that are clinically indistinguishable from viral warts. A skin biopsy is required to establish diagnosis.

Leser-Trélat Sign

Leser-Trélat sign is characterized by the sudden eruptions and rapid increase in size, and number of seborrheic keratoses in the settings of malignancies of gastrointestinal tract, lung, kidney, liver, or pancreas.

Paraneoplastic Dermatomyositis

Paraneoplastic dermatomyositis occurs before the onset of proximal skeletal muscle weakness in 10–25% cases of malignancies of breast, ovary, lung, and prostate.

- Patient presents with purplish skin rashes on upper eyelids, erythematous rash on the face, neck, chest and shoulders, and scaly eruption over the phalangeal joints.
- Diagnosis of dermatomyositis is suggested by an elevated level of **creatine phosphokinase**, electromyography, and muscle biopsy. Histologic examination of muscle biopsy shows mixed B cell and T cell perivascular inflammatory infiltrate and perifascicular muscle fiber atrophy.
- Patients with dermatomyositis must be screened for malignancies by imaging modalities of the chest, abdomen, and pelvis.
- Dermatomyositis is treated by administration of glucocorticoids and immunotherapy.

Hypertrophic Osteoarthropathy

Hypertrophic osteoarthropathy is characterized by periostitis ad subperiosteal new bone formation along the shaft of long bones and distal phalanges (digital clubbing), joint swelling and pain in 10% cases of bronchogenic carcinoma.

- Vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and prostaglandin E_2 are possible contributors to hypertrophic osteoarthropathy.
- Bone scan demonstrates symmetric and concentrated tracer uptake along these bones.
- The symptoms of hypertrophic osteoarthropathy resolve with successful cancer therapy. Other treatment options include nonsteroidal anti-inflammatory drugs (NSAIDs), bisphosphonates, opioid analgesics, and localized palliative radiation.

Vascular Manifestations

Vascular manifestations of paraneoplastic syndromes occur in patients with adenocarcinomas and metastatic disease without male or female predominance.

Leukocytoclastic Vasculitis

Leukocytoclastic vasculitis of paraneoplastic syndrome occurs most commonly in patients with hematologic malignancies, lung carcinoma, gastrointestinal tract carcinoma, and urinary tract carcinoma.

- Patient presents with palpable purpura over the lower extremities accompanied by pain, burning and pruritus. Constitutional symptoms such as fever and malaise are also common.
- Paraneoplastic leukocytoclastic vasculitis is attributed to circulating tumor-associated antigens, which leads to antigen–antibody complex deposits in small blood vessels, complement fixation resulting in vasculitis.
- Treatment of the malignancy leads to resolution of inflammation of small blood vessels.
- In addition, other treatment options include colchicine and corticosteroids for mild to moderate disease. Methotrexate, azathioprine or IVIg may be considered for resistant disease.

Thrombotic Microangiopathy

Thrombotic microangiopathy of paraneoplastic syndrome has been associated with mucin-secreting gastric adenocarcinoma, mucin-secreting colon adenocarcinoma, lung carcinoma, breast carcinoma, hepatocellular carcinoma, pancreatic carcinoma, cholangiocarcinoma, prostatic carcinoma, renal cell carcinoma, ovarian carcinoma, and hematologic malignancies (Hodgkin's disease, non-Hodgkin's disease, acute lymphoblastic leukemia, multiple myeloma).

- Thrombotic microangiopathy is characterized by microvascular thrombosis, thrombocytopenia, and ischemic end-organ damage. Hemolytic uremic syndrome and thrombotic thrombocytopenic purpura are the two subtypes of thrombotic microangiopathy.
- Activation of coagulation system cascade and the ensuring procoagulant state in cancer occurs due to release of procoagulant factor from CSCs, defective anticoagulant and fibrinolytic mechanisms, and damaged vascular endothelium.
- Solid malignant tumor may express different procoagulant molecules including tissue factor, which complexes with factor VIIa to activate factor IX and factor X. Cancer procoagulant activates factor X. Mucin secreting adenocarcinoma can induce the formation of platelet microthrombi.
- Endothelial damage can increase the risk of thrombosis by increasing von Willebrand factor, dysfunctional vascular endothelium, impaired coagulation system, and defective fibrinolysis.

Trousseau's Syndrome (Migratory Thrombophlebitis)

Trousseau's syndrome is defined as a migratory thrombophlebitis of paraneoplastic syndrome (venous thromboembolism accompanied by inflammation) in the context of malignancy. **Pancreatic adenocarcinoma** synthesizes thromboplastin-like substance that results in migratory thrombophlebitis.

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation of paraneoplastic syndrome is an occasional complication of solid malignant tumors.

- Disseminated intravascular coagulation is characterized by the systemic activation of coagulation and results in the formation of thrombin throughout the microvasculature leading to consumptive coagulopathy. Widespread fibrin deposition within microvasculature leads to organ ischemia, and thrombotic microangiopathy.
- Disseminated intravascular coagulation is triggered by release of thromboplastic substances into the circulation in the settings of acute promyelocytic leukemia, mucin secreting gastrointestinal carcinoma, and prostatic carcinoma.
- Disseminated intravascular coagulation (DIC) has two consequences, i.e. (a) widespread fibrin deposition within microvasculature leads to organ ischemia and thrombotic microangiopathy, and (b) consumptive coagulopathy, and release of plasminogen activators.
- Acute DIC results in bleeding tendencies, whereas chronic DIC presents with thrombotic phenomena.
  - Patient presents with cyanosis, cerebral stroke, convulsions, acute renal failure, breathlessness, shock and coma.
  - Laboratory findings in DIC include thrombocytopenia and increased values of activated partial thromboplastin time (APTT), prothrombin time (PT) and D-dimers.
  - DIC is medical emergency that requires immediate administration of anticoagulants (e.g. heparin) and fresh frozen plasma (FFP).

Nonbacterial Thrombotic Endocarditis of Paraneoplastic Syndrome

Nonbacterial thrombotic endocarditis of paraneoplastic syndrome is characterized by the presence of sterile cardiac vegetations in aortic or mitral valves associated with systemic arterial thromboembolism. Nonbacterial thrombotic endocarditis is a common complication of lung adenocarcinoma, mucus secreting gastric

carcinoma, colorectal carcinoma, pancreatic carcinoma, breast carcinoma, cervical carcinoma, multiple myeloma, leukemias and non-Hodgkin's lymphoma.

Hematologic Manifestations

Hematologic manifestations of paraneoplastic syndrome are rarely symptomatic, which are usually detected after diagnosis of metastatic disease, which may improve with successful treatment of the underlying malignancy.

Secondary Eosinophilia

Secondary eosinophilia in hematology is a manifestation of paraneoplastic syndrome occurs as a result of production of eosinophil growth factors such as GM-CSF, IL-2, IL-3, and IL-5 in the settings of leukemias, non-Hodgkin's lymphoma, lung carcinoma and gynecologic malignancies.

- In contrast, primary eosinophilia is a separate hematologic clonal neoplastic disorder associated with gene rearrangements involving FIP1L1, PDGFRA, PDGFRB and FGFR1.
- Paraneoplastic eosinophilia is typically asymptomatic; however, some patients can develop dyspnea and wheezing, which may respond to corticosteroid therapy.

Agranulocytosis

Agranulocytosis is a hematologic manifestation of paraneoplastic syndrome occurs in about 15% of patients with solid malignant tumors of lung, breast, gastrointestinal tract, brain, and kidney including gynecologic cancers.

- White blood cell count ranges from 12 to 30×10^9/L, but in some patients exceed 5×10^9/L. Some solid malignant tumors have been shown to produce colony-stimulating activity.
- Alternatively, leukocytosis may result from bone marrow involvement by solid malignant tumor. Agranulocytosis manifestation of paraneoplastic syndrome does not require treatment.

Pure Red Cell Aplasia

Pure red cell aplasia (PRCA) with hematologic manifestation of paraneoplastic syndrome is most often associated with **thymoma**. Ineffective eradication of autoreactive T cells by neoplastic thymic tissue results in an autoimmune attack on red blood cell precursors.

- Pure red cell aplasia (PRCA) can also occur in settings of **leukemia** and **lymphoma** due to autoimmune dysfunctional erythropoiesis. It is worth mentioning that PRCA can occur in the settings of myelodysplasia and nonmalignant disorders such as infections with parvovirus B19, HIV, HBV, HCV, and herpesviruses.
- Bone marrow examination demonstrates failure of erythropoiesis but preservation of granulocyte lineage and megakaryocytes.
- Pure red cell aplasia with hematologic manifestation of paraneoplastic syndrome is treated by cancer therapy and immunosuppression. Corticosteroids, azathioprine, antithymocyte globulin, cyclophosphamide, cyclosporine A and monoclonal antibodies (e.g. rituximab and alemtuzumab) are being administered in these patients. Plasma exchange and androgen therapy are also used to treat these cases.

Thrombocytosis

Thrombocytosis occurs in about 35% of cancer patients. Thrombocytosis is defined by platelet count $>400 \times 10^9$/L.

- Thrombocytosis occurs due to malignant tumor production of cytokine IL-6. Serum IL-6 levels are analyzed to distinguish paraneoplastic syndrome-associated thrombocytosis, and reactive thrombocytosis processes from clonal etiologies (e.g. essential thrombocytopenia, polycythemia vera, myelodysplastic syndrome, acute leukemias and chronic leukemias).
- JAK2 V617F gene mutation is present in 50% cases of essential thrombocytopenia but not present in cases of reactive thrombocytosis.
- Thrombocytosis in paraneoplastic syndrome is usually associated with advanced cancer disease.

Autoimmune Hemolytic Anemia

Autoimmune hemolytic anemia (AIHA) of paraneoplastic syndrome is associated with malignant solid tumors (e.g. non-Hodgkin's lymphoma).

- Patients with AIHA as paraneoplastic syndrome manifestation are often refractory to corticosteroid therapy. AIHA occurs due to production of autoantibodies against tumor antigens, which cross react with erythrocyte antigen band 3.
- These patients are treated by targeted immunotherapy against autoimmune hemolytic anemia and solid tumors.

Renal Manifestations

Renal manifestations of paraneoplastic syndrome indirectly compromise glomerular and tubular function by electrolyte imbalance, hormone producing tumors or deposition of antigen–antibody complexes in the glomeruli. An early diagnosis and effective treatment

might improve quality of life and alter prognosis of these patients.

- Immune complex-mediated secondary glomerulonephritis has been associated with renal cell carcinoma, lung carcinoma, colon carcinoma, Hodgkin's disease, and non-Hodgkin's lymphoma.
- Tumor-derived antigen combined with antibody to form immune complexes, which are deposited in the glomeruli leading to glomerular injury. Patient presents with features of nephrotic syndrome, which is characterized by massive proteinuria (≥3.5 g/24 hours), hypoproteinemia, hypercholesterolemia, and generalized edema.

HOST DEFENSE AGAINST CANCER

The immune system can recognize and destroy nascent cancer stem cells in a process termed cancer immunosurveillance, which functions as important defense against cancer.

- Potential strength of immune surveillance is based on the knowledge that CSCs express tumor-associated antigens, and tumor-specific antigens (TSAs) of cancer that can be recognized by the immune system as foreign elements.
- Tumor-associated antigens (TAAs) and TSAs of cancer are categorized in four groups: (a) unique antigens specific for an individual malignant tumor, (b) overexpression of peptides or proteins that are found in normal tissues, (c) shared tumor antigens, and (d) viral antigens that may be shared among different cells, but confined to malignancy.
- Natural killer cells and gamma-delta T cells (γδ-T cells) are capable of specifically recognizing and killing CSCs. Cancer patients generate CD4+ helper T cells, CD8+ cytotoxic T cells and B cells specific for these tumor antigens.
- Tumor-specific lymphocytes are found infiltrating malignant tumors and in the peripheral blood of cancer patients. Macrophages are also part of the nonspecific natural immune response to CSCs, which are activated by interferon-gamma (IFN-γ).
- Development of cancer can be explained by the ability of CSCs to evade immune destruction either due to failure of immune system to be adequately generated or by induction of immune tolerance or other inhibitory mechanisms that allows malignant tumor to escape immune detection and destruction.
- In contrast to malignant tumors induced by carcinogens, in which each malignant tumor has unique antigenic specificity regardless of their morphologic appearance of malignant tumors induced by viruses consistently express antigens that cross-react with other malignant tumor induced by the same or similar viruses even though their morphologic appearance may differ.
- The importance of the immune system in preventing cancer is reflected in the increased frequency of malignancies in immunosuppressed and immunodeficient patients. Cancer immune evasion is a major block in designing effective anticancer therapeutic strategies.

CANCER IMMUNOSURVEILLANCE AND IMMUNOEDITING

Carcinogenesis is a multistep process resulting from cross-talk of CSC intrinsic factors and host system effects. During cancer immunosurveillance, immunoediting process consists of three phases: elimination, equilibrium, and escape.

- **Immunoediting elimination phase:** Immunoediting elimination phase corresponds to cancer immunosurveillance in which nascent CSCs are successfully recognized and eliminated by the immune system, thus returning the tissues to their normal state of function.
- **Immunoediting equilibrium phase:** Cancer stem cells (CSCs) that elude the surveillance phase will progress to immunoediting equilibrium phase, where malignant tumor expansion and metastasis are minimal (tumor dormancy), and usually occur without symptoms. In a second scenario, constant interaction of immune system leads to immunoselection of malignant tumor that has been shaped into less-immunogenic state.
- **Immunoediting escape phase:** As immune system may eventually eliminate CSCs during immunoediting equilibrium phase. CSCs are no longer susceptible to immune attack leading to immunoediting escape phase.

Innate Immune System Role in Tumor Immunity

Innate immune system plays an important role in resistance against the development and progression of malignant tumor growth. Macrophages and mast cells activate vascular and fibroblasts, which eliminate cancer cells and initiate local tissue repair.

- Dendritic cells, on the other hand, take up tumor antigens and migrate to lymphoid organs, where dendritic cells process peptides and present to T cells for the induction of specific antibody by B cells and cell-mediated immune responses.
- Natural killer cells also play key role in cellular cross-talk between innate and adaptive immune cells through their ability to interact bidirectionally with dendritic cells.
- Natural killer cells regulate dendritic cells by two mechanisms: (a) elimination of immature dendritic

cells and (b) promotion of maturation and activation of dendritic cells. Induction of efficient adaptive immune response requires direct interactions with tumor-associated antigen-presenting cells (APCs) and a strong proinflammatory milieu.

Adaptive Immune System Role in Tumor Immunity

Tumor-associated antigens (TAAs) consist of short amino acid peptide segments, which are recognized by T cells through T cell receptors (TCRs) in the context of MHC-I molecule on the surface of CSCs and MHC-II molecule on the surface of antigen-presenting cells (APCs). Tumor-associated antigens are processed by exogenous and endogenous pathways.

- MHC (HLA) class I molecule is recognized by CD8+ cytotoxic T cells. MHC (HLA) class II molecules are chiefly found on immunocompetent professional APCs such as macrophages, dendritic cells (DCs), Langerhans cells, B cells, and CD4+ helper T cells.
- Antigen-presenting cells (APCs) present antigen to naïve CD4+ helper T cells, because of their ability to process endocytosed antigens, and partly possessing cell surface proteins, that bind to T cell surfaces. CD4+ helper T cells become activated to synthesize an array of cytokines. Activated CD4+ helper T cells mature and differentiate into CD4+ helper T cell populations (**Th1, Th2, Th17** and **Treg**). Activated CD4+ helper T cells promote cell-mediated immunity and antibody production through B cell interaction.
- Tumor-associated antigens (TAAs) shed from CSCs are endocytosed by resident dendritic cells (DCs) in the tumor microenvironment, and following cognate presentation of MHC-I molecule or MHC-II to CD8+ cytotoxic T cells and CD4+ helper T cells (Th17) activation respectively.
- There is clonal expansion of antitumor CD8+ cytotoxic T lymphocytes (CTLs), that secrete proangiogenic factors (e.g. VEGF) and chemotactic factors for recruitment of dendritic cells (DCs). Activation of Th17 cells synthesize IL-17 that induces CSCs to secrete chemotactic factors (e.g. CXCL9 and CXCL10) leading to the recruitment of additional cytotoxic effector cells (i.e. natural killer cells and CD8+ cytotoxic T cells).

Cross Presentation and Priming of Tumor Antigens

Antigen-presenting cells (APCs) process and present tumor antigens to naïve T cell, and induce antitumor immune responses. T cell activation requires two distinct signals (signal 1 and signal 2).

- **Signal 1** is transmitted by the interaction between T cell receptor (TCR) and antigenic peptide fragments on MHC molecules on APCs.
- **Signal 2** is transmitted by one of co-stimulatory molecules, i.e. binding of CD28 molecule on T cells with B7 family molecules on APCs. CSCs frequently stimulate signal 1 alone and inefficiently signal 2 activate naïve T cells in a process called cross-priming. Accordingly, cancer cells preferentially induce T cell unresponsiveness or immune tolerance.

CD8+ Cytotoxic T Cells and Natural Killer Cells Role in Killing Cancer Stem Cells

Antigen-presenting cells (APCs) process cancer-peptide-loaded MHC-I molecule and present to CD8+ cytotoxic T cells leading to effective CSC killing.

- During transformation of a normal cell to CSC, the CSCs may lose MHC-I molecule on its cell membrane as a part of its evasion strategy to elude its destruction by CD8+ cytotoxic T cells. In the event of loss of MHC-I molecule expression by CSCs, natural killer cells interaction with CSCs can only occur through killer-activating receptor (KAR) ligand, leading to their destruction.
- Natural killer cells can also destroy CSCs by antibody-dependent cytotoxicity (ADCC) mechanism, where the Fab portion of an IgG antibody produced by B cells binds to surface tumor-specific antigen and bridges to the Fc receptor on the natural killer cells.

CD4+ Regulatory T Cells (Treg Cells) Role in Tumor Immunity

CD4+ regulatory T cells (Treg cells) are recruited in the periphery of the tumor microenvironment. Treg cells suppress the immune responses directed against tumor-associated antigens (**TAAs**) by influencing the activity of antigen-presenting cells (APCs) and dendritic cells (DCs), which become dysfunctional by the elaboration of immunoregulatory cytokines IL-10 and TGF-β in the tumor microenvironment.

- T cells are divided into three populations: (a) classic CD4+ CD25+ FOXP3+ regulatory T cells, (b) CD4+ IL-10+ FOXP3– regulatory T cells, and (c) CD4+ TGF-β+ T cells.
- CD4+ regulatory T cell-mediated immune system suppression evades CSCs immune destruction is crucial and it is the main hurdle for the successful tumor immunotherapy. CSCs secrete CCL22 chemokine, that binds to CCL4 chemokine receptor on recruited Treg cells.
- Following cytokines release from CSCs, TAAs are taken up by DCs resulting in activation of effector CD4+ CD25– T cells.
- Cancer stem cells (CSCs) secrete TGF-β that converts CD4+ CD25– T cells into CD4+ CD25+ Treg cells leading to further immunosuppression activity. VEGF secreted by CSCs stimulates angiogenesis.

Antibody-mediated Cancer Stem Cells Destruction

In addition to indirect beneficial role of antibody in the ADDC mechanism of CSCs destruction, and can play a direct role in tumor immunity. Antibody directed to TAAs together with activation of complement system can destroy the CSCs by cytolytic action of the C5b–C9 **MAC** complement cascade. TAA alone or as antigen–antibody complexes can interfere with the CD8+ cytotoxic T cell destruction of CSCs by blocking its activity.

CANCER STEM CELLS EVASION OF IMMUNE RESPONSE

Cancer stem cells (CSCs) escape immune destruction by many potential mechanisms: (a) downregulation of potential target tumor-associated antigens, (b) inhibition of the T cell response, and (c) direct modulation of pro-inflammatory cytokines. These CSC evasion mechanisms may serve as novel targets for cancer therapy.

Role of Immunoregulatory Enzyme Indoleamine 2,3-Dioxygenase

Cancer stem cells (CSCs) escape immune destruction resulting from activation of immunoregulatory enzyme **indoleamine 2,3-dioxygenase (IDO)** produced by macrophages and other immunoregulatory cells. Upregulation of enzyme IDO leads to conversion of tryptophan to kynurenine, which are involved in malignant tumor growth and immunosuppression by blunting T effector cell function as well as recruitment of Treg cells. Enzyme IDO dysregulation induces malignant tumor growth through its ability to inactivate tumor suppressor gene **Bin-1**, as well as by incapacitating effective tumor immunosurveillance mechanisms.

Inflammatory Cytokines Synthesis in Tumor Microenvironment

The inflammatory tumor microenvironment consists of numerous cells, cytokines, enzymes and signaling pathways. Cancer-related inflammation possesses both pro-tumor and anti-tumor features. Activated immune cells induce the production of pro-inflammatory cytokines, which bind to cognate receptors and enzymes, which inhibit malignant tumor growth.

- TNF-α contributes to epithelial–mesenchymal transition (EMT), CSC proliferation and angiogenesis. TGF-β contributes to EMT, evasion of immune destruction of CSCs, and suppresses apoptosis. IL-6 activates STAT proteins, suppression of apoptosis leading to release of reactive oxygen species (ROS) and reactive nitrogen species (RNS).
- Cancer-related inflammation is also involved in initiation, promotion, and progression by various mechanisms.
  - The initiation of carcinogenesis requires a series of genetic mutations and epigenetic modifications, that lead to the activation of tumorigenic pathway as well as loss of tumor suppression.
  - In inflammatory tumor microenvironment, immune cells (i.e. macrophages and neutrophils) synthesize reactive oxygen species (ROS) as well as reactive nitrogen species (RNS), which can cause DNA damage leading to initiation of tumorigenesis process.
- Furthermore, genetic alterations can be induced by cytokines secreted by immune cells in the inflammatory tumor microenvironment. Excess production of TNF-α and other proinflammatory cytokines induce systemic effects of fever, weight loss, malaise, cachexia in cancer patients with extensive disease.

Chronic Inflammation in Tumor Immunity or Progression

Chronic inflammation perturbs innate or adaptive immune responses that initiates tumorigenesis in the settings of ulcerative colitis, rheumatoid arthritis, and pancreatitis. Some infectious pathogens can initiate tumorigenesis.

- *Helicobacter pylori* causes chronic gastritis , metaplasia and dysplasia in infected hosts leading to development of gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma.
- Hepatitis B virus (HBV) or hepatitis C virus (HCV) are linked to chronic hepatitis, cirrhosis, and hepatocellular carcinoma.
- Human papillomavirus (HPV 16, 18) has been linked to development of cancers of uterine cervix, vulva, vagina, anus, and penis.
- Unresolved inflammation resulting from exposure to carcinogenic agents, ultraviolet rays, cigarette smoking, gastroesophageal reflux disease (GERD), and failure of elimination of injurious agents have been linked to development of cancers.

Myeloid-derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells, which have potent mechanism to inhibit T cell and natural killer cell activity to promote malignant tumor growth, development of premetastatic niche, and contribute to resistance to immunotherapy.

- In the bone marrow, hematopoietic stem cells (HSCs) can differentiate into common progenitor cells, and then to immature myeloid cells under the influence of several growth factors (i.e. GM-CSF, M-CSF, SCF, IL-3 and FLT-3). The immature myeloid cells migrate into peripheral tissues, where they can differentiate and participate in either physiologic or pathologic conditions according to the local environment.

- In pathologic conditions there is a partial block in the differentiation of immature myeloid cells, which leads to abnormal expansion of myeloid-derived suppressor cells (MDSCs).
  - MDSCs express STAT-induced immune suppressive factors, such as arginase 1, inducible nitric oxide synthase (iNOS) and reactive oxygen species.
  - MDSCs also induce downregulation in expression of cell surface molecules like MHC-I, and subsequent suppression of antigen-specific T cell activation.

Innate Lymphoid Cells

Innate lymphoid cells (ILCs) lack rearranged antigen-specific receptor (TCR or BCR). In response to tissue damage, ILCs contribute to immunity via the secretion of signaling molecules (cytokines IL-4, IL-5, IL-13, IL-22), and regulation of both innate and adaptive immune responses. ILCs are abundant in tissues of skin, lung, liver, and gastrointestinal tract.

- Innate lymphoid cells (ILCs) are immune cells derived from common lymphoid progenitors (CLPs), which differentiate to produce committed ILC progenitors leading to formation of cytotoxic ILCs (e.g. CD8+ cytotoxic cells, NK cells), and helper T cell-like ILCs (i.e. ILC1, ILC2, ILC3 subsets).
- CD8+ cytotoxic ILCs secrete IFN-γ that kill CSCs and inhibit their proliferation. CD8+ cytotoxic ILCs also stimulate NK cells and upregulate the expression of MHC class I and MHC class II molecules on CSCs, as well as adhesion molecules and transcription factors in T cells that mediate CSCs killing. IFN-γ also induces the expression of CXCL10/IP-10 and MIG, a member of the CXC subfamily of chemokines in endothelial cells, both of which inhibit tumor angiogenesis.

TUMOR MARKERS: DIAGNOSTICS IN CLINICAL PRACTICE

Current clinical practice in oncology has a growing impetus on screening, early diagnosis, proper prognostication, staging, evaluation in postoperative cases, monitoring therapeutic response, surveillance for recurrence, and targets for therapeutic intervention.

- Cancer stem cells (CSCs) produce substances, many of which are proteins, which are helpful in diagnosis and monitoring of treatment. Tumor markers include a variety of substances like cell surface antigens, cytoplasmic proteins, enzymes, isoenzymes, hormones, oncofetal proteins, receptors, oncogenes, and their products. Detection of tumor markers can be done either in tissues or body fluids like pleural fluid, ascitic fluid, serum, urine, and cerebrospinal fluid.

Table 6.107 Characteristics of an ideal tumor marker

Characteristics	Comments
Highly sensitivity tumor marker	Tumor marker is detected in patients with malignant tumor and not detectable in physiologic or benign disease states
Long lead-time over clinical diagnosis	Sufficient time for alteration of natural course of disease
Levels correlate with malignant tumor burden	Prognostic and predictive utility of the tumor marker
Short half-life of tumor marker	Frequent serial monitoring of the tumor marker levels after 5–6 half-lives
Simple and cheap tumor marker test	Applicability as screening test to detect cancer
Easily obtainable specimens for testing	Acceptability of the tumor marker by target population

- An ideal tumor marker has important characteristics: (a) it should be highly-specific to a given malignant tumor, (b) it should provide a lead time over clinical diagnosis, and (c) it should be highly-sensitive to avoid false-positive results. In addition, tumor marker should correlate reliability related to malignant tumor burden, accurately reflecting disease progression and/or regression by doing serial tumor marker analysis. Characteristics of an ideal tumor marker are given in Table 6.107.
- Carcinoembryonic antigen (CEA) represents dedifferentiation process of cancer. HER2/neu and prostate-specific antigen (PSA) reflect increased cellular proliferation. Some tumor markers such as β-hCG and α-fetoproteins are estimated to monitor the response to therapy in germ cell tumors of testes.

MOLECULAR BASIS OF TUMOR MARKERS

Genetic alteration in malignant tumor can be reflected at various levels from viral genome incorporation to genetic defects, which form the molecular basis of tumor markers. Molecular basis of tumor markers is given in Table 6.108.

TUMOR MARKERS: DETECTION METHODS

Detection methods of tumor markers can be classified into six major groups. The most common method in use today is serological enzyme assays. Methods of detection of tumor markers are given in Table 6.109.

- Immunological detection of tumor markers relies on monoclonal antibodies, that specifically bind to epitopes on CSCs and are in turn tagged for

Table 6.108 Molecular basis of tumor markers

Levels of Classification of Tumor Markers	Examples of Tumor Markers
Deoxyribonucleic acid (DNA) level	
Epigenetic alterations	Promoter hypermethylation, e.g. DAP in lung cancers, p15, p16 in hepatocellular carcinoma
Endogenous alterations	Mutations, e.g. NADH dehydrogenase 4
Mitochondrial alterations	ND4 analysis in urine in patients with urinary bladder carcinoma
Gene level	
Oncogene	KAS mutation in pancreatic carcinoma, microsatellite alterations in head and neck cancers
Exogenous viral oncogene	EB virus in nasopharyngeal carcinoma and Burkitt's lymphoma; HPV in cervical carcinoma
Ribonucleic acid (RNA) level	
Cell based endogenous RNA	Tissue-specific markers (PSA mRNA in prostatic carcinoma, cytokeratin 20 mRNA in breast carcinoma)
Cell-free based endogenous RNA	Circulating mRNA, e.g. tyrosinase mRNA in melanoma
Exogenous viral RNA	Viral RNA, e.g. EB virus coded RNA in nasopharyngeal carcinoma
Translational protein level	
Native protein (conventional tumor marker)	Prostate-specific antigen (PSA) in prostatic carcinoma, and CEA in colon carcinoma
Glycan	Aberrant glycosylation in hepatocellular carcinoma

AFP: α-Fetoprotein; CEA: Carcinoembryonic antigen; HPV: Human papillomavirus; mRNA: Messenger RNA; PSA: Prostate-specific antigen.

Table 6.109 Methods of detection of tumor markers

Serology	Assay
Immunological analysis	▪ Immunohistochemistry technique ▪ Radioimmunoassay ▪ Enzyme-linked immunosorbent assay
Flow cytometry	Single or multiple cells suspended in buffered salt-based solution analysis for visible light scatter and fluorescence parameters
Cytogenetic analysis	▪ Fluorescence *in situ* hybridization (FISH) ▪ Spectral karyotyping ▪ Comparative genomic hybridization
Genetic analysis	▪ Sequencing (automated) ▪ Reverse transcription ▪ Gel electrophoresis ▪ DNA microarray analysis
Proteomics	Surface enhanced laser desorption/ionization

identification with dyes in immunohistochemistry (IHC), radioactive tags in radioimmunoassay (RIA) or enzymes in enzyme-linked immunosorbent assay (ELISA). Alternatively, in a suspension, **flow cytometry** can analyze the presence and percentage of antibody tagged CSCs. These diagnostic methods are highly sensitive and can detect quantities in the nanogram to picogram range.

- Of these diagnostic techniques, the most used technique today is immunohistochemistry. Uses of immunohistochemistry technique in clinical oncology include categorization of undifferentiated malignant tumors, leukemia and lymphomas, determination of site of origin of metastatic tumors, and detection of molecules of prognostic or therapeutic significance, e.g. estrogen/progesterone receptors (ER/PR) in breast cancer.

TUMOR MARKERS: CLASSIFICATION AND USES

Tumor markers can be detected in tissues (tissue tumor markers, e.g. in solid malignant tumors, lymph nodes, bone marrow or circulating CSCs in the bloodstream or in the body fluids such as pleural fluid, ascitic fluid, cerebrospinal fluid or serum (serological tumor markers). Monoclonal antibodies are used to detect tumor antigens of specific malignant tumor. These serum tumor markers are most useful for monitoring response to therapy and detecting early relapse.

- Tissue tumor markers are of prime importance to a diagnostic pathologist, while serological markers are more often used by clinical oncologist depending on the clinical scenario ranging from initial clinical presentation, differential diagnosis and recurrence of malignant tumor.
- Laboratory-based tests are potentially useful in screening and early detection, diagnostic confirmation, prognosis, and prediction of therapeutic response or resistance and monitoring therapy in advanced cancer and recurrence. **Ki-67** (proliferative index marker) may help in prognostication and choice of therapy in cancer patients.

- Currently, cancer detection methods are not 100% specific to screen apparently healthy population for occult malignancies using a tumor marker as many tumor markers may be elevated in benign conditions. Some of the most useful tumor markers include: prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), CA 125, carcinoembryonic antigen (CEA), α-fetoprotein (AFP), human chorionic gonadotropin and CA 19-9. CEA and AFP are normally expressed during fetal development, but do not occur normally in the tissues or sera of children and adults.
- Carcinoembryonic antigen (CEA) tumor marker is analyzed in occult blood in early colorectal carcinoma. CEA is increased in malignant epithelial tumors of colon, pancreas, lung, stomach, and breast.
- Expression of AFP by CSCs is considered a manifestation of dedifferentiation. The undifferentiated CSCs tend to resemble their embryonic counterparts. AFP is increased in hepatocellular carcinoma and yolk sac tumor (endodermal sinus tumor) of testis and ovary. AFP is also increased in fetal anencephaly and other neural tube defects.
- CA 125 tumor marker is analyzed to monitor therapy in patients with ovarian surface epithelial carcinoma. Estrogen receptors (ERs) are analyzed to predict response to hormonal therapy (tamoxifen drug). Human epidermal growth factor receptor 2 (HER2) analysis in women with breast carcinoma is likely to respond to trastuzumab (Herceptin).
- Tumor markers in cancers of head and neck, lung, mesenchymal and paraneoplastic syndromes are shown in Fig. 6.128. Tumor markers in cancers of liver, pancreas, colorectal region, gastric region and adrenals are shown in Fig. 6.129. Tumor markers and associated malignancies are given in Table 6.110. Some of the recommended uses of tumor markers in clinical practice are given in Table 6.111. Tumor markers in cancers of breast, kidney, urinary bladder, prostate, testes, ovaries, endometrium, and cervix are shown in Fig. 6.130.

RECOMMENDATIONS FOR ADVISING TUMOR MARKER TESTS

Serum tumor markers are not recommended for screening asymptomatic patients for malignancy, because serum tumor markers lack specificity and sensitivity. Many patients may have an elevated rserum tumor marker level due to benign disease. Many patients with malignancy can have a normal serum tumor marker level.

- Single serum tumor marker test is unreliable by itself. It is worth mentioning in most situations, elevation of serum tumor markers in benign conditions are most often transient, whereas elevations of serum tumor marker with cancer either remain constant or continuously remain elevated.
- Serial serum tumor testing can help in detection of false positivity due to transient elevation. Therefore, treatment of patients cannot be initiated without undisputable documentation of disease on histologic examination.

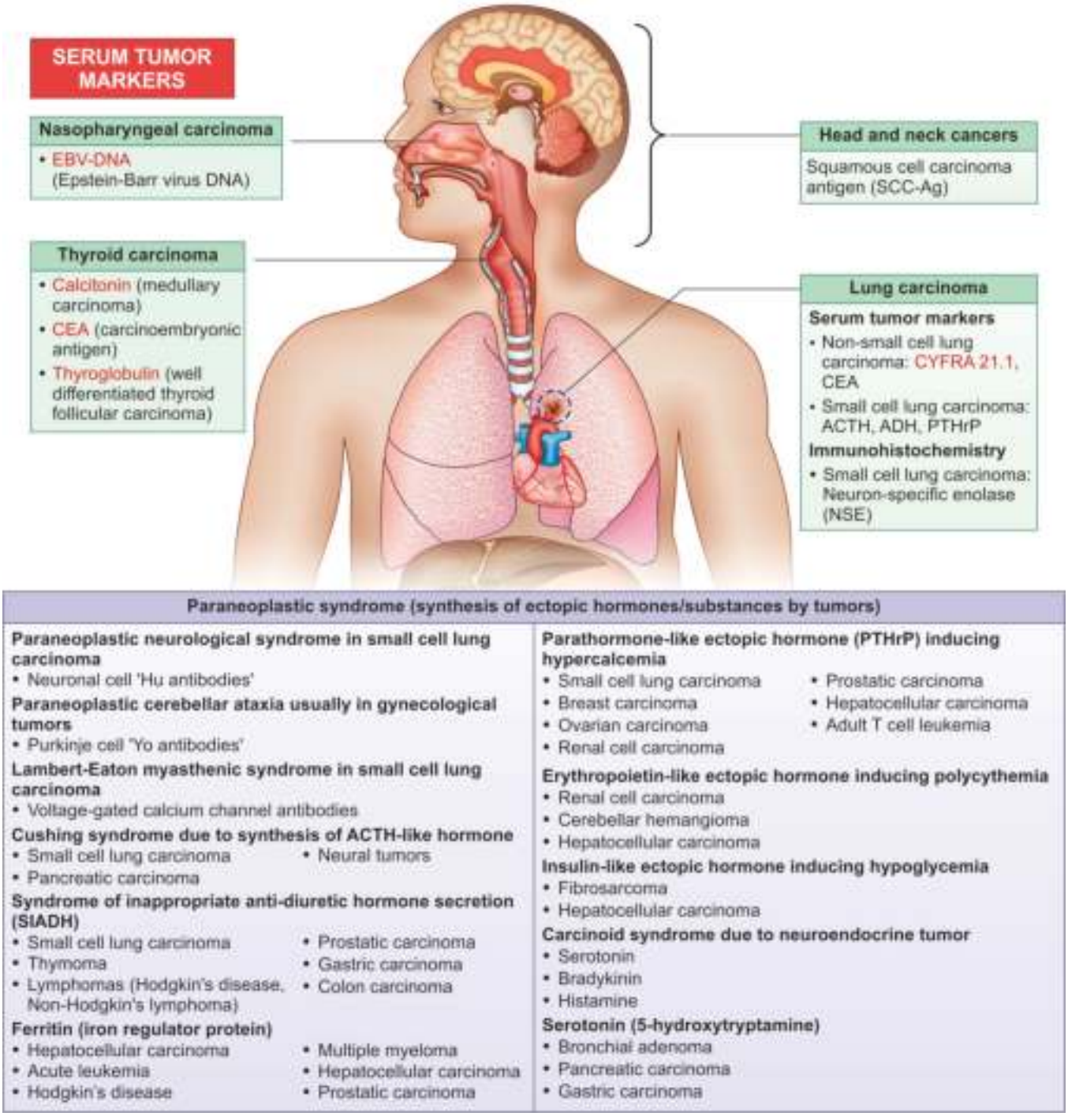
DIAGNOSTIC APPROACH OF CANCER

Cancer diagnostics is concerned with diagnostic approach of cancer taken into consideration of clinical history, physical examination, radiographic evaluation of possible metastatic disease, laboratory blood and urine tests including tumor markers, cytologic examination of cells (e.g. exfoliative cytology, fine needle aspiration cytology, liquid-based cytology), pathologic evaluation of malignant tumors, e.g. gross morphology (tumor characteristics), histologic examination, different immunohistochemical panels to analyze specific histologic dilemma, histochemistry, electron microscopy, and molecular markers for prognosis and predictive value.

CLINICAL HISTORY

The taking of an initial clinical history from the cancer patients, presenting symptoms, lifestyle and his/her family members is challenging as compared with the standard clinical medical history.

- Oncologist should listen to the cancer patient's complaints carefully, and not interrupting and without leading questions.
- Analyzing complaints and collecting anamnesis are the most important factors, and no detail should be overlooked.
- Physical examination of cancer patients should be strictly systemic and based on consistency.
- There are five cancer-specific steps that are essential for completing clinical history and physical examination for a new patient in a cancer care visit: (a) obtaining information according to malignant tumor type, (b) determining cancer diagnostic approach up-to-date, (c) obtaining a relevant cancer-focused clinical history, (d) performing detailed head-to-toe physical examination, and (e) performing appropriate organization of the data to prepare a professional presentation and complete appropriate documentation.



Paraneoplastic syndrome (synthesis of ectopic hormones/substances by tumors)

Paraneoplastic neurological syndrome in small cell lung carcinoma
- Neuronal cell 'Hu antibodies'

Paraneoplastic cerebellar ataxia usually in gynecological tumors
- Purkinje cell 'Yo antibodies'

Lambert-Eaton myasthenic syndrome in small cell lung carcinoma
- Voltage-gated calcium channel antibodies

Cushing syndrome due to synthesis of ACTH-like hormone
- Small cell lung carcinoma
- Neural tumors
- Pancreatic carcinoma

Syndrome of inappropriate anti-diuretic hormone secretion (SIADH)
- Small cell lung carcinoma
- Thymoma
- Lymphomas (Hodgkin's disease, Non-Hodgkin's lymphoma)
- Prostatic carcinoma
- Gastric carcinoma
- Colon carcinoma

Ferritin (iron regulator protein)
- Hepatocellular carcinoma
- Acute leukemia
- Hodgkin's disease
- Multiple myeloma
- Hepatocellular carcinoma
- Prostatic carcinoma

Parathormone-like ectopic hormone (PTHrP) inducing hypercalcemia
- Small cell lung carcinoma
- Breast carcinoma
- Ovarian carcinoma
- Renal cell carcinoma
- Prostatic carcinoma
- Hepatocellular carcinoma
- Adult T cell leukemia

Erythropoietin-like ectopic hormone inducing polycythemia
- Renal cell carcinoma
- Cerebellar hemangioma
- Hepatocellular carcinoma

Insulin-like ectopic hormone inducing hypoglycemia
- Fibrosarcoma
- Hepatocellular carcinoma

Carcinoid syndrome due to neuroendocrine tumor
- Serotonin
- Bradykinin
- Histamine

Serotonin (5-hydroxytryptamine)
- Bronchial adenoma
- Pancreatic carcinoma
- Gastric carcinoma

Fig. 6.128: Tumor markers in cancers of head and neck, lung, mesenchymal and paraneoplastic syndromes.

- Oncologist gathers detailed account of the patient's current symptoms such as change in bowel or urinary bladder habits, non-healing sore, unusual bleeding, or discharge, lump, obvious change in a mole, and persistent cough or hoarseness. General signs and symptoms of advanced and metastatic cancer include: fatigue, unexplained weight loss, and shortness of breath. It should be remembered that pain is not characteristic of early cancer disease. In the presence of malignant tumor related to gynecology, it is essential to clarify the state of menstrual cycle (i.e. time of onset of menstruation, duration, periodicity, and age at which menopause began).
- Examination of cancer patients should be strictly based on consistency, analyzing complaints, and collecting personal history are the most important factors and no detail should be overlooked.

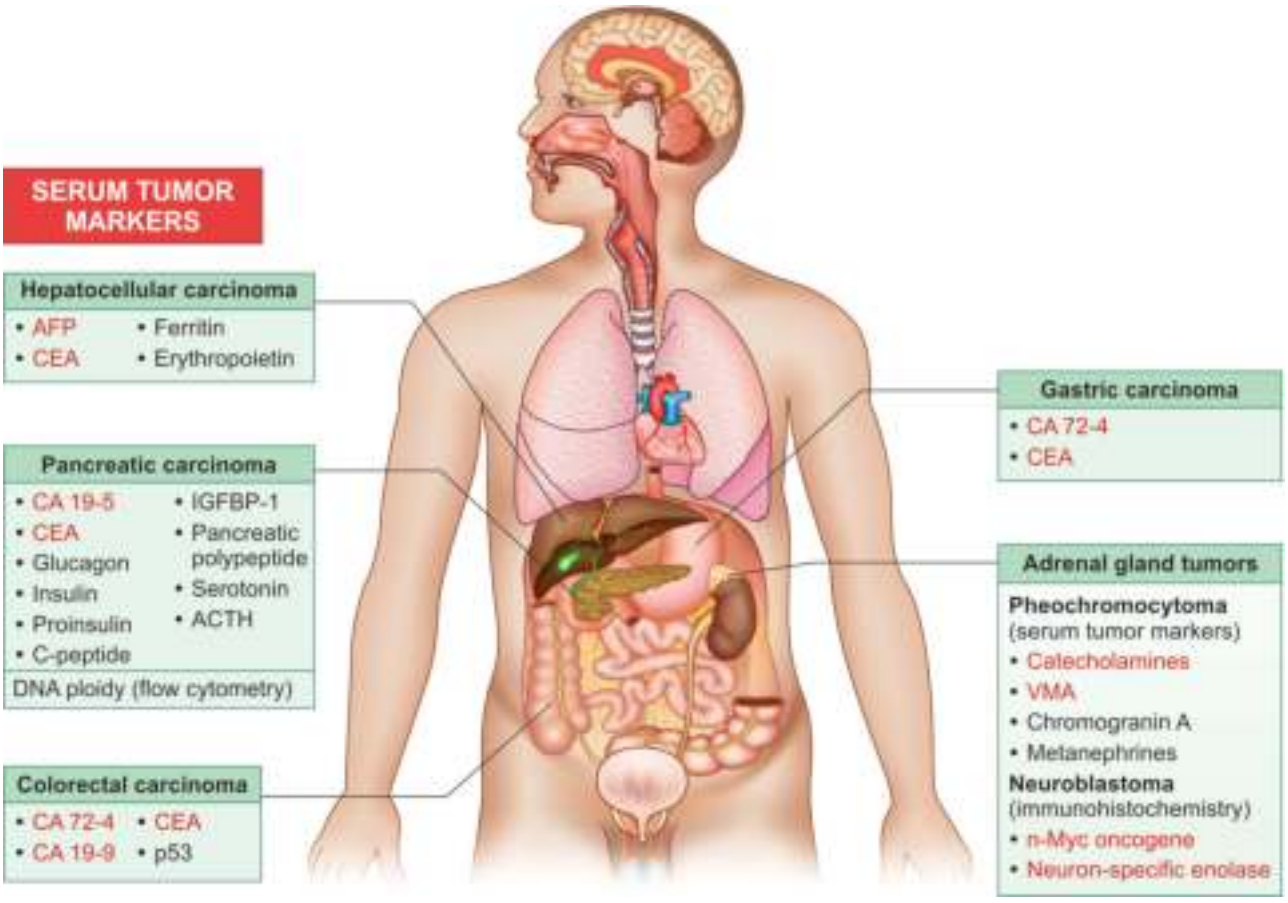


Fig. 6.129: Tumor markers in cancers of liver, pancreas, colorectal region, gastric region and adrenals.

Table 6.110 Tumor markers and associated malignancies

Serum Tumor Markers	Associated Malignancies and Other Disorders
Oncofetal antigens	
α-Fetoprotein (AFP)	▪ Hepatocellular carcinoma (75%) ▪ Yolk sac tumor (endodermal sinus tumor) of testis or ovary ▪ Hepatoblastoma ▪ Benign conditions (pregnancy, hepatitis and cirrhosis, inflammatory bowel disease)
Carcinoembryonic antigen (CEA)	▪ Non-seminomatous germ cell tumors of testes ▪ Colon carcinoma ▪ Gastric carcinoma ▪ Pancreatic carcinoma ▪ Lung carcinoma ▪ Breast carcinoma ▪ Benign conditions (hepatitis, pancreatitis, cigarette smokers, benign nodular hyperplasia of prostate, inflammatory bowel disease and hemolytic anemia)
Hormones	
Human chorionic gonadotropin (hCG) in blood and urine	▪ Choriocarcinoma ▪ Seminoma with syncytiotrophoblastic element ▪ Dysgerminoma with syncytiotrophoblastic element ▪ Benign conditions (pregnancy, tobacco smokers, and marijuana use in patients)
Calcitonin	▪ Medullary thyroid carcinoma ▪ Hepatocellular carcinoma ▪ Renal cell carcinoma
Ectopic adrenocorticotropic hormone (ACTH)	▪ Small cell lung carcinoma ▪ Pancreatic carcinoma ▪ Neural tumors

Contd...

Table 6.110 Tumor markers and associated malignancies (*Contd...*)

Serum Tumor Markers	Associated Malignancies and Other Disorders
Antidiuretic hormone/atrial natriuretic hormone (ADH/ANH)	▪ Small cell lung carcinoma (most common) ▪ Intracranial neoplasms ▪ Thymoma ▪ Prostate carcinoma ▪ Gastric carcinoma ▪ Colon carcinoma ▪ Hodgkin's disease ▪ Non-Hodgkin's lymphoma
Ectopic parathyroid hormone-related protein (PTHrP)	▪ Small cell lung carcinoma ▪ Squamous cell lung carcinoma ▪ Breast carcinoma ▪ Ovarian surface epithelial carcinoma ▪ Renal cell carcinoma ▪ Prostatic carcinoma ▪ Adult T cell lymphoma ▪ Hepatocellular carcinoma ▪ Tumors with neuroendocrine differentiation
Ectopic insulin hormone	▪ Fibrosarcoma ▪ Mesenchymal sarcoma ▪ Hepatocellular carcinoma
Ectopic erythropoietin hormone	▪ Renal cell carcinoma ▪ Cerebellar hemangioma ▪ Hepatocellular carcinoma ▪ Uterine leiomyomas
Serotonin	▪ Bronchial adenoma ▪ Pancreatic carcinoma ▪ Gastric carcinoma
Ferritin	▪ Hepatocellular carcinoma ▪ Acute leukemia ▪ Hodgkin's disease ▪ Multiple myeloma ▪ Malignant lymphoma ▪ Prostatic carcinoma
TSH-like (causing hyperthyroidism)	▪ Hydatidiform mole ▪ Choriocarcinoma ▪ Lung carcinoma (in some cases)
Enzymes	
Prostatic acid phosphatase (PAP)	▪ Prostatic carcinoma ▪ Prostatic acid phosphatase is also increased in prostatitis and benign prostate hyperplasia
Lactate dehydrogenase	▪ Germ cell tumors ▪ Acute leukemias ▪ Malignant lymphoma ▪ Metastatic carcinomas of colon, breast, and lung
5'-Nucelotide phosphodiesterase	▪ Lung carcinoma ▪ Liver metastases
Neuron-specific enolase (NSE)	▪ Small cell lung carcinoma ▪ Neuroblastoma
Terminal deoxynucleotidyl transferase (TdT)	Acute lymphoblastic leukemia
Thymidine kinase	▪ Hodgkin's disease ▪ Certain leukemias ▪ Small cell lung carcinoma
Isoenzymes	
CK-BB (nonspecific marker)	▪ Prostatic adenocarcinoma ▪ Lung adenocarcinoma ▪ Gastric adenocarcinoma
Type 2 macro-CK (oligomeric mitochondrial CK)	▪ Metastatic liver carcinoma ▪ Various malignancies
Type 1 macro-CK (complex between CK-BB and IgG)	Various neoplastic diseases
Mitochondrial CK-IgA complex	Various carcinomas (prognostic role in advanced cancers)
Galactosyltransferase II	▪ Ovarian carcinoma ▪ Hepatocellular carcinoma ▪ Esophageal carcinoma
Placental-type alkaline phosphatase (PLAP), Regan isoenzyme	▪ Germ cell tumors (specific marker) ▪ Advanced colorectal carcinoma (not very specific) ▪ Ovarian carcinoma (not very specific)
Bone alkaline phosphatase (bone ALP)	▪ Osteosarcoma ▪ Bone metastasis
Lactic dehydrogenase 1 (LD1)	Testicular germ cell tumors (i.e. seminomas, yolk sac tumor)
Lactic dehydrogenase 4 and 5 (LD-4 and LD-5) isoenzymes	Most cancers in advanced stage

Contd...

Table 6.110 Tumor markers and associated malignancies (*Contd...*)

Serum Tumor Markers	Associated Malignancies and Other Disorders
Specific proteins	
Immunoglobulin heavy and light chains, Bence-Jones proteins in blood and urine	▪ Multiple myeloma ▪ Other gammopathies
β_2-Microglobulin	Multiple myeloma
Prostate-specific antigen (PSA)	Prostatic carcinoma Prostate-specific antigen is also increased in prostatitis and benign prostate hyperplasia
Thyroglobulin	Follicular thyroid carcinoma (well-differentiated)
Insulin-like growth factor binding protein 2 (IGFBP-2)	Prostatic carcinoma
Human epididymis protein 4 (HE4)	Ovarian carcinoma
Nuclear matrix protein 22 (excreted in urine)	Urothelial carcinoma
Cytokeratin fragment 21-1 (Cyfra 21-1)	Non-small cell lung carcinoma
Synaptophysin (synaptic vesicle membrane protein)	Neuroendocrine tumors (e.g. small cell lung carcinoma)
Chromogranin A	▪ Pheochromocytoma ▪ Neuroblastoma ▪ Small cell lung carcinoma ▪ Carcinoid tumors
Calretinin	Mesothelioma
Mucins and glycoproteins	
CA 125	▪ Ovarian carcinoma ▪ CA 125 level is also increased during menstruation, endometriosis, pelvic inflammatory disease, pregnancy, peritonitis, pancreatitis, and hepatitis
CA 19-9	▪ Pancreatic carcinoma ▪ Gastric carcinoma ▪ Colon carcinoma ▪ Breast carcinoma ▪ CA 19.9 level may also be increased in pancreatitis and ulcerative colitis
CA 72-4	▪ Gastric carcinoma ▪ Colon carcinoma ▪ Pancreas carcinoma ▪ Ovarian carcinoma
CA 15-3	Breast carcinoma
CA 27-29	Breast carcinoma
TAG-72 (new marker)	▪ Gastric carcinoma ▪ Colorectal carcinoma ▪ Lung carcinoma ▪ Pancreatic carcinoma ▪ Ovarian cancers
Viruses	
HPV-DNA	Cervical carcinoma
Epstein-Barr virus (EBV)	▪ Burkitt's lymphoma ▪ Nasopharyngeal carcinoma ▪ Some lymphoproliferative disorders
Metabolic products	
Vanillylmandelic acid (VMA) is a metabolite of catecholamines excreted in urine	Pheochromocytoma
Catecholamines	Pheochromocytoma
5-Hydroxyindoleacetic acid (5-HIAA) is metabolite of 5-hydroxytryptamine in urine	Metastatic carcinoid tumors
Osteoclast activating factor (OAF)	Multiple myeloma

Lipid associated sialic acid in plasma (LASA-P) is expressed in various carcinomas, leukemias/lymphomas, Hodgkin's disease.

Table 6.111 Some of the recommended uses of tumor markers in clinical practice

Malignancy	Tumor Marker(s)	Tumor Marker Detection Method	Tumor Marker Uses
Breast carcinoma			
Breast ductal carcinoma	CA 15-3, CA 27-29	Serology/tissue immunohistochemistry	▪ Monitoring ▪ Recurrence
	ER/PR, HER2/neu, gross cystic disease fluid protein-15 (GCDFP-15), mamma-globin, Ki-67, p53, CK7, GATA3	Tissue immunohistochemistry	▪ Response to therapy ▪ Diagnosis
	DNA ploidy, S phase	Flow cytometry	▪ Diagnosis ▪ Monitoring
	EGFR	Fluorescence *in situ* hybridization (FISH)	▪ Diagnosis ▪ Monitoring
Breast lobular carcinoma	GCDFP-15 (+100%), E-cadherin (–)	Tissue immunohistochemistry	Diagnosis
Breast medullary carcinoma	Triple negative (ER/PR, HER2/neu)	Tissue immunohistochemistry	Diagnosis
Breast metaplastic carcinoma	CK903, p63, CAM 5.2 (most reliable marker), CK7 (most reliable marker)	Tissue immunohistochemistry	Diagnosis
Breast undifferentiated carcinoma	NY-BR1 (highly specific marker)	Tissue immunohistochemistry	Diagnosis
Female genital system			
Ovarian surface epithelial carcinoma	CA 125, CEA, HE4	Serology/tissue immunohistochemistry	▪ Monitoring ▪ Diagnosis ▪ Recurrence
Cervical carcinoma	HPV-DNA, SCC antigen (SCC-Ag), CEA	Serology	▪ Diagnosis ▪ Monitoring
Endometrial carcinoma	Urinary gonadotropin peptide	Urine	▪ Diagnosis ▪ Monitoring
Male genital system cancers			
Choriocarcinoma	β-hCG	Serology/tissue immunohistochemistry	▪ Diagnosis ▪ Prognosis ▪ Monitoring
Germ cell tumors	α-Fetoprotein (AFP), β-hCG	Serology/tissue immunohistochemistry	▪ Diagnosis ▪ Prognosis ▪ Monitoring
	Lactate dehydrogenase (LDH), placental alkaline phosphatase (PLAP) in seminoma	Serology	▪ Prognosis ▪ Monitoring
Prostatic carcinoma	Prostate-specific antigen (PSA), prostatic alkaline phosphatase (PAP), IGFBP2, CEA	Serology/tissue immunohistochemistry	▪ Screening ▪ Monitoring ▪ Diagnosis
Renal cell carcinoma	Erythropoietin, PTHrP, renin, prolactin, neuron-specific enolase (NSE)	Serology	▪ Screening ▪ Monitoring
Urinary bladder carcinoma	Nuclear matrix protein 22	Urine	▪ Diagnosis ▪ Prognosis
	DNA ploidy, S phase	Flow cytometry	▪ Diagnosis ▪ Prognosis
Gastrointestinal tract, pancreas, liver, and adrenal gland cancers			
Gastric carcinoma	CEA, CA 72-4	Serology/tissue immunohistochemistry	▪ Prognosis ▪ Monitoring

Contd...

Table 6.111 Some of the recommended uses of tumor markers in clinical practice (*Contd...*)

Malignancy	Tumor Marker(s)	Tumor Marker Detection Method	Tumor Marker Uses
Colorectal carcinoma	Carcinoembryonic antigen (CEA)	Serology	▪ Prognosis ▪ Monitoring
Pancreatic carcinoma	CA 19-5, CA 50, CEA, glucagon, IFBP-1, pancreatic polypeptide, serotonin, ACTH, γ-glutamyl transpeptidase	Serology	▪ Prognosis ▪ Monitoring
Insulinoma	Insulin, proinsulin, C peptide, IGF-1 binding protein	Serology	▪ Screening ▪ Diagnosis ▪ Prognosis ▪ Monitoring
Hepatocellular carcinoma	α-Fetoproteins, CEA, ferritin, erythropoietin, γ-glutamyl transpeptidase	Serology	▪ Screening ▪ Diagnosis ▪ Prognosis ▪ Monitoring
Carcinoid tumor	Chromogranin A, 5-hydroxyindoleacetic acid (5-HIAA)	Serology/urine	▪ Monitoring ▪ Recurrence
Pheochromocytoma (adrenal medulla)	Metanephrines (catecholamines metabolic products), vanillylmandelic acid (VMA)	Serology/urine	▪ Screening ▪ Diagnosis
Hematolymphoid system cancers			
Leukemia	TdT, ALK, ferritin, LDH, myelin basic protein, adenosine deaminase	Serology	▪ Screening ▪ Monitoring
Lymphomas	β-Microglobulin, TdT, LASA-P, lactic acid dehydrogenase (LDH)	Serology	▪ Diagnosis ▪ Prognosis
Multiple myeloma	Immunoglobulins heavy and light chains, Bence-Jones proteins, β-microglobulin	Serology/urine	▪ Diagnosis ▪ Prognosis
Head and neck cancers			
Nasopharyngeal carcinoma	EBV-DNA (Epstein-Barr virus-DNA)	Serology	▪ Diagnosis ▪ Prognosis
Squamous cell carcinoma of head and neck, cervix, lung	Squamous cell carcinoma-antigen (SCC-Ag)	Serology	▪ Diagnosis ▪ Prognosis
Follicular thyroid carcinoma (well-differentiated)	Thyroglobulin	Serology/tissue immunohistochemistry	▪ Screening ▪ Monitoring
Medullary thyroid carcinoma	Calcitonin, neuron-specific enolase (NSE)	Serology	▪ Screening ▪ Monitoring ▪ Prognosis
Lung cancers			
Small cell lung carcinoma	ACTH, ADH, PTHrP	Serology	▪ Screening ▪ Monitoring
Nonsmall cell lung carcinoma	Cyfra 21-1, CEA	Serology	▪ Screening ▪ Monitoring
Miscellaneous cancers			
Melanoma	Tyrosinase	Serology	Diagnosis
Sarcoma	Cytogenetic alterations	Genetic analysis	Diagnosis

- Once the cancer diagnosis is established, the oncologist can refer to evidence-based National Comprehensive Cancer Network (NCCN) guidelines needed for the complete staging of specific malignant tumor type, size, specific lymph node evaluation and imaging techniques such as ultrasonography, X-rays, CT and MRI performed for possible metastatic disease.
- The National Comprehensive Cancer Network (NCCN) guidelines are recognized standard

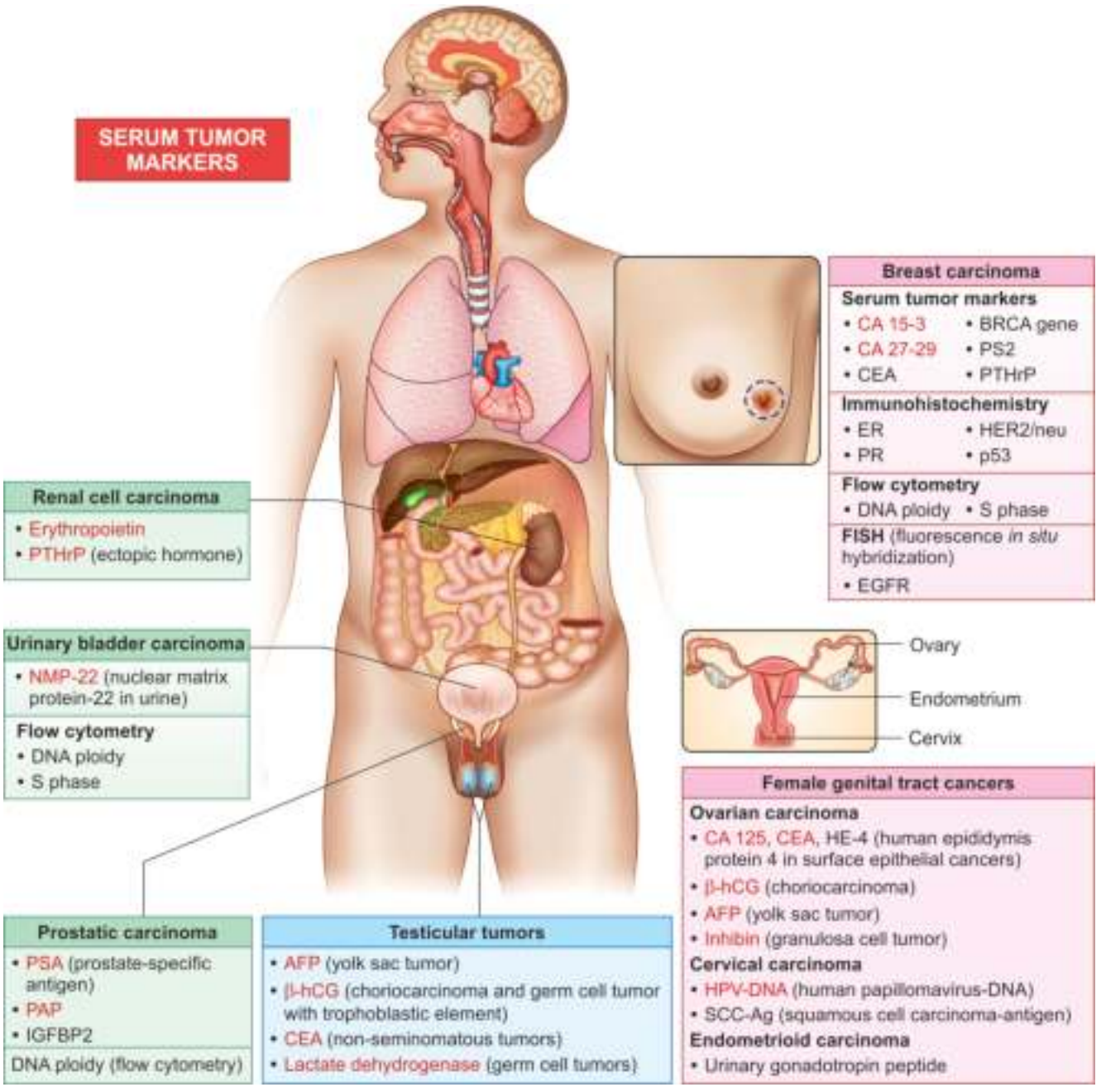


Fig. 6.130: Tumor markers in cancers of breast, kidney, urinary bladder, prostate, testes, ovaries, endometrium, and cervix.

for screening, diagnosis, and treatment of cancer patient.

Clinical Pearls: Some Common Alerts in Oncology History Taking

- Unexplained weight loss
- Persistent pain
- Night sweats
- Dysphagia, change in bowel or urinary bladder habits (blood in the stool, diarrhea, constipation)
- Unresolved cough or hoarseness
- Abnormal lump(s) or mass(es)
- Personal history or family history of cancer
- Abnormal bleeding
- Neurological symptoms
- Suspected paraneoplastic syndromes (endocrine abnormalities)

PAST MEDICAL HISTORY

Taking the patient's past medical history helps in identification of preexisting conditions and highly potential genetic factors that may impact cancer treatment decisions. In the presence of malignant tumors related to gynecology (breast carcinoma, endometrial

carcinoma, cervical carcinoma, ovarian carcinoma), it is essential to clarify the state of menstrual cycle: time of onset of menstruation, duration, periodicity of menstrual cycle and age at which menopause began.

LIFESTYLE, SOCIAL HISTORY AND ENVIRONMENTAL FACTORS

Oncologist should evaluate lifestyle history such as tobacco smoking (duration, number of cigarettes per day), alcohol consumption, dietary habits, and exercise, along with exposure to occupational and environmental carcinogenic agents, which provides insights into potential risk factors. When residing in a territory contaminated with radionucleotides, it is essential to specify the locality, the period of residence, and the time of relocation.

SYSTEMIC EVALUATION OF THE BODY SYSTEMS

Conduction of systemic evaluation of the body systems helps in detection of symptoms related to primary malignant tumor and its treatment. Oncologist should conduct physical examination (palpation, percussion, auscultation), and systemic evaluation of the body systems, and organs including peripheral lymph nodes.

BLOOD TESTS

Blood tests play important role in diagnosing and treating malignant tumors. Renal and liver functions are useful to determine overall health of cancer patients. A complete blood count (CBC) test measures the number of red blood cells, white blood cells and platelets circulating in the bloodstream. CBC test also measures hemoglobin in red blood cells (RBCs) that carries oxygen and hematocrit, the ratio of the red blood cells to plasma. Some cancer treatments may temporarily lower blood counts, oncologists often advise CBC tests throughout treatment to closely monitor a patient's complete blood counts.

URINE ANALYSIS

Urine analysis may be useful in detection of malignant tumors by analyzign various components (e.g. red blood cells, white blood cells, sugars, and proteins) and CSCs, which can detect the presence of certain cancers. Detection of cells in a urinary sediment is called urine cytology. Urine analysis is done to detect malignant epithelial tumors of urinary bladder, prostate, and cervix. Additionally, urinalysis is performed in multiple myeloma and is suspected to look for myeloma protein in the urine. If myeloma protein is detected, additional urine testing is called urine proteins electrophoresis and immunofixation will likely be conducted.

IMAGING TECHNIQUES

Imaging techniques may aid in early detection of malignant tumor in tissue/organ, staging and determination of therapy. Imaging techniques include ultrasonography, X-rays, CT scan, MRI, radioactive isotope imaging, positron emission tomography (PET), and mammography.

Ultrasonography

Ultrasonography uses high-frequency sound waves to detect changes in the density of tissues, thus helps to differentiate cysts from solid malignant tumors.

Conventional Radiography

X-rays use low doses of radiation to identify and evaluate changes in tissue densities such as location of tumor or metastasis.

Computed Tomography

Computed tomography (CT) evaluates successive layers of tissue by using narrow beam X-ray to provide a cross-sectional view of the structure. It also can reveal different characteristics of tissues within a solid organ.

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) uses fields and radio-frequencies to demonstrate a cross-sectional view of the body organs and structures.

Radioactive Isotope Scanning

Radioactive isotope scanning involves the use of specialized camera which detects radioactive isotopes that are injected into blood stream or ingested. Radiologist analyzes distribution of radioactive isotopes throughout tissues and organs.

Positron Emission Tomography

Positron emission tomography (PET) scan is an imaging technique, that can help reveal the metabolic function of tissues and organs. The PET scan used a radioisotope drug called a radiotracer to demonstrate both physiologic and pathologic metabolic activity in tissues and organs. The PET imaging can be useful whether a tumor is benign or malignant.

Mammography

Screening mammography is a low-dose X-ray imaging method to examine the breast for early detection of malignant tumor and other diseases such as fibroadenomas or complex cysts. A radiologist examines a mammogram to search for high density regions of unusual configuration that look different from normal tissue. Three recent advances in mammography include digital mammography, computer-aided detection system, and digital breast tomosynthesis.

- **A digital mammography** uses the same X-ray technology as conventional mammograms, but instead of using film, solid-state detectors are used to record X-ray pattern passing through the breast.
- **Computer-aided detection** highlights mammographic images for normal areas of density mass, or calcification that may indicate the presence of breast cancer.
- **Digital breast tomosynthesis**, also called three-dimensional mammography for breast cancer screening in which multiple radiographs of the breast are taken at different angles to generate thin cross-sections.

CYTOLOGIC EXAMINATION

Cytology is mainly used to diagnose or screen for cancer. Diagnosing diseases by looking at single cells/small clusters of cells is called cytology. Methods of obtaining cells for cytologic examination are simple, cost effective and minimally invasive. Cells shed naturally into body fluids, e.g. sputum, urine, cerebrospinal fluid, fluid in pleural and peritoneal cavities.

- The cells to be examined may be taken through various methods: (a) scraping and brushing tissue from the surface of Papanicolaou stained cytologic smears for screening cervix and gastrointestinal tract, (b) exfoliative cytology, i.e. collection of cells shed naturally in body fluids, e.g. sputum, urine, cerebrospinal fluid, fluid in pleural and peritoneal cavities, (c) fine needle aspiration cytology (FNAC) to sample superficial malignant tumors in lymph node, breast, thyroid gland, axillary region, abdomen, and extremities.
- Cytologic smears are prepared and stained by Papanicolaou stain or May-Grünwald-Giemsa (MGG) stain, and examined under light microscopy. Immunohistochemistry is performed on these smears.
- Comparison between air-dried May-Grünwald-Giemsa stained and wet-fixed Papanicolaou stained smears is given in Table 6.112.

Fine Needle Aspiration Cytology

Fine needle aspiration cytology (FNAC) is commonly done using a narrow 22 gauze percutaneous thin hollow needle to collect a sample of solid malignant tumors of breast, thyroid, and pancreas under aseptic conditions and draining lymph nodes for microscopic examination of smears fixed in fixative (95% ethyl alcohol and 2% acetic acid), and stained by May-Grünwald-Giemsa (MGG) stain. In some cases, this technique limits to make a definitive diagnosis. Diagnosing metastatic or recurrent malignancy by FNAC generally has a high specificity and sensitivity, which is ideal for confirming metastasis from a clinically or radiologically suspected primary site. FNAC is used as a diagnostic tool and in the follow-up of certain patients with known lumps.

Scraping and Brushing Tissue Surface of Papanicolaou Stained Cytology Smears

Scraping and brushing tissue surface and examination of Papanicolaou stained cytology smears are prepared from suspected region with endoscope from cervix and gastrointestinal tract. Scrap cytology technique can be useful for rapid intraoperative diagnosis of ovarian mass that acts as an adjunct to frozen section examination, where facility for frozen section is not available. Scrap cytology smears are obtained from tobacco-related oral leukoplakia lesions.

Conventional Exfoliative Cytology

Cancers involving or protruding from the surface of organs continuously shed exfoliative cells into the surrounding space. The exfoliative cells are obtained from cervix (cervical carcinoma), sputum (lung carcinoma), pleural fluid (lung carcinoma), peritoneal fluid (various cancers), cerebrospinal fluid (brain tumors) and urine (transitional cell carcinoma). Cells are obtained during endoscopic procedures. Smears are prepared and stained by Papanicolaou stain and examined under light microscope.

Liquid-based Cytology

Liquid-based cytology (LBC) is two-step procedure that involves the fixation of the fine needle aspiration material in an alcohol-based solution (methanol or ethanol depending on the technique), and automated processing

Table 6.112 Comparison between air-dried May-Grünwald-Giemsa stained and wet-fixed Papanicolaou stained smears

Air-dried May-Grünwald-Giemsa Stained Smears	Wet-fixed Papanicolaou Stained Smears
Good fixation but presence of artefacts	Good fixation
Cell morphology is poorly defined due to heavily stained ground substance	Individual cell morphology is clearly defined
Cytoplasmic details are well demonstrated	Cytoplasmic details are poorly demonstrated
Morphology of nuclear details is variable	Morphology of nuclear details is excellent
Morphology of nucleoli is always not well defined	Morphology of nucleoli is always well defined
Stromal components are well defined	Stromal details are poorly defined

of the material to obtain a thin layer of representative cells with the aim to improve diagnostic accuracy.

- In liquid-based cytology, the brush head of the device is immerged in vial of fixative solution (methanol or ethanol), where it is vigorously rotated several times to ensure release of the sample cells and kept for 30 minutes.
  - The brush head of the device is then removed from the vial. Excess mucus and blood are eliminated by means of washing through succession of centrifugation and resuspension of the sample in mucolytic and hemolytic agents.
  - Subsequently, slides may be stained routinely by Papanicolaou stain or used for molecular diagnostics.
- Two methods are used for cervical smear cytology. The first one is the conventional Papanicolaou (Pap), and the second one is liquid-based cytology, which provides optimal viewing of cellular features attributed to the reduction in the air-dried artifacts obscuring background elements and this reduces the number of unsatisfactory smears.
  - Cervical liquid-based cytology is now widely accepted as superior technique to diagnose cervical carcinoma than conventional technique used in high-risk population.
  - Following pelvic examination and liquid-based cytology from brushings at screening, women with CIN-II or CIN-III then undergo colposcopy and biopsy. Similarly, liquid-based cytology has been applied to thyroid cytopathology.

SURGICAL PATHOLOGY

Surgical pathology is the study of tissues removed in living patients during surgery to diagnose a disease and determine a treatment plan.

- Biopsies can be performed in several different ways. Biopsies should be professionally taken without error and sufficient biopsy from appropriate malignant tumor site for accurate reporting. Some biopsies involve removing a small amount of tissue with a needle, while others involved surgically removing an entire lump or nodule that is suspicious. Biopsies can be safely performed with imaging guidance such as ultrasonography, X-ray, computed tomography (CT), or magnetic resonance imaging (MRI). Breast biopsy is used to determine whether the patient has benign or malignant tumor.
  - **Endoscopic biopsy:** Endoscopy provides a direct view of a body cavity or passage way to detect abnormalities. It is applied to detect lesions in gastrointestinal, respiratory, genital, and urinary tracts. During endoscopy, the medical professionals can excise small tumors, aspirate fluid, or obtain tissue samples for histologic examination. Endoscopic biopsy is performed through fiberoptic endoscope through a natural orifice or a small incision. The clinician can insert the endoscope into gastrointestinal tract (GI endoscopy), urinary bladder (cystoscopy), abdominal cavity (laparoscopy), joint cavity (arthroscopy), mid-portion of the chest (mediastinoscopy), or trachea and bronchial system (laryngoscopy and bronchoscopy).
  - **Tissue biopsy needle:** Needle biopsy is used with a cutting needle to sample suspected lesion in the tissues including those in brain with the guidance of ultrasonography, computed tomography (CT), fluoroscopy, magnetic resonance imaging (MRI). A biopsy needle is generally several inches long, which can capture the tissue specimen. **A core needle biopsy**, also called an automatic spring-loaded, which consists of an inner needle connected through, or shallow receptacle covered by a sheath and attached to a spring-loaded mechanism. Small size biopsy measuring 1–2 mm wide and 2 cm long biopsy can make histopathologic interpretation difficult.
  - **Incisional tissue biopsy:** Scalpel is used to take a biopsy from a lesion. Sample is variable in size depending on nature of lesion. It is applied to surgically accessible lesions only.
  - **Excision tissue biopsy:** Whole abnormal lesion is surgically removed and fixed in 10% buffered neutral formalin to obtain paraffin sections. Sampling of large specimen would include malignant tumor (for histogenetic pattern of differentiation, staging and grading), resection margins, lymph nodes and background tissue. Hematoxylin and eosin-stained sections are studied by light microscopy. Freshly prepared 2% buffered glutaraldehyde is recommended for fixation of tissues for electron microscopy. Prompt refrigeration is required for study of hormone receptors.
- Surgical pathology includes gross examination of processed tissue under a microscope and may be supported by immunohistochemistry and other tests.
- The surgical pathologist's interpretation of a biopsy determines the patient's treatment plan, which provides critical information if the tumor is benign, borderline, or malignant, staging the extent of malignant disease and pinpointing the activity of specific molecular pathways in the malignant tumor.
- Normal cells will appear uniformly organized and have similar sizes, but in the case of malignant tumors, CSCs will be less organized and have different sizes. The surgical pathologist supports the oncologists by determining if the entity of a diseased region has been

removed during surgery and provided information that guides for postoperative treatment. Surgical pathologists diagnose diseases other than cancers.

Pathology Pearls: Frozen Section and Cryostat Techniques

- Frozen section and cryostat techniques are used for rapid diagnosis of malignancy to confirm cytologic diagnosis of malignancy, before proceeding to definite surgery.
- Assessment of excision margins for a wide local excision to ensure complete excision. Assessment of draining lymph nodes is done to identify patients who are lymph node negative and who require only a limited dissection.
- Tissues are quickly frozen, sectioned, mounted on slides, stained, and interpreted within a few minutes, which enable surgeon to take appropriate decisions about the extent of surgery.

Tumor Macroscopic Morphology

Biopsy/surgical specimen of tumor is fixed in **10% buffered neutral formalin** (containing sodium dihydrogen phosphate, disodium hydrogen phosphate, distal water, formalin, and pH 7.2–7.4 and volume of fixative 10–20 times of the specimen) and examined with naked eye, which includes the general color, weight, gross appearance, size, shape, consistency, and cut surface with prominent hemorrhage and necrosis. In mastectomy specimens, lymph nodes should be isolated and processed.

- Histopathology laboratory should be well equipped, which include automatic tissue processor, paraffin wax dispensers, embedded work stations, cryostat, hot plates, oven, weighing machine, knives of different sizes, blade, ruler, board, and staining machines. Grossing room should be well ventilated with an air extractor.
- Tissue processing is the technique by which fixed tissues are made suitable for embedding within supportive paraffin medium and consists of sequential steps: dehydration, clearing, impregnation, and embedding each of as designated duration to ensure completion of the procedure. All hard tissues (bone) should be sent for decalcification.
- Tissue sections obtained are stained by hematoxylin and eosin, mounted, and then microscopically evaluated. Tissue shrinkage is less in microwave-processed tissue as compared to routine manual method and rapid manual routine methods.

Histopathologic Examination

The single most important diagnostic tool is the biopsy for direct histologic study of the tumor tissue. Histopathology is defined as the diagnosis and study of the tissues and/or cells from suspicious lumps under light microscope to diagnose diseases. Histopathologist confirms the diagnosis of cancers and helps clinicians to manage a patient's care. Tissue embedded in paraffin can be sectioned at 4–8 microns in thickness with a microtome having thick metallic knife and stained with hematoxylin and eosin stain and examined under a microscope.

- The concept of quality control in histopathology means standard reagents and equipment, high quality tissue sections, well-stained tissue sections with hematoxylin and eosin, air bubble-free, artifact-free, and well-demonstration of tissue elements and accurate reporting. Primary aim of quality control is to monitor, detect errors, prevent pre-analytic, analytic and post-analytic errors and reliable result/diagnosis.
- First step is to classify the type of malignant tumor based on cellular differentiation and growth patten based on the light microscopic examination of paraffin-embedded tissue by hematoxylin and eosin-stained slides. Microscopic description describes morphology of CSCs, differentiation, invasiveness/noninvasiveness, grade, mitoses, tumor margins, and lymph node status.
- After determining that the lesion is neoplastic in nature, the next step is to analyze whether the malignant tumor is of epithelial origin or mesenchymal origin. The main difference between malignant epithelial tumors and malignant mesenchymal tumors include: (a) tumor cells are oval–round to polygonal in malignant epithelial tumors, but the tumor cells are generally spindle-shaped in malignant mesenchymal tumors, (b) malignant epithelial tumors generally form tumor cell nests, but malignant mesenchymal tumors are arranged in diffuse sheets, without forming tumor cell nests, (c) in malignant epithelial tumors, desmoplastic stroma is well formed between tumor cell nests, but mesenchymal tumors lack desmoplastic stroma, and (d) blood vessels open in the supporting stroma in malignant epithelial tumors, but open directly between CSCs in malignant mesenchymal tumors.
- Next step is to decide, whether the tumor is benign or malignant in nature, based on various characteristics: (a) differentiation, (b) growth rate, (c) growth pattern, and (d) metastasis.
  - A benign tumor is well-differentiated, grows slowly, that shows expansile growth with capsule, and does not metastasize.
  - Malignant tumor is most often poorly differentiated that grows rapidly without a capsule, and frequently metastasizes. In general, malignant tumors show high cellularity, tumor necrosis and nuclear alterations, which include nuclear enlargement with a high nuclear/cytoplasmic ratio, pleomorphism, hyperchromatism, prominent nucleoli and frequent atypical tripolar or quadripolar mitoses.

- For the correct diagnosis of the malignant tumor, immunohistochemistry technique, molecular diagnostic tools such as fluorescence *in situ* hybridization (FISH), and polymerase chain reaction (PCR) technique to map the genetic alterations in tissues or tumors, or possibly electron microscopic evaluation may be required. After establishing diagnosis of malignancy, one should then consider the relevant important prognostic factors (e.g. tumor grade and TNM stage) to be mentioned in the histopathology report.

Grading and Staging Systems

Both grading and staging systems are used to classify malignant tumors for prognostic evaluation and planning of clinical management. Malignant tumor ≥2 cm has inherent ability to metastasize to distant tissues/organs. In general, higher the grade or the stage of malignant tumors, patients have worse prognosis.

- Oncologists use grading and staging systems to describe the characteristics of the malignant tumor to determine prognosis. Staging system is more important than grading of cancers and has great significance to predict prognosis and effective management in clinical practice.
- Some malignant tumors such as melanoma and gastrointestinal carcinomas are staged by how deeply these tumors extend beyond the basement membrane. Invasion of the dermis by melanoma or invasion of gastrointestinal carcinomas through the lamina propria, muscularis mucosa, and serosa represent progressively more extensive invasion and higher risk of metastasis. Grading and staging of breast carcinoma is shown in Fig. 6.131.

Grading System

Grading system is based on histologic cellular and differentiation of malignant tumors (i.e. how much malignant tumor resembles the tissue in which it grows), considers of architectural and nuclear features.

- Well-differentiated (low-grade) carcinoma resembles normal parent tissue. Poorly-differentiated (high-grade) carcinoma does not resemble apparent tissue. Moderately-differentiated carcinoma partially resembles parent tissue.
- Grading is important for determining prognosis. Poorly-differentiated (high-grade) carcinoma is more aggressive than well-differentiated (low-grade) carcinoma.
- Poorly-differentiated is high-grade anaplastic carcinoma that does not resemble parent tissue, which may be difficult to determine without immunohistochemical stains directed against squamous cell-specific proteins.

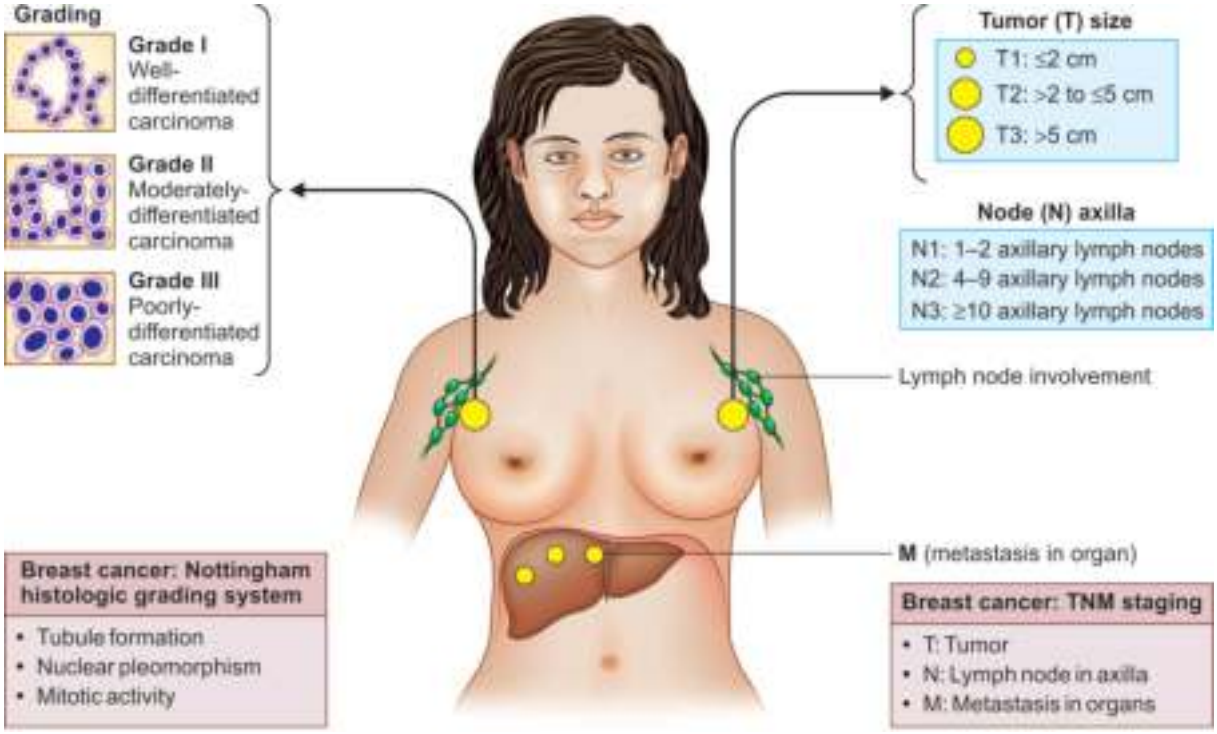


Fig. 6.131: Grading and staging of breast carcinoma. TNM stands for tumor, lymph node metastases and distant metastases [grading classifies tumor according to their light microscopic characteristics. Staging is a clinical exercise that classifies tumors according to their size, invasiveness and spread. Tumors are staged according to the TNM system (T: Tumor size, N: Lymph node involvement, M: Metastases to distant organs). Schemes for TNM classification vary according to tumor type and organ involved].

Staging System

Staging system is based on size of primary malignant tumor, extent of local invasion into surrounding tissue and systemic metastasis to distant organ(s) via lymphatic, hematogenous or transcoelomic routes.

- Staging system generally correlates with prognosis than does histopathologic grading; however, both approaches are useful.
- Staging system is exemplified by TNM system that evaluates the size and/or depth of invasion of tumor into the surrounding tissues, and systemic metastasis.
  - **T**—tumor (size and/or depth of invasion) refers to carcinoma *in situ* (T) followed by T1–T4.
  - **N**—denotes lymph node involvement. N0 denotes absence of lymph node followed by N1–N3 increasing in number of lymph nodes.
  - **M**—denotes systemic metastases (e.g. liver, lung, bone, and brain). M0 refers to absence of metastasis followed by M1 for metastasis.
- Metastasis is single most important prognostic factor. The most employed staging guidelines are those laid out by the American Joint Committee on Cancer (AJCC).

Electron Microscopy

Electron microscopy is a useful diagnostic technique to supplement morphologic, immunohistochemical, cytogenetic, and molecular analysis of tissues, although immunoperoxidase techniques have largely replaced electron microscopy for malignant tumor diagnosis in surgical pathology. Tissue is fixed in 2% glutaraldehyde.

- Electron microscopy plays significant role in the diagnosis of poorly differentiated carcinoma, sarcoma or lymphoma, whose classification is difficult by routine light microscopy.
- Desmosomes and specialized junctional complexes are demonstrated in carcinoma, while absent in sarcoma and lymphoma.
- Carcinoma exhibits short microvilli, blunt with terminal web, while microvilli of lymphoid and mesenchymal tumors do not show a terminal web.
  - Cells of ectodermal origin often have many cilia.
  - Malignant epithelial tumors show presence of bundles of tonofilaments, whereas slender microfilaments are common in malignant mesenchymal tumors.
  - Malignant melanoma shows presence of melanosomes.
  - Small membrane-bound granules with dense core are features of endocrine neoplasms.
- Demonstration of microvillous core rootlets in glandular epithelium of intestines help in diagnosis of metastatic deposits from gastrointestinal carcinoma.

Immunohistochemistry Technique

As the cells differentiate into different tissues like epithelial tissue, mesenchymal tissue and hematolymphoid tissue (T cells and B cells). The cells specialize and express specific proteins (e.g. intermediate filaments), which can be analyzed by purified monoclonal antibodies against tumor antigens aiding identification of the cell of origin of cancer.

- Immunocytochemistry in conjunction with immunofluorescence is a useful diagnostic tool in the identification and classification of tumors arising from T and B cells, and mononuclear phagocytic cell. Immunohistochemistry technique as a diagnostic tool in cancers is given in Table 6.113.
- Immunohistochemical staining may be performed to diagnose, classify, and characterize cellular differentiation of tumors. Cytokeratin immunostaining is helpful to detect metastatic carcinoma to an axillary lymph node, diagnose round cell tumors and metastatic disease in bone marrow and lymph nodes, particularly when small samples of tissues are submitted for diagnosis, because distinct protein expression patterns define different entities.
- Immunohistochemistry technique begins with hybridizing monoclonal antibody solution directed against specific proteins in cell membrane, cytoplasm, and nuclear membrane within the tissue section aiding identification of the cell of origin of a lesion. It is then hybridized with a secondary monoclonal antibody conjugated to a reporter enzyme that precipitates a signal in the cells that are positive for the antigen (using peroxidase-antiperoxidase method).
- **Cytokeratin** is used to differentiate between carcinoma and lymphomas. **Cytokeratin** positivity is seen in carcinoma and mesothelioma. **Desmin** and **myoglobin** are specific for malignant smooth and striated muscle tumors. **Vimentin** is demonstrated in malignant mesenchymal tumors and some carcinomas.
- Immunohistochemical staining may be performed using frozen tissue, formalin-fixed paraffin-embedded tissue sections (routine pathology), or freshly suspended cells (flow cytometry) using either enzyme reporters or fluorescence.
- A growing array of monoclonal antibodies is routinely used. Despite its potential, immunohistochemical staining results must be interpreted with care. The expression pattern of tumor antigens may change as individual CSC dedifferentiate and begin to express an antigen profile unlike original cellular lineage.
- The specificity of a particular immunomarker varies significantly from one malignant tumor type to another.

Table 6.113 Immunohistochemistry technique as a diagnostic tool in cancers

Intermediate Filaments	Tissue Origin of Tumor	Tumors
Cytokeratin	Epithelial tissue	▪ Squamous cell carcinoma of skin ▪ Renal cell carcinoma ▪ Mesothelioma
Vimentin	Mesenchymal tissue	Sarcoma
Desmin	▪ Skeletal muscle ▪ Smooth muscle	▪ Rhabdomyosarcoma ▪ Leiomyoma
Neurofilament	▪ Neurons (brain) ▪ Neural crest derivatives	▪ Brain tumors (e.g. ganglioglioma, gangliocytoma, neurocytoma) ▪ Neuroendocrine tumors (e.g. neuroblastoma, pheochromocytoma, small cell lung carcinoma)
Glial fibrillary acidic protein (GFAP)	Neuroglial cells	▪ Glial tumors of brain ▪ Breast carcinoma
Prostate-specific antigen (PSA)	Prostatic epithelium	Prostatic adenocarcinoma
Estrogen receptor (ER)	Breast epithelium	Breast carcinoma
Thyroglobulin	Thyroid follicular cells	Thyroid carcinoma
Chromogranin	Neuroendocrine cells	Small cell lung carcinoma and carcinoid tumor

Renal cell carcinoma expresses both cytokeratin and vimentin.

- Location of immunoreactivity directed against specific antigens located in cell membrane, cytoplasm, and nuclear membrane of CSCs must be considered.
- For example, monoclonal antibody to thyroid transcription factor 1 (TTF-1) stains the nuclei of small cell lung carcinoma (specific), but it stains the cytoplasm of hepatocellular carcinoma (not specific).
- Immunohistochemistry has been discussed in details in Chapter 14: Cellular–Molecular Diagnostic Techniques in Clinical Practice.

PD-L1 Biomarker Immunohistochemistry Technique

Programmed cell death protein ligand 1 (PD-L1) biomarker immunohistochemistry (IHC) technique is indicated in patients with specific advanced cancers to predict their responses to treatment with PD-L1 inhibitors.

- PD-L1 on CSCs plays a vital role in suppressing immune responses, and promoting self-tolerance through modulating the activity of PD-1 positive CD8+ cytotoxic T cells, activating apoptosis of antigen-specific T cells, and inhibiting apoptosis of CD4+ regulatory T cells.
- PD-L1 is a transmembrane protein on the CSCs that combines with programmed cell death protein 1 (PD-1) positive CD8+ cytotoxic T cells, and inhibits apoptosis of CSCs.
- Monoclonal antibodies that target either PD-1 or PD-L1 can block this binding, and boost the immune response against CSCs. PD-1/PD-L1 axis inhibitors are checkpoint inhibitors as frontline treatment of human cancers.
- PD-1/PD-L1 signaling pathway in cancer is shown in Fig. 6.132.

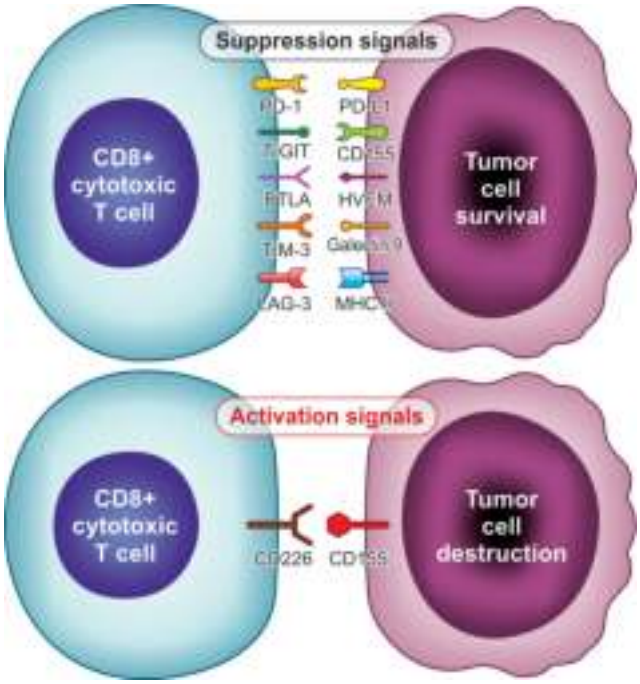


Fig. 6.132: PD-1/PD-L1 signaling pathway in cancer. Interaction between PD-1 and PD-L1 occurs in the tumor microenvironment. PD-1 is expressed on CD8+ cytotoxic T cells and the ligand, PD-L1 is expressed on specific malignant neoplastic cells and antigen-presenting cells. PD-1/PD-L1 axis subsides immune responses and promotes self-tolerance through suppressing the activity of T cells. Blocking the binding of PD-L1 to PD-1 with an immune checkpoint inhibitor (anti-PD-L1 or PD-1) allows the T cells to kill malignant neoplastic cells.

Anaplastic Lymphoma Kinase Mutations Biomarker Immunohistochemistry Technique

Most mutations of the anaplastic lymphoma kinase (ALK) gene are in the form of translocation with another partner NPM gene resulting in formation of NPM-ALK fusion oncogene, which then becomes overexpressed in malignant tumor, and activates many downstream signaling pathways resulting in unrestricted CSCs proliferation and survival. Subsequent ALK-rearrangement has been identified in non-small cell lung carcinoma and diffuse large B cell lymphoma. ALK biomarker of immunohistochemistry technique is used as a predictive biomarker of an underlying ALK translocation to identify patients with specific cancer types who can potentially benefit from ALK inhibitors.

ROS1 Rearrangement Biomarker Immunohistochemistry Technique

Immunohistochemistry technique is a screening diagnostic tool for detection of ROS1 rearrangement in non-small cell lung carcinoma. ROS1 rearrangement occurs in 1–2% of patients in non-small cell lung carcinoma, and it is predictive of treatment response to tyrosine kinase inhibitor. Fluorescence *in situ* hybridization (FISH) is the gold standard method to detect ROS1 rearrangement. ROS1 immunohistochemistry technique has been proposed as a cost-effective screening test for detection of ROS1 rearrangement.

BRAF V600E Mutations Biomarker Immunohistochemistry Technique

BRAF V600 mutations biomarker can be reliably detected by immunohistochemistry technique in primary and metastatic colorectal carcinoma, papillary thyroid carcinoma and melanoma. Molecular analysis for BRAF V600 mutations can be applied to fresh, frozen, or more commonly formalin-fixed paraffin-embedded (FFPE) malignant tumor. Real-time polymerase chain reaction (PCR) is used in detection of BRAF V600 mutations. BRAF V600 is a promising biomarker of prognosis in stage II cancer patients.

TREATMENT MODALITIES FOR TREATING CANCER

Targets for cancer suppression are to reduce CSCs proliferation and promoting apoptosis. Administration of receptor tyrosine kinase inhibits block signal pathways, that induce unrestricted CSC proliferation upregulation of pathways that cause apoptosis. Angiogenesis inhibitors are administered to cut off blood supply of CSCs. The treatment modalities for cancers include surgery, chemotherapy, and radiation therapy.

- Targeted drug therapy uses medicines that targets specific genes and proteins that help CSCs, grow, spread, and survive longer.
  - Two main groups of targeted drug therapy are **monoclonal antibodies** and **small molecule inhibitors**, which have side effects different from chemotherapy.
  - In about 15–20% of breast carcinomas, CSCs make too much of a growth promoter protein known as HER2.
  - The HER2 positive breast carcinomas tend to grow faster and disseminate more aggressively than HER2 negative breast carcinomas.
  - Different types of drugs such as 'Herceptin' have been developed, that target the HER2 protein in breast carcinomas.
- Cancer therapy varies by the patient's tumor type, and location. Site-specific cancer treatments are given in Table 6.114. Therapeutic targeting of the hallmarks of cancer drugs are shown in Fig. 6.133.

SURGICAL TREATMENT

Surgical oncology is the field of cancer care that focuses on surgery to may be used to determine location, remove the malignant tumor, as well as nearby draining lymph nodes and tissues containing CSCs, restore the body's appearance or function (reconstructive surgery) and manage some cancer-related symptoms (palliative surgery). Mastectomy is a surgical procedure to remove one or both breasts and lymph nodes, usually to treat breast cancer. There are different types of mastectomies and the options depend on the histologic type of breast carcinoma: simple total mastectomy, partial mastectomy (skin-sparing mastectomy, nipple-sparing mastectomy), and modified radical mastectomy.

RADIATION THERAPY

Radiation therapy is a cancer treatment that uses high doses of high energy beam radiation to kill CSCs and shrink malignant tumors. Radiation therapy may be of two types: external beam radiation (outside the body), brachytherapy, i.e. internal beam radiation (placed inside the body).

- Radiation therapy may be used in the early stage of cancer (curative radiation therapy). It can be combined with chemotherapy or used before surgery (neoadjuvant radiation therapy).
- Radiation therapy is of three types: (a) proton radiation therapy uses a beam of protons to deliver radiation directly to the malignant tumor, (b) magnetic resonance imaging (**MRI**) linear accelerator is used to track soft tissue-based malignant tumors in real-time during radiation, and

Table 6.114 Site-specific cancer treatments

Treatment Modalities	Method	Aim of Treatment	Site of Cancer
Surgical treatment	Excision of tumor along with lymph nodes	Surgical excision of cancerous growth along with draining lymph nodes is performed	Breast, thyroid gland, lung, colon, ovary, uterus, skin
Radiation therapy	▪ External radiation	▪ Radiation source is outside body	▪ Malignant tumor of brain
	▪ Internal radiation	▪ Radiation source is inside body and radioactive material (capsule, needles, or seeds) is placed inside malignant growth in the organ	▪ Prostatic carcinoma
	▪ Systemic radiation	▪ Radioactive substance (radioactive iodine) is absorbed by thyroid cancers	▪ Thyroid carcinoma
Immunotherapy	▪ Checkpoint inhibitors drugs	▪ Blocking checkpoint proteins from binding with their partner proteins	▪ Melanoma, lung carcinoma
	▪ Adoptive cell therapy (T cell transfer therapy)	▪ T cells are cultured in laboratory	▪ Cervical carcinoma, cholangiocarcinoma
	▪ Cancer vaccines	▪ Boost immune system response	▪ Different types of cancer
	▪ Synthetic monoclonal antibodies	▪ Monoclonal antibodies bind to proteins and transmit signal to immune system that destroy CSCs	▪ Trastuzumab (Herceptin) to treat breast carcinoma ▪ Rituximab to treat chronic lymphocytic leukemia, NHL ▪ Cituximab (Erbitux) to treat bowel cancer and head and neck cancer
	▪ Immune system modulators	▪ Immune system modulators enhance immune system response that kill CSCs	▪ Renal cell carcinoma, leukemia, lymphoma, melanoma, and sarcoma
Hormonal therapy	Hormone therapy in cancer patients	Hormone therapy may slow or inhibit by blocking the body's ability to produce hormones or changing how hormone receptors behave in the body	Breast, prostate, and endometrium
Bone marrow transplantation	Bone marrow transplant	Bone marrow transplant is done by transferring stem cells from one person to another	Leukemia, multiple myeloma, lymphoma

Breast, lung, uterus, cervix, lymphoma (many cancers need combined surgery and radiation therapy).

(c) stereotactic radiation therapy (**SBRT**) has a narrower beam of radiation. Radiation alone is never right for lymphomas.

- Radiation therapy increases the risk for solid malignant epithelial tumors of breast, thyroid, and lung. Radiation therapy also increases the chance of premature coronary artery disease. Risk for acute leukemia, MDS and NHL as a complication of chemotherapy is about 1% per year.

HORMONAL THERAPY

Hormonal therapy is used to treat cancer patients that removes, blocks, or add specific hormones to the body. It is also called hormonal therapy, anti-hormonal therapy, or endocrine therapy. Breast and prostatic carcinomas are commonly treated with hormonal therapy.

- Most cases of breast carcinoma have either estrogen receptors (ERs) or progesterone receptors (PRs) or both, which means these breast carcinoma cases need these hormones to grow and disseminate to distant organs. Tamoxifen, selectively estrogen receptor modulator blocks estrogen in breast carcinoma. Tamoxifen may be recommended after surgery for early ER positive breast carcinoma.
- Aromatase inhibitor works by inactivating aromatase used to make estrogen in the ovaries and other tissues. Aromatase inhibitor is primarily used in premenopausal young women to suppress ovarian function in patients with breast carcinoma before surgery to shrink.
  - Fulvestrant binds to estrogen receptor, completely stopping the hormone from attaching to the receptors.

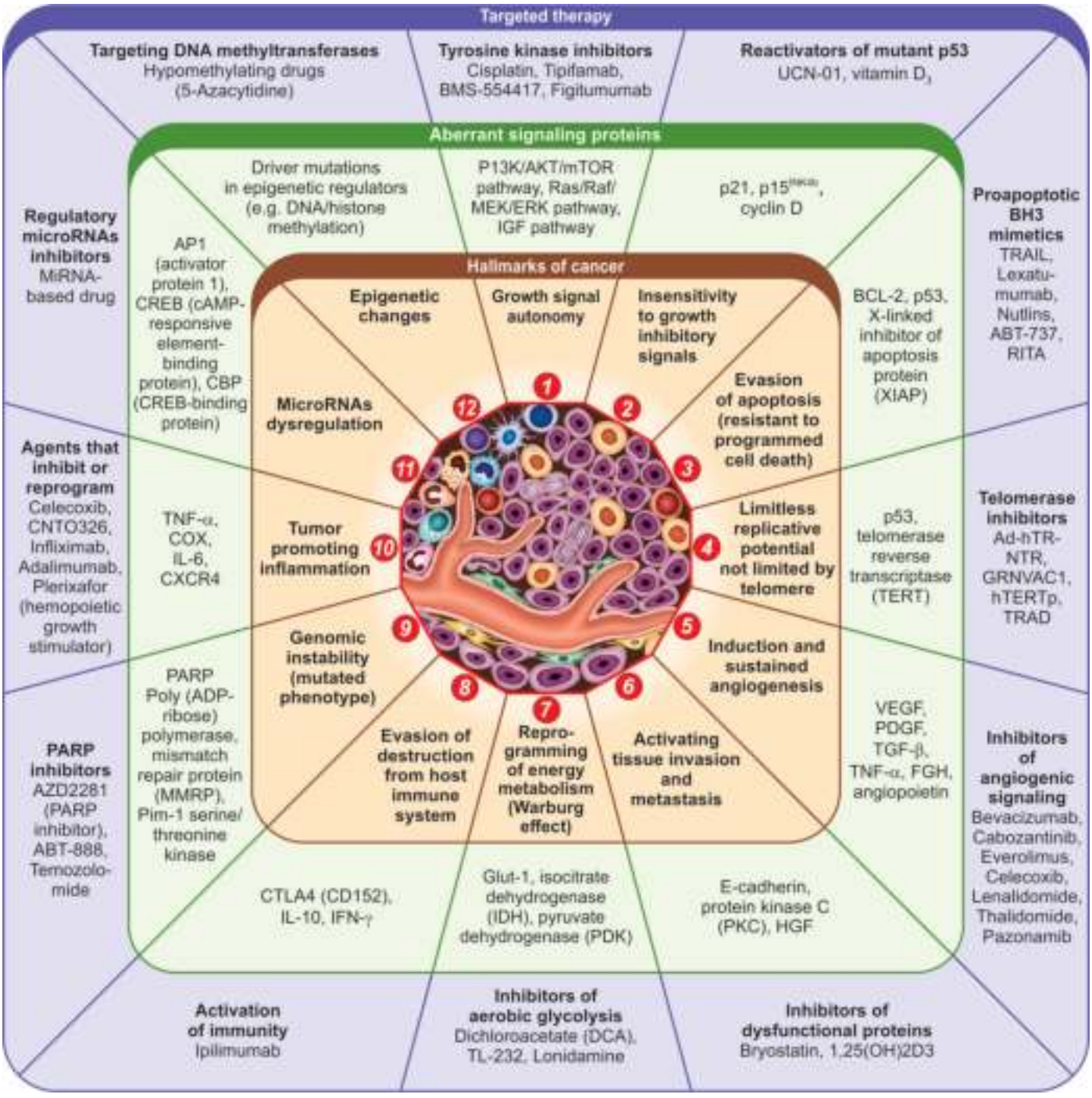


Fig. 6.133: Therapeutic targeting of the hallmarks of cancer drugs that interfere with each of the hallmark capabilities and hallmark-enabling processes have been developed and are in preclinical and/or clinical testing, and in some cases, approved for use in treating certain forms of human cancer. A focus on antagonizing specific hallmark capabilities is likely to yield insights into developing novel, highly effective therapeutic strategies. PARP: Poly (ADP-ribose) polymerase; VEGF: Vascular endothelial growth factor, HGF: Hepatocyte growth factor.

- Fulvestrant is recommended for women who have advanced ER positive breast carcinoma that spreads following treatment with estrogen therapy in ER positive breast carcinoma. Ovarian suppression may involve surgical oophorectomy, drugs, and radiation therapy.
- On the contrary, patient with prostatic carcinoma needs testosterone and other male sex hormones, such as DTH, to grow and metastasize to distant organs. Hormonal therapy may help patient with prostatic carcinoma, so that testosterone and DTH hormones are less available for growing CSCs.

CHEMOTHERAPY

Chemotherapy is an aggressive form of chemical drug therapy meant to destroy rapidly growing and dividing CSCs in the body. Chemotherapeutic agents are classified according to their mechanism of action and include alkylating agents, antimetabolites, topoisomerase inhibitors, antibiotics, mitotic inhibitors, and protein kinase inhibitors.

- Chemotherapeutic agents used in treating cancer patients are given in Table 6.115.
- Chemotherapy is most often administered with surgery, radiation, or hormonal therapy. The use of combination therapy depends on the location, histologic type, and stage of malignant tumors.
- Chemotherapy has been proven to effectively attack CSCs, but it can induce serious side-effects, that can have severe impact on quality of life.
- Chemotherapeutic agents generally affect DNA synthesis, DNA repair, and promote CSCs apoptosis. Cell cycle-specific chemotherapeutic agents prevent DNA synthesis, and act on the synthetic (S) phase of the cell cycle. Cell cycle-independent chemotherapeutic agents act at all phases of cell cycle, and include alkylating agents, which bind to DNA, and anthracyclines, which induce breakage of DNA strand.
- Chemotherapy is associated with wide-range of adverse effects (e.g. nausea, vomiting, immunosuppression, impaired growth, and proliferation of healthy cells and tumor lysis syndrome). Some chemotherapeutic agents increase the risk of development of secondary malignancy. Adverse effects of chemotherapeutic agents are given in Table 6.116.

Table 6.115 Chemotherapeutic agents used in treating cancer patients

Chemotherapeutic Agents Categories	Chemotherapeutic Agents	Mechanism of Action
Microtubule inhibitors	▪ Paclitaxel ▪ Vinblastine ▪ Vincristine ▪ Eribulin	Inhibits cellular mitosis phase
Cell cycle-independent drugs	▪ Platinum agents (e.g. cisplatin) ▪ Alkylating agents (e.g. busulfan, cyclophosphamide, chlorambucil, melphalan, ifosfamide)	Cross-linkage in DNA prevents DNA replication and transcription during G1–G0 phase
Antimetabolites	▪ Methotrexate ▪ Cytosine arabinoside ▪ Azathioprine ▪ Gemcitabine ▪ Fludarabine ▪ Cladribine ▪ 6-Fluorouracil ▪ Hydroxyurea ▪ 6-Mercaptopurine	Block essential enzymes and interfere in the synthesis of DNA and RNA during synthetic (S) phase
Topoisomerase inhibitors	▪ Etoposide ▪ Teniposide ▪ Irrinotecan ▪ Topotecan	Inhibit DNA synthesis and double check repair during G2 phase and S phase
Antibiotics	▪ Bleomycin ▪ Mitomycin ▪ Nitrosoureas	Drug binds to guanosine–cytosine-rich portions of DNA and breaks DNA strand during G2 phase
Receptor tyrosine kinase (RTK) inhibitors	▪ EGFR inhibitors (e.g. gefitinib, erlotinib, cetuximab) ▪ Kit/BCR-ABL inhibitors (e.g. imatinib/dasatinib)	Receptor tyrosine kinase (RTK) inhibitors are potent antineoplastic agents which target protein tyrosine kinases that are altered in CSCs and that account for some of their abnormal growth

Table 6.116 Adverse effects of chemotherapeutic agents

Chemotherapeutic Agent	Toxicity
Doxorubicin (Adriamycin)	Cardiomyopathy analyzed by nuclear ventriculogram for assessing left ventricular ejection fraction. Use the MUGA scan to determine prior to the development of symptoms
Vincristine	Neuropathy
Bleomycin	Pulmonary fibrosis
Cyclophosphamide	Hemorrhagic cystitis
Cisplatin	Nephrotoxicity and ototoxicity

- Oncologist administers some detoxifying agents to avert side-effects of some chemotherapeutic agents in cancer patients such as **leucovorin** administered in the treatment of methotrexate toxicity, and chemotherapy regimens, and mesna injection to lower risk for inflammation, and bleeding of urinary bladder, who receive ifosfamide.

Pathology Pearls: Chemotherapy-induced Secondary Malignancies

- Systemic anticancer treatment with chemotherapy and hormonal therapy are associated with increased risk of screening for secondary malignant neoplasm (**SMN**).
- Prior cytotoxic treatment-related acute myelogenous leukemia (**AML**), and myelodysplastic syndrome (**MDS**) are the most established examples.
- Alkylating agents, topoisomerase II inhibitors and antimetabolites have the highest leukemogenic potential.
- Administration of chemotherapy also increases the risk of development of solid malignancies after 10 years of therapy.
- Exposure to alkylating agents increase the risk of second malignancies such as lung carcinoma, thyroid carcinoma, gastrointestinal carcinoma, urothelial carcinoma, andsarcoma.
- Exposure of urothelium to metabolic product of alkylating agent (e.g. cyclophosphamide) increases the risk of urothelial carcinoma.
- Direct exposure of gastrointestinal epithelium to procarbazine increases the risk of development of treatment-related secondary gastrointestinal tract malignancies.
- Administration of lenalidomide immunomodulator also increases the risk of development of treatment-related AML.

Pathology Pearls: Tumor Lysis Syndrome

- Tumor lysis syndrome is the most common oncologic emergency prevalent in patients undergoing chemotherapy within hour of chemotherapy with characteristic findings of hyperuricemia, hyperkalemia, hyperphosphatemia, and hypocalcemia leading to end-organ damage.
  - High uric acid can deposit as crystals in kidneys and impair functioning of kidneys. High phosphatase can also impair functioning of kidneys. High potassium can cause nausea, vomiting, diarrhea, cardiac arrhythmias, and heart attack.
  - Uremia impairs functioning of kidneys and can cause adverse effects on bones, muscles, and blood vessels.
  - Hypocalcemia can cause changes in cardiac rhythm, muscle cramps, contusions, numbness and tingling sensations and potentially cardiac arrest.
- Tumor lysis syndrome is most common in patients diagnosed with **leukemia**, who have a very high white blood cell count. Tumor lysis syndrome also occurs in patients with **diffuse large B cell lymphoma (DLBCL), Burkitt's lymphoma, neuroblastoma** and **hepatoblastoma**.
- Most of symptoms observed in patients with tumor lysis syndrome are related to the release of intracellular chemical substances into the bloodstream that cause impaired functions of target organs (i.e. acute kidney injury, fatal cardiac arrhythmia) and even death.
- ECG is a part of the workup for patients with tumor lysis syndrome to check for findings associated with hyperkalemia and hypocalcemia.
- Blood urea nitrogen (BUN) and creatinine are also elevated in tumor lysis syndrome.

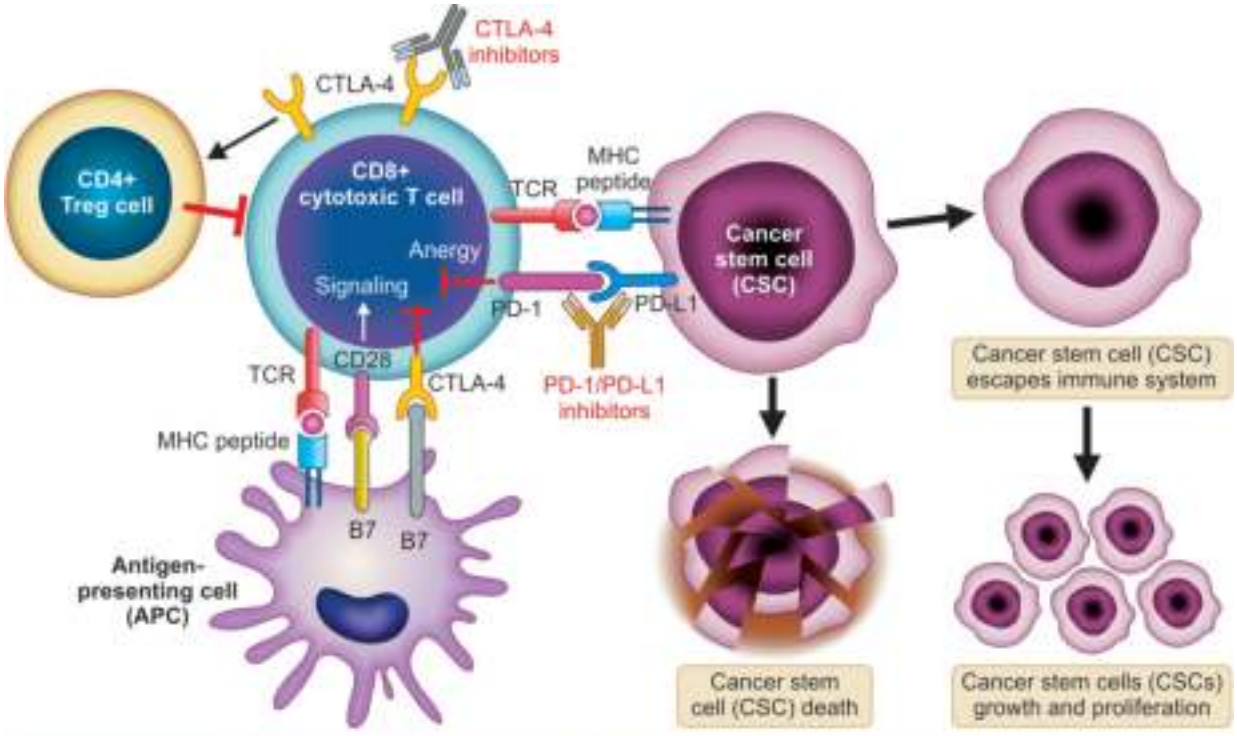
IMMUNOTHERAPY

Immunotherapy is a type of treatment designated to boost immune response, that attacks CSCs by targeting several immune checkpoint pathways. There are five types of immunotherapies: checkpoint inhibitors, adoptive cell therapy (T cell transfer therapy), cancer vaccines, monoclonal antibodies, and immune system modulators. Immunotherapy currently uses antibodies made in the laboratory to boost immunity against malignancies of breast, urinary bladder, cervix, head and neck, liver, kidney, stomach, colorectum and lymph node (Hodgkin's disease). Common side-effects of immune checkpoint inhibitors include skin rashes, diarrhea, and fatigue.

Immune Checkpoints and their Blockade in Cancer Therapy

Normal cells express surface proteins, that bind to immune checkpoint proteins to turn them off, whereby immune system does not attack normal cells. Checkpoint proteins and other proteins regulate flow of signals to T cells telling the cells when to 'turn on' or 'turn off''. T cell 'turning on' kill the CSCs. Immune checkpoint inhibitors in cancer treatment are shown in Fig. 6.134.

- Cancer stem cells (CSCs) can also utilize same protective mechanism by expressing surface proteins that interact with checkpoint proteins on immune cells to overcome the immune surveillance.
- Immunotherapy drugs called immune checkpoint inhibitors work by blocking checkpoint proteins from binding with their partner proteins. These immunotherapy drugs block different checkpoint proteins such as PD-L1 (programmed cell death ligand 1), PD-1 (programmed cell death protein 1), and CTLA-4 (cytotoxic T lymphocyte-associated protein 4). CTLA-4 and PD-1 are found on T cells.
- PD-L1 is present on CSCs. Blocking the binding of PD-L1 to PD-1 with an immune inhibitor (anti-PD-L1) allows the T cells to kill CSCs.
- In clinical oncology, immune checkpoint inhibitors are used to treat cancer patients, which include: monoclonal antibodies blocking CTLA-4 (ipilimumab,



Immunosuppressive mechanisms in the tumor microenvironment (TME)	
Immunosuppressive cells in the tumor microenvironment (TME)	
• CD4+ regulatory T cell (Treg) • Bone marrow-derived suppressor cell (MDSC) • Tumor-associated macrophage (TAM-M2 polarized)	• Tumor-associated neutrophils (TAN) • Extracellular matrix (ECM) restricts activated CD8+ cytotoxic T cells to invasive tumor margin
Immune checkpoints in the tumor microenvironment (TME) and immune checkpoint inhibitors in cancer	
• **PD-1/PD-L1:** Antigen-presenting cells (APCs) patrol the body and pick up cancer stem cells (CSCs) and present to CD8+ cytotoxic T cells in the lymph nodes. CD8+ cytotoxic T cells become activated, undergo proliferation and head out to hunt for CSCs. – PD-1/PD-L1 acts to slow-down CD8+ cytotoxic T cell activity and fails to kill CSCs. – Blocking PD-1 or PD-L1 checkpoints by immune checkpoint inhibitors (monoclonal antibodies) restore CD8+ cytotoxic T cells antitumor activity and relieve immunosuppression leading to destruction of CSCs.	• **CTLA-4/B7:** CTLA-4 acts to slow-down CD8+ cytotoxic T cell activity. Blocking CTLA-4 checkpoints by immune checkpoint inhibitor (monoclonal antibody) allows CD8+ cytotoxic T cell to kill CSCs. • **LAG-3/TIM-3:** LAG-3 checkpoint promotes tumor cell immune escape in the tumor microenvironment. TIM-3 checkpoint plays a role in CD8+ cytotoxic T cell exhaustion, loss of their effector functions, expression of multiple inhibitory receptors and altered transcriptional programme in cancer. Blocking LAG-3/TIM-3 checkpoint inhibitors (monoclonal antibodies) restore CD8+ cytotoxic T cells antitumor activity and relieve immunosuppression leading to destruction of CSCs.

Fig. 6.134: Immune checkpoint inhibitors in cancer treatment. Inability to activate CD8+ cytotoxic T lymphocytes (CTLs) in tumor microenvironment through the suppressive effect or regulatory T cells (Tregs) through immune checkpoints allow CSCs to escape immune attack, survive and grow. B7 ligands expressed on APCs bind to CD28 receptor on CTL leading to T cell amplification and immune response. Alternately, binding of B7 ligands to CTLA-4 expression on T cells suppresses their activity. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) also enhances the activity of Tregs leading to immunosuppressive activity. Programmed cell death 1 (PD-1) is expressed on activated T cells. PD-1 binds to its programmed cell death ligand 1 (PD-L1) leading to the anergy of CTLs further promoting inhibitory signals. Pharmacological inhibition of immune checkpoints with monoclonal antibodies restores CTL antitumor activity and relieves immunosuppression.

tremelimumab), PD-1 inhibitors (nivolumab, pembrolizumab) and PD-L1 inhibitor (atezolizumab).

Cytotoxic Lymphocyte Antigen 4 (CTLA-4) Immune Checkpoint Inhibitor

Cytotoxic lymphocyte antigen 4 (CTLA-4) is a member of immunoglobulin superfamily and expressed on some types of immune cells. CTLA-4 is a regulatory molecule normally acts as type of switch '**turning off**' that suppresses CD8+ cytotoxic T cell effector function following initial activation by costimulatory signals such as TNF-α and interferon-γ (IFN-γ). Fully human monoclonal antibodies such as ipilimumab (Yervoy) and tremelimumab (Imjudo) targeting CTLA-4 have been

shown to increase T cell function, and antitumor responses by elimination of CSCs in patients with advanced metastatic melanoma.

- CD8+ cytotoxic T cells in the tumor microenvironment (TME) are most often supported by CD4+ helper T cells (Th1 cells) that produce IL-2 and IFN-γ. Other CD4+ helper T cells (Th2 cells) support the B cell activity by releasing IL-4, IL-5 and IL-13.
- Moreover, Th17 cells release IL-17A, IL17F, IL-21 and IL-21, which promote malignant tumor growth by fostering an inflammatory tumor environment. Precise understanding of the functions of immune cells and their interaction with other stromal cells as well as CSCs in tumor microenvironment (TME) is essential for the development of a sustainable and effective strategy for cancer immunotherapy.

Programmed Cell Death Protein 1 (PD-1) Immune Checkpoint Inhibitor

Programmed cell death protein 1 (PD-1) is a checkpoint cell surface protein on immune cells (T cells, B cells, macrophages, dendritic cells (DCs), and natural killer cells). PD-1 modulates T cell activity in peripheral tissues via interactions with its soluble ligands PD-L1 and PD-L2.

- PD-1 normally acts as a type of switch (**'turning off'**) that helps keep T cells from attacking other cells in the body. PD-1 does this when it attaches to CSCs. When PD-1 binds to PDL-1, it basically conveys the T cell to leave the other cell alone. Some CSCs have large amount of PD-L1, which helps them hide from an immune attack.
- Monoclonal antibodies that target either PD-1 or PD-L1 can block PD-1/PD-L1 binding and boost the immune system response against CSCs.
- Some anticancer drugs, called immune checkpoint inhibitors, are used to block PD-1 and PD-L1 interaction.
  - Examples of checkpoint inhibitor monoclonal antibodies that target PD-1 include: nivolumab (Opdivo), pembrolizumab (Keytruda) and cemiplimab (Libtayo) administered to treat melanoma, Hodgkin's disease and non-small cell lung carcinoma.
  - Nivolumab is used to treat some patients of renal cell carcinoma and head and neck cancers. Pembrolizumab is used to treat cancers of urinary tract.

Programmed Cell Death Protein–Ligand-1 and 2 (PD-L1 and PD-L2) Immune Checkpoint Inhibitor

Programmed cell death protein–ligand 1 (PD-L1) is a transmembrane protein that plays a major role in suppressing adaptive immune system. PD-L1 drives PD-1-mediated immune inhibition and is consequently expressed on T cells, B cells, macrophages and APCs, heart and lungs parenchymal tissues, and CSCs.

- PD-L1 is also detected in low levels on cardiac endocardium, pancreatic islets and syncytiotrophoblasts in the placenta, highlighting a role of PD-L1 in immunological tolerance.
- Examples of checkpoint inhibitor monoclonal antibodies that target PD-L1 include atezolizumab (Tecentriq), avelumab (Bavencio) and durvalumab (Imfinz). PD-L1 blockade can also be demonstrated efficacy in lung, urinary bladder, and other cancers.
- Programmed cell death protein–ligand 2 (PD-L2) is also an immune checkpoint inhibitor. The engagement of PD-1 by PD-L2 dramatically inhibits TCR-mediated proliferation, and cytokine production by CD4+ helper T cells. Programmed cell death protein–ligand 2 (PD-L2) proteins are being investigated in clinical trials.

LAG-3/TIM-3 Immune Checkpoint Inhibitor

LAG-3 is an immune checkpoint protein expressed on some types of immune cells, that normally suppresses CD8+ cytotoxic T cell effector function.

- On contrary, LAG-3 encourages differentiation of regulatory T cells (Tregs). Relatimab is a monoclonal antibody that attaches to LAG-3 and stops it from working. This can help boost the immune system response against CSCs.
- TIM-3 is an immune checkpoint receptor that suppresses antitumor response by negatively regulating the activity of CD8+ cytotoxic T cell and APCs. Anti-TIM-3 immune checkpoint monoclonal antibody boots immune system response against CSCs.

Pathology Pearls: Immune Checkpoint Inhibitors in Cancer Treatment

- Antigen-presenting cells (APCs) patrol the body and pick up evidence of threat-like CSCs.
- APCs present evidence of CSC to CD8+ cytotoxic T cells in the lymph nodes.
- CD8+ cytotoxic T cells become activated and undergo proliferation and head out to hunt for CSCs. CTLA-4 acts to slow down CD8+ cytotoxic T cell activity. Blocking CTLA-4 by checkpoint inhibitor monoclonal body allows CD8+ cytotoxic T cell killing CSCs.
- CD8+ cytotoxic T cells recognize and kill CSCs. PD-1/PD-L1 acts to slow down CD8+ cytotoxic T cell activity and fails to kill CSC. Blocking PD-1 or PD-L1 by checkpoint inhibitor monoclonal body restores CD8+ cytotoxic T cells anti-tumor activity and relieve immunosuppression leading to destruction of CSCs.

Adoptive T Cell Therapy (T Cell Transfer Therapy)

Adoptive T cell therapy (T cell transfer therapy) with tumor infiltrating lymphocytes or gene modifies T cells expressing novel T cell receptors is a treatment that uses a cancer patient's autologous T cells with anti-tumor activity, expanded *in vitro* in laboratory. The process of growing own T cells in the laboratory can take two to eight weeks. During this time, cancer patients (e.g. acute lymphoblastic leukemia/diffuse large B cell lymphoma, multiple myeloma) are treated with chemotherapy and radiation therapy to get rid of other immune cells. Cultures with high anti-tumor reactivity are expanded to $>10^{10}$ cells and reinfused into the cancer patient (melanoma) following the administration of a conditioning of lymphodepleting interleukin-2 (IL-2).

Cancer Vaccines

Cancer vaccines are used to treat patients with cancers expressing viral antigens called immunotherapy, which work to boost immune response and can destroy CSCs and inhibit metastasis to distant organs. Some cancers are caused by viruses. Cancer vaccines can provide protection against viral infections inducing cancers. Vaccinating children and certain young adults against human papillomavirus (HPV) provide protection against cancers of cervix, anal region, throat, vagina and penis.

Monoclonal Antibody-based Drugs

Monoclonal antibodies are laboratory-produced molecules engineered with plant or bacterial toxin to form immunotoxin complex, that can enhance or modify immune system's attack on the target CSCs only, but sparing normal cells. The monoclonal antibodies kill CSCs, interfere with angiogenesis, and suppress local production of growth factors.

- The monoclonal antibody-based drugs are currently employed in the treatment of breast carcinoma, colorectal carcinoma, and NHL. Selected monoclonal antibody-based drugs used in various cancers are given in Table 6.117.
- Monoclonal antibodies are designed to function in different ways: (a) CSCs coated with monoclonal antibodies are easily detected by immune system and targeted for destruction, (b) monoclonal antibodies can trigger immune system, that destroys membrane of CSCs, (c) monoclonal antibodies block the interaction of CSCs, with proteins, that promote cell growth and unrestricted CSCs proliferation, (d) as blood supply is essential for growth of malignant tumor, hence some monoclonal antibodies block protein–cell interactions, hence inhibit tumor angiogenesis, (e) certain monoclonal antibodies attack and destroy CSCs, (f) certain monoclonal antibodies combined with a small radioactive particle assists in transport of radiation treatment directly to CSCs, and thus minimize the effect of radiation on healthy cells, (g) similarly, monoclonal antibodies combined with chemotherapeutic agents, assist in transport of chemotherapeutic agent directly to CSCs, and thus minimize the effect of radiation on healthy cells, and (h) some drugs combined two monoclonal antibodies, one monoclonal antibody that attaches to CSCs, and

Table 6.117 Selected monoclonal antibody-based drugs used in various cancers

Agent	Target	Process Targeted	Cancer Type
Monoclonal antibody (mAb)			
Bevacizumab (Avastin)	Vascular endothelial growth factor (VEGF)	Angiogenesis	▪ Colorectal carcinoma (metastatic) ▪ Lung carcinoma
Cetuximab	Epidermal growth factor receptor	Growth factor signaling	Colorectal carcinoma
Trastuzumab (Herceptin)	HER2 growth factor receptor	Growth factor signaling	Breast carcinoma
Rituximab	CD20 Receptor	Induction of cell death	Non-Hodgkin's lymphoma (B cell)
Gemtuzumab ozogamicin (Mylotarg)	CD33	Blocks the growth of leukemic cells	Acute myelogenous leukemia
Small molecule inhibitors			
Imatinib	BCL-ABL fusion protein; kit	Growth factor signaling (tyrosine kinase inhibitor)	▪ Chronic myelogenous leukemia ▪ Gastrointestinal stromal tumors
Erlotinib	Epidermal growth factor receptor	Growth factor signaling	Non-small cell lung carcinoma
Bortezomib	Proteosome	Multiple processes affected	Multiple myeloma

Note that these names are coded to end in mAb for short of monoclonal antibody.
Herceptin is the brand name for trastuzumab

other monoclonal antibody, that attaches to immune system cells. This interaction may promote immune system attacks on the CSCs.

Immunomodulators used to Boost Immune System

Immunomodulators are used to boost immune response in cancer patients, which include cytokines (IFN-γ, interleukins), and immunoregulatory drugs (thalidomide, lenalidomide, pomalidomide, imiquimod).

- Cytokines drive communication between cells of immune system and coordinate attacks on specific CSCs and send signals that may help in survival of normal cells.
- Interferon-γ (IFN-γ) activates cellular immunity and stimulates subsequent anti-tumor immune response. Laboratory-prepared IFN-γ affects malignant tumors by virtue of its anti-angiogenic and antiproliferative properties.
- Interleukins bind to high-affinity receptors, and modulate cell growth, differentiation, and activate immune responses during inflammatory. IL-2 increases number of white blood cells especially T cells and B cells in the body.
- Laboratory-made interleukins are used to treat renal cell carcinoma and melanoma. Immunoregulatory drugs, also called biologic response modifiers, are medications, that boost immune system.

Pathology Pearls: Minimal Residual Disease

- Minimal residual disease (MRD) refers to presence of small number of CSCs especially in leukemias/lymphomas that remains occult within the patient after cancer treatment but eventually leads to relapse.
- In patients with acute lymphoblastic leukemia (**ALL**) and acute myelogenous leukemia (**AM**L) monitoring of minimal residual disease (**MRD**) offers a way to precisely assess early treatment.
- Polymerase chain reaction (**PCR**) and next generation sequencing (**NGF**) are performed on bone marrow samples at the time of initial diagnosis to look for genetic mutations in leukemic cells.
- Genetic mutations are reassessed again following treatment to determine minimal residual disease, even if leukemic cells can no longer be observed under the microscope.

PROGNOSIS OF SOLID CANCERS

Several solid malignant tumors can occur in several places such as organs, bones, muscles in adults. Most common solid malignant tumors in children are brain tumor, neuroblastoma, Wilms tumor, retinoblastoma, osteosarcoma, and Ewing sarcoma. Solid malignant tumors are carcinomas and sarcomas and are often treated with surgery. Prognosis of solid malignant tumors varies depends on origin in various organs. Prognosis of solid malignant tumors is given in Table 6.118.

Table 6.118 Prognosis of solid malignant tumors

Prognosis	Solid Malignant Tumors
Good prognosis	▪ Seminoma/dysgerminoma ▪ Basal cell carcinoma
Intermediate prognosis	▪ Breast carcinoma ▪ Colorectal carcinoma ▪ Laryngeal carcinoma ▪ Endometrial carcinoma ▪ Melanoma ▪ Teratoma testes ▪ Osteosarcoma
Poor prognosis	▪ Lung carcinoma ▪ Pancreatic carcinoma ▪ Gastric carcinoma ▪ Esophageal carcinoma ▪ Hepatocellular carcinoma ▪ Malignant mesothelioma

Pathology Pearls: Spontaneous Regression of Malignant Solid Tumors

- Spontaneous regression of pathologically confirmed solid malignant tumors, including those with metastases, is extremely rare but is documented for some malignant tumors of infancy and childhood and of adults.
- Some tumors may regress spontaneously such as renal cell carcinoma, neuroblastoma, retinoblastoma, malignant melanoma, leukemias, lymphomas, choriocarcinoma, hepatocellular carcinoma, cholangiocarcinoma, and osteosarcoma.
- Notably, in this regard is neuroblastoma in infants and childhood, which originates from neuroblasts of the adrenal medulla and usually has a highly aggressive course but, remarkably, in a small proportion of cases regresses by maturation/differentiation to a benign ganglioneuroma or disappears.

PREVENTION MODALITIES OF CANCERS

Cigarette smoking is usually associated with lung carcinoma. In cigarette smokers, there is also increased incidence of cancers of oral cavity and urinary bladder. Alcohol abstinence reduces squamous cell carcinoma of oropharynx and upper/middle esophagus, pancreatic and hepatocellular carcinomas. Increased adipose tissue increases aromatase conversion of androgens to estrogens, which increase risk for endometrial carcinoma and breast carcinoma. Therefore, reduction of body weight decreases risk for endometrial carcinoma and breast carcinoma.

- **Hepatitis B vaccination:** Immunization against HBV decreases the risk for hepatocellular carcinoma due to hepatitis B induced post-necrotic cirrhosis.
- **Screening procedures:** Cervical Papanicolaou smear examination detects cervical dysplasia (preneoplastic lesion), which can be surgically removed.
  - **Colonoscopy** detects numerous polyps that are precancerous.
  - **Mammography** detects non-palpable masses in breasts.
  - **Prostate-specific antigen (PSA)** detects prostatic carcinoma, but lacks specificity as it is increased in benign hyperplasia of prostate.
- **Treatment of conditions that predispose to cancer:** Eradication of *Helicobacter pylori* decreases the risk for developing MALT lymphoma and gastric adenocarcinoma. Treatment of gastroesophageal reflux disease (GERD) decreases the risk for adenocarcinoma arising from Barrett's esophagus.

MOLECULAR DIAGNOSTICS IN ONCOLOGY

Molecular diagnostics in oncology are tests that detect alterations in chromosomes, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, and metabolites that provide information about screening of high-risk populations, cancer diagnosis, prognosis and severity of disease, therapy selection, molecular stratification of otherwise identical malignant tumors for the purpose of treatment and efficient management, monitoring the clinical course of disease, predicting response to therapy, prevention of cancer, diagnosis of hereditary predisposition to cancer, detection of minimal residual disease (MRD), establishment of baseline values for assessment of future change and clinical research studies. Proteins released by malignant tumors into the serum can be used to screen populations for cancer and to monitor recurrence following treatment.

- Gene consists of three types of nucleotide base pair sequences: (a) coding regions, called exons, which specify a sequence of amino acids, (b) noncoding regions, called introns, which do not specify amino acids, and (c) regulatory sequences, which play a role in determining when and where the protein is produced. Enhancer region of DNA is far away, whereas promoter region near to gene. Transcription factors and mediator proteins complex 'turn on' the gene and help RNA polymerase to the reads the gene.
- Each organ contributes wild-type DNAs to the circulation, while organ metastatic seeds will shed mutant DNA.
- Integrated analysis of DNA, RNA and methylation next generation sequencing will help elucidate all relevant genetic alterations in malignant tumors: gene structural variation, gene copy number variation, gene mutational profile, gene mutational spectrum, gene mutational rate, gene arrangements, DNA methylation, RNA expression, and signaling pathways analysis.
- Cytogenetic, molecular diagnostics and epigenetic biomarkers can be performed for diagnosis and prognosis of cancer and its epidemiologic studies.
- Circulating small noncoding RNAs (e.g. microRNAs), long noncoding RNAs and exosomal RNAs thus reflect the overall host–tumor cross-talks.
  - Exosomal RNAs induce angiogenesis in human cancers.
  - Biomarkers are analyzed on samples obtained from blood plasma/serum, urine, cerebrospinal fluid, saliva, and malignant tumor tissue.
- One or more cancer-specific molecular markers are analyzed to study genetic or epigenetic pathways regulating cellular proliferation and differentiation or apoptosis. Some biomarkers are specific and highly sensitive for detection of malignant tumors.
- Learning some basic facts about DNA, RNA, and proteins, it is essential to understand the importance of biomarkers in human malignant tumors. Biomarkers are analyzed on samples obtained from blood plasma/serum, urine, cerebrospinal fluid, saliva, and tumor tissue.
- Some biomarkers are specific and highly sensitive for detection of malignant tumor. Recently, our knowledge on biomarkers has evolved significantly for improving the detection and efficient management of cancer patients.
- Selected examples of molecular diagnostics in malignant tumors are given in Table 6.119. Molecular diagnostics in oncology is shown in Fig. 6.135. Kindly refer to Chapter 14: 'Cellular–Molecular Diagnostic Techniques in Clinical Practice'.

PURPOSE TO ANALYZE CANCER BIOMARKERS

In general cancer biomarkers are classified by their different functions. Even within the biomarker categories, molecules that can trigger abnormal cell growth

Table 6.119 Selected examples of molecular diagnostics in malignant tumors

Molecular Biomarker	Associated Cancers
Human papillomavirus (HPV)	Cervical carcinoma
BRCA1/BRCA2 mutation	Inherited breast carcinoma and ovarian carcinoma
CA125	Ovarian carcinoma
Prostate-specific antigen (PSA)	Prostatic carcinoma
HER2/neu mutation	Breast carcinoma
DNA mismatch repair (MLH1, MSH2, MSH3, MSH6, PMS1 and PMS2) mutations	Hereditary nonpolyposis colorectal carcinoma (HNPCC) also called Lynch syndrome
c-KIT mutation	Acute myelogenous leukemia (AML), gastrointestinal stromal tumors, seminoma, and sinonasal NK cell lymphoma
EFGR mutation	Head and neck cancers, and lung carcinoma
EGFR, EML4-ALK, ROS1, K-RAS mutations	Non-small cell lung carcinoma
BRAF mutation	Colorectal carcinoma, melanoma, thyroid carcinoma, lung carcinoma, brain gliomas and hairy cell leukemia
BCR-ABL1 mutation	Chronic myelogenous leukemia (CML), and B cell acute lymphoblastic leukemia (B-ALL)
IGH-BCL-2 mutation	B cell lymphoma
K-RAS, N-RAS mutation	Colorectal carcinoma
TP53 mutation	Li-Fraumeni
ATRX mutations	Brain gliomas, neuroendocrine tumors, osteosarcoma, liver angiosarcoma, leiomyoma

Molecular diagnostics are most performed on samples of blood, saliva, or tumor tissue.

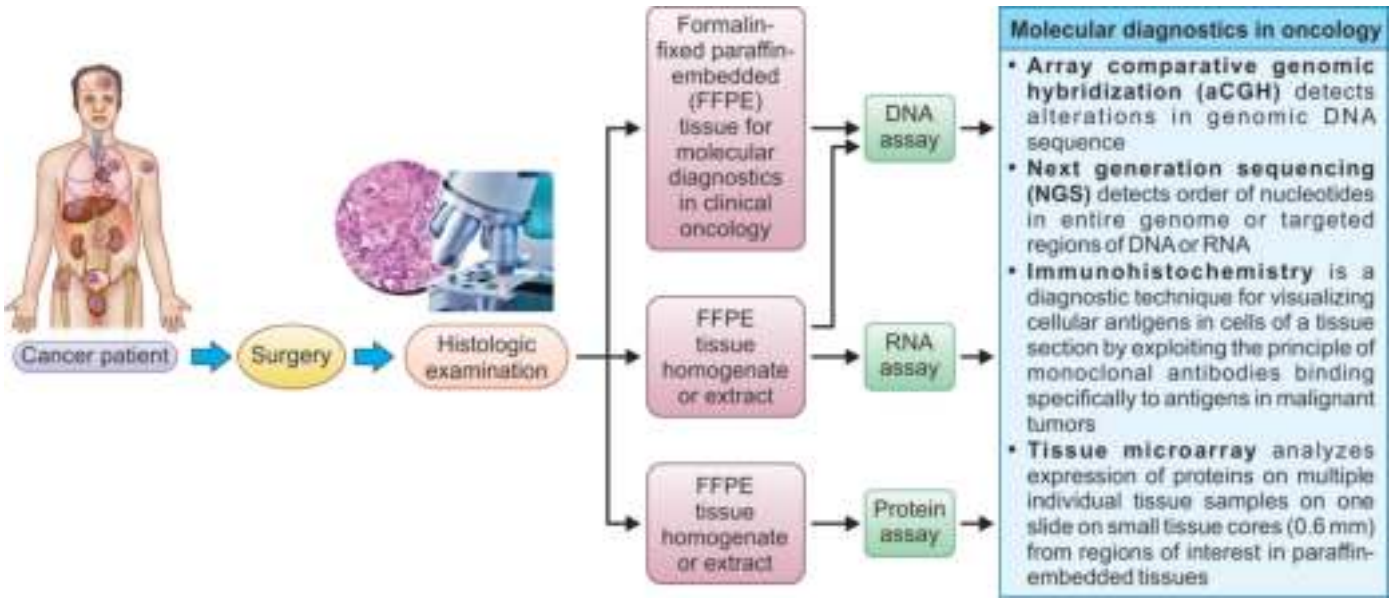


Fig. 6.135: Molecular diagnostics in oncology. There are multiple applications of molecular tests in clinical oncology. Mutation analysis is now routinely performed for the diagnosis of hereditary cancer syndromes and healthy carriers of cancer predisposing germline mutations. Mutation analysis is performed to detect somatic mutations. Tumors almost always shed their fragments (i.e. single cells or their clusters, DNA, RNA, and proteins) or some other tumor-derived molecules hold a great promise for non-invasive monitoring of cancer disease, analysis of drug-sensitizing mutations and early cancer detection through screening. Some tumor- or tissue-specific mutations and medically relevant DNA, RNA and protein-base markers expression can be efficiently utilized for the diagnosis of cancers of unknown primary origin.

and proliferation can come from a gene mutation or extra copies of an otherwise healthy gene within the tumor's DNA.

- **Analysis of unrestricted cell proliferation:** HER2/neu-positive breast cancer that rests positive for protein called epidermal growth factor receptor 2 (EGFR2, i.e. HER2) leading to unrestricted CSC proliferation and progressive disease. Targeted 'Herceptin therapy' is known to disrupt the HER2 signaling pathway, that inhibits cell growth, proliferation, and progression of disease.
- **Analysis of cellular changes in cancer:** SPARC (secreted protein, acidic, rich in cysteine) protein regulates interactions between cells and their surrounding extracellular matrix (ECM), and collagen in bones.
  - SPARC protein governs cell adhesion, proliferation, and differentiation. Cell migration and metastasis greatly contribute to the progression of cancers.
  - In highly malignant tumors (i.e. glioblastoma multiforme, melanoma, breast carcinoma and prostatic carcinoma), SPARC protein promotes bone metastasis and transforming growth factor-β (TGF-β) induced epithelial–mesenchymal transition (EMT).
- **Analysis of efficacy platinum-based chemotherapeutic agents:** Platinum-based chemotherapeutic drugs (**cisplatin, carboplatin** and **oxaliplatin**) are widely used for eradication of CSCs. However, there is a protein-encoded by **ERCC1** (excision repair 1, endonuclease non-catalytic unit) gene, that repairs tumor DNA. If cancer biomarker testing detects high levels of ERCC1 protein in a patient's malignant tumor, platinum-based chemotherapeutic agents are not likely to be very effective for that patient.

SPECIMEN REQUIREMENTS FOR MOLECULAR DIAGNOSTICS IN CANCER

Biomarkers are analyzed on samples obtained from blood plasma/serum, urine, cerebrospinal fluid, saliva, and tumor tissue.

- Fresh tissue is required for cell culture and subsequent G-banded metaphase cell preparations or metaphase FISH procedures and special karyotyping multicolor FISH techniques.
- DNA can be extracted from the fresh, frozen, or formalin-fixed paraffin-embedded tumor tissue for comparative genomic hybridization platforms.
- Cytologic touch imprints are prepared from fresh or frozen malignant tumor tissue. Formalin-fixed paraffin-embedded malignant tumor tissue is used to analyze interphase FISH.
- Blood and bone marrow samples should be drawn into anticoagulated tubes containing ethylene-diaminetetra-acetic acid (EDTA) and transported at ambient temperature for DNA sequencing.

MOLECULAR DIAGNOSTICS USES IN ONCOLOGY

In the field of oncology, biomarkers have several potential applications in oncology, including risk assessment, differential diagnosis, prognosis, prediction of treatment response, pharmacokinetics, monitoring treatment response and monitoring of disease progression and cancer recurrence.

- **Biomarker use to recognize genetic variations:** Genetic variations are nonpathogenic and present in 1% of healthy population. Term '**polymorphism**' is used to describe nonpathogenic genetic variation, and mutation is a change in DNA sequence from errors in DNA replication during cell division.
  - Conversion of a proto-oncogene to oncogene is called activation, which results from transduction, insertional mutagenesis, point mutation (single nucleotide substitution), deletions, insertions, amplification, copy number variations, and chromosomal rearrangements. Mutations can be classified according to their effects on structure of gene, i.e. gene promoters, splicing regions, and coding regions (missense, nonsense, and salient mutations). K-RAS, BRAF and HER2/neu gene mutations are common in lung cancers. ALK gene activated by point mutation in neuroblastoma, and chromosomal translocation in non-small cell lung carcinoma (NSCLC) and anaplastic large cell lymphoma.
  - Tumor suppressor genes inhibit cell proliferation and malignant tumor development. Tumor suppressor genes require two mutational hits, i.e. inactivation/biallelic loss to induce malignant tumors. Point mutations or deletions in tumor suppressor TP53 and CDKN2A genes are linked to many cancers.
- **Biomarker use in risk assessment of cancer:** Molecular diagnostics refers to molecular profiling, that can be used to assess whether an individual is at risk for development of certain histologic type of malignant tumor. Molecular testing can also be used to decide whether an individual should undergo more intensive screening or take preventive measures. For example, blood sample is used to analyze BRCA1 and BRCA2 genes in patient and close relatives (e.g. mother, aunt). BRCA1 and BRCA2 gene alterations can increase risk of breast carcinoma and ovarian carcinoma.

- **Biomarker use in differential diagnosis of cancer:** Molecular diagnostics plays pivotal role in clinical oncology in establish diagnosis, tumor origin and histologic subtypes, that affects the clinical course. The cancer's subtype may have implications for treatment.
  - Chromosomal translocations are one of the biomarkers for cancer diagnosis (e.g. BCR-ABL1 gene fusion in chronic myelogenous leukemia), and heavy immunoglobulin–Myc gene fusion in Burkitt's lymphoma.
- Molecular diagnostics is widely used to diagnose and determine clinical course of acute myelogenous leukemia (AML) and acute promyelocytic leukemia (APL).
  - Acute myelogenous leukemia (AML) is classified into poor, intermediate and favorable risk categories based on evaluation of chromosomes. Patients with intermediate risk of AML should undergo molecular testing for different gene mutations.
  - Karyotyping detects chromosomal translocations in various malignancies such as in papillary thyroid carcinoma (RET-PTC gene fusion), prostatic carcinoma (TMPRSS-ETS gene fusion) and other solid malignant tumors.
- **Biomarker use to assess prognosis in cancer patients:** Prognosis is defined as the natural course of disease in the absence of treatment or a predicted clinical outcome of cancer patients from medical treatment.
  - Some malignant tumors are naturally more aggressive than others, malignant tumors and knowing, this can help the clinician to select line of treatment. For example, alteration in FLT3 gene in AML is associated with aggressive clinical course. FLT3 inhibitor is administered to treat such AML cases. Molecular diagnostics can also be used to evaluate the response of AML to treatment in relation to its recurrence.
  - Several molecular diagnostics are available to predict the clinical course of breast carcinoma in female with early stage disease, lymph node negative, estrogen receptor (ER) positive, and treated with tamoxifen 'anti-estrogen' therapy. These molecular tests analyze multiple genes in CSCs obtained from a tissue sample of the breast carcinoma.
- **Biomarker use in prediction of treatment response in cancer patients:** Molecular diagnostics can be used to predict response to treatment. Molecular testing is done to analyze HER2/neu gene amplification, which directs CSCs to produce excess of human epidermal growth factor receptor 2 (EGFR2, i.e. HER2), leading to rapid cell proliferation and hence breast carcinoma. A drug known as 'trastuzumab' sold under the brand name 'Herceptin' is a monoclonal antibody, that inhibits the activity of HER2 protein.
- **Biomarker use in pharmacokinetics in cancer patients:** Pharmacokinetics is the aspect of pharmacology dealing with how drugs are absorbed, distributed, metabolized, and eliminated from the body.
  - Depending on the genetic constitution of individuals, therapeutic drug is metabolized at a faster rate in some individuals. Therapeutic drug '**irinotecan**' (topoisomerase 1 inhibitor) is used to treat colon carcinoma. Irinotecan is converted to an active metabolite SN-38, which is then inactivated, and detoxified by UDP-glucuronosyltransferase **(UGT)** enzyme encoded by the UGT1A1 gene in the body. UDP-glucuronosyltransferase (UGT) enzymes are responsible for glucuronidation, a process that transforms lipophilic metabolites into water-soluble metabolites that can be excreted from the body.
  - Patient of colon carcinoma with a genetic pattern designated 'UGT1A1-28' metabolize 'irinotecan' more slowly than those without this pattern. To prevent the accumulation of 'irinotecan' in the body, these persons must be administered a lower dose of therapeutic drug than normal.
- **Biomarker use in monitoring treatment response in cancer patients:** Molecular diagnostics monitors response to neoadjuvant chemotherapy by analyzing blood biomarkers at multiple times over the course of the treatment of many human cancers.
  - Patients with chronic myelogenous leukemia (CML) are treated by imatinib for years. Such CML patients can develop resistance to imatinib due to change in the sequence of genetic material over time.
  - Consequently, CML patients, who no longer respond to imatinib may undergo molecular diagnostic testing to determine whether the gene has changed. Successfully treated CML patients are monitored at regular intervals for signs of recurrence of disease. It is essential to develop better molecular diagnostics that can accurately monitor cancer recurrence, whose disease has been successfully treated.

Pathology Pearls: Molecular Diagnostics Applications in Oncology

There are multiple applications of molecular tests in clinical oncology, which utilizes molecular-based assays CTCs, ctDNA, RNA and proteins.

- **Herediatry cancer syndromes testing:** Mutational analysis is routinely utilized for the diagnosis of hereditary cancer syndromes for identification of persons at-risk, risk of second malignancy and for personalization of systemic treatment.

- Healthy carriers of cancer-predisposing mutations benefit from close medical surveillance and various preventable interventions.
- Cancers caused by germline mutations in corresponding genes most often require significant modification of treatment strategy.
- Germline mutations in BRCA-1 and BRCA-2 genes are associated with probability of cancers of breast and ovary.
- Analysis of mutation-related to breast cancer risk includes BRCA1, BRCA2, PALB2, TP53, CHEK2, ATM, BLM, NBS/NBN, PTENRAD51CC, RAD51D, RECQL, FNACC, FANCM, BRAD1, and BRIP1.
- Hereditary nonpolyposis colorectal carcinoma (**HNPCC**) syndrome, also known as Lynch syndrome, is caused by germline mutations in MLH1, MSH2, MSH6, PMS2, and EPCAM genes.
- Molecular testing detects recurrent mutations (PCR), single-gene analysis (Sanger sequencing), multigene analysis (next-generation sequencing), and whole exome sequencing.

- **Predictive molecular markers:** There are several predictive tests (DNA, RNA, proteins, cells, and tissue slices) involving either the analysis of individual drug targets (EGFR, BRAF, ALK, ROS1 and PARP inhibitors) or identification of specific tumor phenotypes such as high level microsatellite instability (**MSI-H**), BRCA gene and mutation burden, which aid the choice of anticancer drugs.
- **Circulating tumor fragments and molecular tests:** Malignant tumors almost always shed their fragments (single cells or in clusters, DNA, RNA, and proteins) into various body fluids.
  - Monitoring of malignant disease can be achieved through molecularly driven detection of residual tumor fragments. It is anticipated that liquid biopsy will serve as an instrument for early cancer diagnosis and screening in the future.
  - Liquid biopsy has many clinical applications in oncology by analyzing circulating free tumor DNA (ctDNA)/viral DNA (virus-related malignancies), circulating free nucleic acids (cfNA), extracellular vehicles (EVs) and tumor-educated platelets (TEPs).
- **Carcinomas of unknown origin at primary site:** Recent developments are in the mutation testing and RNA analysis novel tools for diagnosis of cancers of unknown primary site. Tissue specific markers (RNA and proteins) and genetic markers (point mutation, gene rearrangements and gene copy variants) are analyzed to detect carcinoma of unknown primary tumor.

MOLECULAR TECHNIQUES: METHODOLOGIES

Extraction of intact, moderately high quality DNA or RNA is essential for molecular assays. Depending on the type of molecular diagnostics, molecular testing is currently performed on the malignant tumor itself, by analyzing circulating tumor cells (CTCs) and circulating free tumor DNA (ctDNA), chromosomal translocations, and other structural chromosomal abnormalities, gene structure, gene amplification, gene copy number variation (gains or losses), single nucleotide base pair substitutions, small deletions/insertions, single nucleotide polymorphisms (SNPs), and epigenetic alterations in gene expression, which facilitate diagnosis, subclassification, prognosis and monitoring response to therapy. On receipt of specimen, pathologist reviews the specimen adequacy, extracts DNA or RNA, analyzes and interprets results.

- **Nucleic acid hybridization:** Nucleic acid hybridization is based on the ability of single-stranded nucleic acid sequence (either DNA or RNA) to specifically hybridize/anneal (reestablishment of hydrogen bonds) to a complementary strand.
  - **Southern blotting technique and Northern blotting technique:** Southern blotting technique and Northern blotting technique use a radioactive or fluorescent probe apply the principle of nucleic acid hybridization (DNA and RNA sequences). Probes are the key tools in the hybridization techniques used to identify the complementary sequence in question. The important steps involved in blotting techniques include: (a) digestion of DNA or RNA in small fragments using restriction enzymes, (b) separation of nucleic acid based on size by agarose gel electrophoresis, (c) denaturation of nucleic acids, (d) transfer of nucleic acid from agarose gel to nylon membrane, (e) hybridization of the single nucleic acid probe to the filter-bound nucleic acid, and (f) development of the membrane to visualize and analyze hybridized products.
  - **Polymerase chain reaction:** Another example of nucleic acid hybridization molecular technique is polymerase chain reaction (**PCR**) used to amplify a specific nucleic acid sequence such as template nucleic acid (DNA), primer pairs (forward and reverse primers set to hybridize the complementary sequences of template DNA—the portion of sequence flanked between forward and reverse primers is amplified).
- **DNA sequencing:** DNA sequencing determines the exact order of four chemical building blocks called nucleotide base pairs (e.g. adenine, guanine, cytosine and thymine) in DNA strand.
  - Sequencing strategies include whole genome sequencing (involving entire exons and introns), amplification-based polymerase chain reaction, transcriptome RNA sequencing, also known as RNA sequencing, and exon capture transcriptome sequencing.
  - Whole-genome sequencing is the most powerful form of DNA sequencing technology.
  - Shotgun (Sanger) sequencing is more traditional approach, which is designed for sequencing

Table 6.120 Strategies for detection of genomic alterations in cancers

Sequencing Strategies	Applications
Whole genome sequencing (involving entire exons and introns)	Gene point mutation, copy number and structural variants
Whole exome sequencing (involving 20,000 genes constituting 1% of the genome)	Gene point mutation and copy number
Amplification based polymerase chain reaction	Gene point mutation and deletion
Transcriptome RNA sequencing, also known as RNA sequencing	Gene expression, fusion, and splice variants
Exon capture transcriptome sequencing	Gene expression, fusion, and splice variants

entire chromosomes or long DNA strands with more than 1000 nucleotide base pairs by using a purified DNA polymerase enzyme to synthesize DNA chains of varying lengths.

- Strategies for detection of genomic alterations in cancers are given in Table 6.120.

Pathology Pearls: Southern Blot Hybridization Technique

- Southern blot hybridization is used when a DNA sequence variant is too large detected by conventional polymerase chain reaction (PCR) to detect mutations (i.e. deletions, insertions, or duplications) and polymorphisms. This technique requires a very large amount of high molecular weight genomic DNA as the start input.
- DNA is initially digested with one or more restriction enzymes, which are specific for individual, and the digested fragments are run on an agarose gel electrophoresis, which is transferred to a membrane, hybridized with a labeled probe.
  - The denatured single-stranded DNA is then bound to the membrane by a heat or ultraviolet source.
  - The membrane is subsequently hybridized with sequence specific, washed, and exposed on film for imaging.
- Southern blot hybridization technique may be used to detect clonal rearrangement of their receptor genes in T and B cells neoplasms, deletions in the dystrophin gene in Duchenne muscular dystrophy, and trinucleotide repeat diseases (repetitive duplication of three nucleotides of DNA) such as myotonic dystrophy and fragile X syndrome.

LIQUID BIOPSY: CELL-FREE DNA TECHNOLOGY

In recent years, medical oncologists opt for a liquid biopsy over a tissue biopsy for patients with advanced stage cancer disease.

- **Liquid biopsy** is minimally invasive, safe, quick, and accurate, and performed to obtain blood sample.
- **Tissue biopsy** is analyzed to diagnose fundamental histologic characterization of tumors and analysis of several molecular tests. Compared with tissue biopsy, liquid biopsy has broad potential applications for cancer diagnosis and treatment including screening of early diagnosis study of tumor heterogeneity, and clonal evolution detection of minimal residual disease (MRD). Liquid biopsy accurately detect all four classes genomic alterations in over 70 genes most relevant to solid tumors.
- Liquid biopsy can be repeated at different time points, and therefore it can complement both tissue biopsy and imaging techniques during disease. Moreover, liquid biopsy could anticipate disease progression even months before radiological progression of the disease.
- During cancer progression, liquid biopsy may help oncologists to stratify patients for treatment personalization and to monitor the treatment response and resistance development. It can also be used for the tumor molecular characterization, and its minimal invasive nature allows repeat sampling to monitor changes over time without the need for a tissue biopsy.
- During metastatic cascade malignant tumors shed some amount of their fragments into peritumoral space.
  - Tumor fragments may be represented by single/cluster of circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), cell-fee nuclear DNA (cfDNA), mitochondrial DNA, viral DNA, methylated DNA, extracellular vehicles (EVs), exosomes protein-coding regions of genome, RNA, protein microRNA (MiRNA), and tumor-educated platelets (TEPs).
  - **Exosomes** are small stable vesicles released from the cell of origin and carry a plethora of biological molecules encompassing DNA, RNA, microRNA, and proteins. Consequently, these entities can be collected in various body fluids (blood plasma/serum, urine, cerebrospinal fluid, saliva) and serve as tumor markers evaluated during cancer progression.
- A liquid biopsy may be used to detect malignant tumor at an early stage. In the human body, most of the deoxyribose acid (DNA) in a genome is packed inside with the help of specific proteins, protecting it from being degraded.
  - **Circulating tumor DNA (ctDNA)** is a single- or double-stranded DNA released by the malignant tumors cells into the bloodstream and it harbors the

gene mutations of the original primary malignant tumors.
  - Circulating cell-free RNAs (cfRNAs) are cancer-related messenger RNAs or noncoding RNAs, including microRNAs (miRNAs).
  - Extracellular vesicles (EVs) are small membrane-bound vesicles, including microvesicles and exosomes, which carry cargo such as nucleic acids and proteins during tumor metastasis.
- Circulating tumor cell (CTC) tests may be performed to monitor metastatic tumors of breast, colorectal region, and prostate. CTC diagnostic test helps capture, identify, and count CTC in a blood sample.
  - CTCs are CSCs that detach from solid malignant tumors and enter bloodstream. CTC test may be performed prior to the initiation of therapy or during treatment.
  - Several methods have been developed to capture and analyze circulating tumor DNA (**ctDNA**) for specific molecular tumor alterations, including allele specific polymerase chain reaction (PCR), droplet digital PCR (ddPCR) or targeted next generation sequencing and are successful used in several cancer types to monitor genomic alterations in a dynamic way.
  - Cell-free DNA (**cfDNA**) can be detected in either plasma or serum via multiple mechanisms, including active secretion, necrosis, and apoptosis. Cell-free DNA (cfDNA) is an ideal biomarker for real-time monitoring cancer disease progression and therapeutic response.
- During metastatic cascade, different analytes are involved. Unrestricted CSC proliferation of primary malignant tumor releases DNA from necrotic/apoptotic cells. Extracellular vesicles (EVs) are involved in tumor angiogenesis. A test is performed on a sample containing free circulating pieces of DNA (ctDNA) to detect cancers. Circulating viral DNA can be used to identify virus-related cancers.
- During local invasion, detachment, and intravasation, CSCs acquire significant alterations to survive the physical interactions, and the mechanical forces in blood and tissues.
  - Cancer stem cells undergo epithelial–mesenchymal transition (EMT) to move within surrounding connective tissue, and then secrete exosomes that contribute to induce vascular endothelial permeability for intravasation.
  - During embolization and survival, CTCs can survive and undergo adaptation in the bloodstream by forming CTC clusters/microemboli, that display higher metastatic potential by increasing CSC survival and reducing apoptosis. Clustering of CTCs with neutrophils can also promote cell cycle progression and survival of CTCs. The CTCs also gain physical and immune system protection by interacting with tumor-educated platelets.
  - During arrest and extravasation in target organ/tissue, CTCs interact with vascular endothelial cells, mainly capillaries, where they become trapped. CTCs and platelets might release EVs that can modify the cytoskeleton of vascular endothelial cells and increase blood vessel permeability, and allow CTCs extravasation.
  - In micrometastases in organ/tissue, CTCs need favorable pragmatistic niche and exosomes play a key role in its preparation. CTCs form macrometastasis in distant organs/tissues such as liver, lung, bone, and brain.
- Liquid biopsy has many clinical applications in oncology by analyzing CTCs, ctDNA, cfDNA, mitochondrial DNA, viral DNA, methylated DNA, EVs, exosomes, protein-coding regions of genome, RNA, protein MiRNA, and tumor-educated platelets (TEPs).
- Liquid biopsy is used in detection of genetic alterations (i.e. point mutation, insertions, amplification, deletions, copy number variations (CNVs), chromosomal aberrations, chromosomal translocation, chromosomal fusion transcripts, and aberrant protein expression) in cancers.
- Multistep metastatic cascade and biomarkers analysis, and clinical applications of liquid biopsy for treatment strategy in various stages of cancer are shown in Fig. 6.136. Comparison of tissue biopsy and liquid biopsy is given in Table 6.121. Liquid biopsy to assess analytes during metastatic cascade is given in Table 6.122. Clinical applications of liquid biopsy are given in Table 6.123.

Pathology Pearls: Liquid Biopsy Protocol and Steps

- **Isolation of CTCs technique:** Immune-based methods use antibodies to selectively bind surface antigens that distinguishes cancer tumor cells (CTCs) from blood cells. These antibodies may be conjugated to magnetic nanoparticles or immobilized on the walls of microfluidic chips to isolate CTCs. A new generation of CTC antigen-based dual platforms combines immunomagnetic beads with microfluidics.
  - Size-dependent isolation techniques include filtration, microfluidics, centrifugation and inertial focusing.
  - Direct imaging detection methods use specific fluorescent tags to identify and count cancer tumor cells (CTCs) in blood circulation. Each of these techniques is unique in its sample preparation, detection algorithms and fluorophores.
  - Dielectrophoresis technique relies on particles with different polarization that move differently under a non-uniform electric fluid.

- **Enumeration technique:** Enumeration technique is used to count the number of CTCs detected in a sample include impedance, high-throughput imaging, flow cytometry and artificial intelligence.
- **Characterization technique:** Characterization technique is used to characterize CTCs include immunostaining, fluorescent *in situ* hybridization (FISH) sequencing, quantitative reverse transcription polymerase chain reaction (qrt-PCR), expression analysis and cell culture. The molecular techniques used to characterize CTCs include immunohistochemistry, fluorescence *in situ* hybridization (FISH), next generation sequencing, quantitative reverse transcription polymerase chain reaction (qRT-PCR) and cell culture.
  - **Circulating tumor DNA:** Circulating tumor DNA (ctDNA) is a single- or double-stranded DNA released by the tumor cells into the bloodstream and it harbors the gene mutations of the original tumor. In recent, liquid biopsy based on ctDNA analysis has enlightened on the molecular diagnosis and monitoring of cancer disease. Gene mutations in tumor are detected by droplet digital PCR, beads, emulsion, amplification, magnetic (BEAMing), next-generation sequencing-based approaches.
  - **Circulating cell-free DNA (cfDNA):** Circulating cell-free DNA (cfDNA) is degraded fragment released to body fluids such as bloodstream, urine, and cerebrospinal fluid. cfDNA exhibits the genetic and epigenetic alterations of cancers including mutations, copy number variations, chromosomal rearrangements, DNA hypomethylation. Gene mutations in tumor are detected by droplet digital PCR, BEAMing, next generation sequencing-based approaches. The profiling of epigenetic features of cfDNA, such methylation signatures, may provide information about the tissue of origin.
  - **Extracellular vesicles:** Extracellular vesicles (EVs) are small membrane-bound vesicles, including microvesicles and exosomes, which carry cargo such as nucleic acids and proteins during tumor metastasis. EVs detected by liquid biopsy lacks efficient procedures to obtain EVs with high purity. Strategies used to isolate EVs include centrifugation, polymer precipitation, ultrafiltration, size-exclusion chromatography, affinity isolation and microfluidics-based technique.
  - **Circulating cell-free RNA:** Circulating cell-free RNAs (cfRNAs) are cancer-related messenger RNAs or noncoding RNAs, including microRNAs (miRNAs). Profiling cfRNA offers unique opportunities to detect the molecular subtype. RNA-based liquid biopsy technique hinders clinical application due to lack of standardized protocols.

Pathology Pearls: Applications of Liquid Biopsy to Monitor Tumor Progression

- Early diagnosis through screening based on detection of predictive biomarker.
- Diagnosis—stratification and therapeutic intervention with tumor staging in metastatic disease based on detection of biomarkers.
- Monitoring—drug response and resistance in real-time.
- Tumor clonal evolution assessment.
- Minimal residual disease (MRD) detection, prognosis, recurrence, and metastasis and risk of relapse.
- Actionable therapeutic targets and resistance mechanism—tumor heterogeneity and characterization.
- Prognosis—prognosis and risk of recurrence, and metastasis.
- Immunotherapy—determination of microsatellite instability (MSI) status, mutational load and TCR profile-based next-generation sequencing.

Pathology Pearls: Clinical Applications of Liquid Biopsy

- Circulating free tumor DNA (ctDNA) can be exploited to detect the presence of cancer.
- Circulating viral DNA can be used identify virus-related cancers.
- After diagnosis ctDNA allows patients' stratification and can help guiding therapeutic intervention.
- During therapy ctDNA can be used to monitor and resistance in real time as well as to detect minimal residual disease.
- At progression, ctDNA analysis could reveal tumor molecular heterogeneity and actionable targets for further therapeutic interventions.
  - Also, ctDNA analysis could be helpful to monitor microsatellite instability (MSI) status and tumor mutational loads that are valuable parameters for immunotherapy.
  - In addition, analysis of peripheral blood mononuclear cell can be used to profile T cell receptors (TCRs).

Pathology Pearls: Mitochondrial Deoxyribonucleic Acid (DNA) Biomarkers

- Interestingly, besides nuclear DNA aberrations, mitochondrial DNA (mtDNA) alterations have been detected in patients with human cancers, hence mitochondrial DNA could serve as molecular markers for the disease.
- Mitochondrial DNA molecule is the small circular chromosome found inside mitochondria, which can replicate independently of nuclear DNA.
  - Mitochondrial DNA contains 37 genes, which encodes ribosomal RNAs, transfer RNAs and polypeptides.
  - Mitochondrial DNA has higher rate than nuclear DNA due to lack of DNA repair system and histone proteins; and due to its exposure to reactive oxygen species, which are generated during oxidative phosphorylation in the inner mitochondrial membrane.
- Somatic mitochondrial DNA mutations have been implicated in the process of carcinogenesis seen in all human cancers.
- Single gene mutation, insertion, deletion, and mitochondrial microsatellite instabilities have been demonstrated in the breast carcinoma, cervical carcinoma, urinary bladder carcinoma, and head and neck cancers.

TARGETED MUTATION ANALYSIS METHODS

Germline mutations, also called hereditary mutations, are passed on from parents to offspring leading to

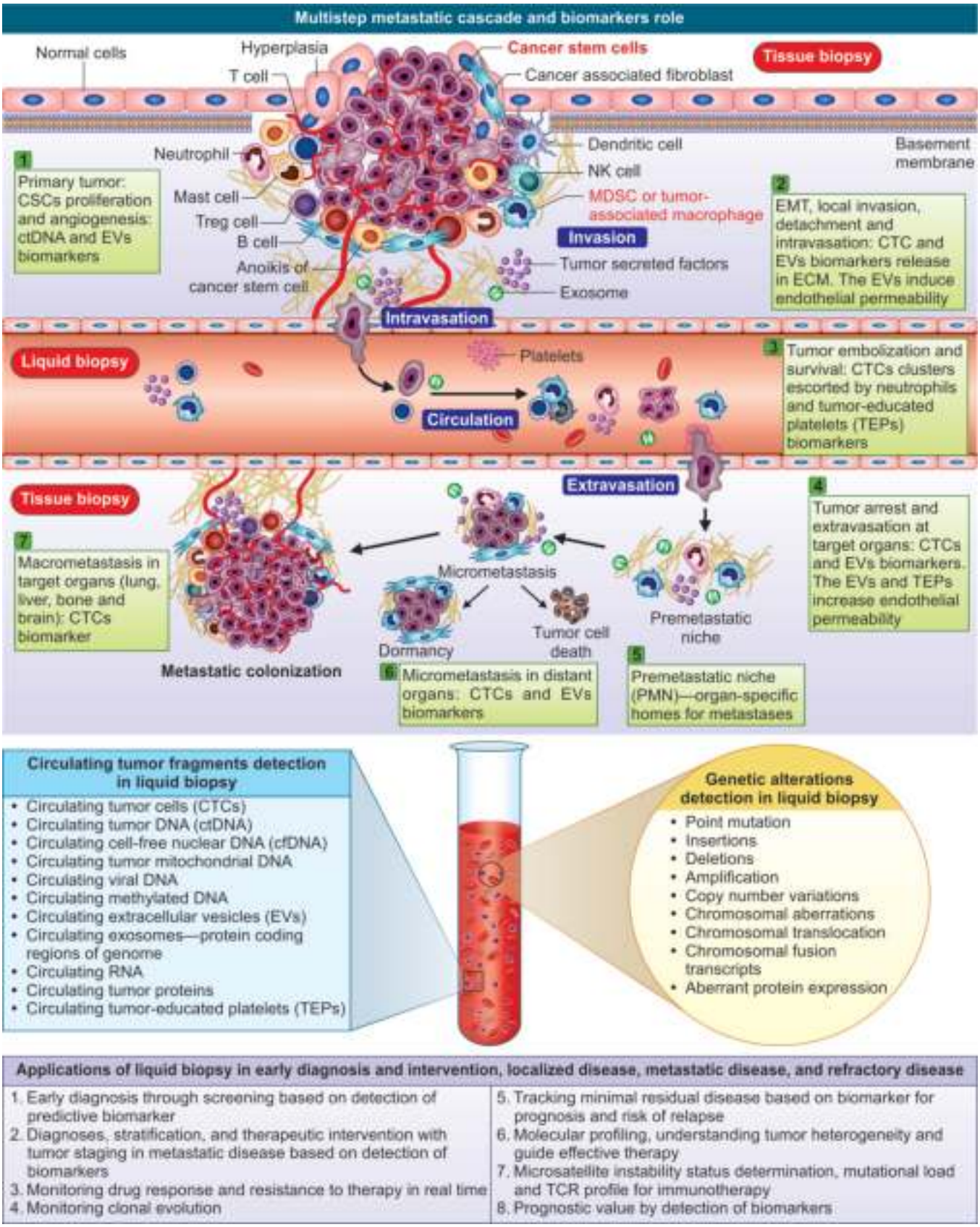


Fig. 6.136: Multistep metastatic cascade and biomarkers analysis, and clinical applications of liquid biopsy for treatment strategy in various stages of cancer. Liquid biopsy has broad potential applications for cancer diagnosis and treatment including early diagnosis through screening, study of tumor heterogeneity and clonal evolution, detection of minimum residual disease, and assessment of treatment response and development.

Table 6.121 Comparison of tissue biopsy and liquid biopsy

Liquid Biopsy	Tissue Biopsy
Liquid biopsy sampling requires venous blood—minimally invasive quick technique	Organ/tissue specific—tissue biopsy often difficult and invasive time-consuming
Liquid biopsy—easily repeatable and highly reproducible	Multiple tissue biopsy samplings—not always feasible
Liquid biopsy does allow tumor histologic type specification and staging of the disease	Tissue biopsy—allows histologic diagnosis and staging of the disease
Liquid biopsy—real-time monitoring of the disease, i.e. minimum residual disease (MRD) and progressive disease	Tissue biopsy—single snapshot, limited for localized cancer over time and space
Liquid biopsy does not provide histologic evaluation	Tissue biopsy provides histologic evaluation
Liquid biopsy—representative of the different localization of the malignant clones (i.e. tumor heterogeneity)	Tissue biopsy—not always representative for the entire variety of malignant clones (i.e. tumor heterogeneity)
Liquid biopsy—multiple real-time assessment and insight for tumor evolution and monitoring of the disease	Tissue biopsy—one-time assessment overtime and space and unable to assess into tumor evolution
Tissue biopsy—real-time monitoring of drug resistance	Tissue biopsy—treatment monitoring not possible without re-biopsy
Liquid biopsy—lacks standardization and it is still used mainly in translational research	Tissue biopsy—still the gold standard for tumor characterization, prognostic, and therapeutic prediction information

Table 6.122 Liquid biopsy to assess analytes during metastatic cascade

Metastatic Cascade	Molecular Events during Metastatic Cascade
Primary malignant tumor—unrestricted CSC proliferation	▪ Circulating tumor cell (CTC) DNA release ▪ Extracellular vesicles (EVs, i.e. apoptosis bodies) release
Primary malignant tumor—angiogenesis	Extracellular vesicles (EVs, i.e. apoptosis bodies) induced angiogenesis
Primary malignant tumor—EMT in some CSCs, local invasion, detachment and intravasation	▪ Tumor EVs induce endothelial permeability ▪ Circulating tumor cell (CTC) subsets survive in the bloodstream ▪ Crosstalk between CTCs and platelets for protection
Malignant tumor—embolization and survival	Higher metastatic and survival of CTC clusters escorted by neutrophils
Malignant tumor—circulating CSCs arrest extravasation at target organs	▪ Circulating tumor cell (CTC) interaction with endothelial cells ▪ Increased permeability of vessels
Malignant tumor—micrometastases in organs	Pre-metastatic niches prepared by exomes for CTCs being settle down in a favorable niche

Table 6.123 Clinical applications of liquid biopsy

Clinical Applications	Comments
Screening of disease	Screening is done to identify predictive biomarker, i.e. ctDNA for early detection of cancer; and circulating viral DNA in virus-related cancers
Diagnosis of disease	Circulating free tumor DNA (ctDNA) allows patients' stratification and can help guiding therapeutic intervention with staging of cancer
Monitoring of disease	Circulating free tumor DNA (ctDNA) can be used to monitor response and resistance to therapy in real time
Detection of minimal residual disease and progression	Circulating free tumor DNA (ctDNA) can be used to detect minimal residual disease (MRD), prognosis and risk of relapse
Actionable targets	At progression, ctDNA analysis could reveal tumor molecular heterogeneity and actionable targets for further effective therapeutic interventions
Immunotherapy	Circulating free tumor DNA (ctDNA) can be used to monitor microsatellite instability (MSI) status, mutational load for immunotherapy, and T cell receptor (TCR) profile for analysis of immune repertoire status to determine progression of cancer

cancer risk and susceptibility. Knowledge of these germline mutations in the DNA sequence can lead to the development of preventive measures to reduce the likelihood of developing malignant tumors. Somatic mutations may drive carcinogenesis and acquisition of subsequent mutations in the DNA sequence but are only present in specific cells. Oncogenes act in a dominant fashion: a gain-of-function mutation in a single copy of the cancer-critical gene can drive normal cell toward CSC. Tumor suppressor genes are recessive at the cellular level, with inactivation/loss of both alleles typically found in malignant tumors. Inheritance of a solitary mutant allele increases tumor susceptibility because only a single additional inactivating event is essential from complete loss of gene function. DNA biomarkers include mutation in single nucleotide, loss of heterozygosity, changes in gene copy number, and MSI.

- **Changes in single nucleotide base pair analysis:** Molecular testing is done to detect mutation in single nucleotide of genes responsible for human cancers such as BRCA1, BRCA2, and RAD1 genes in breast carcinoma, TP53, XRCC1 and ATM genes in head and neck carcinomas, and APC gene in colorectal carcinoma.
- **DNA copy number variation:** Most DNA diagnostics rely on DNA copy number variation analysis or DNA sequencing. Polymerase chain reaction (PCR) technique allows an exponential amplification of a DNA segment from genome, allowing multiple procedures on a single pure DNA sequence.
  - Microarray-based (e.g. oligonucleotide arrays, comparative genomic hybridization array) copy number variation analysis detects small abnormalities in the structure of chromosomes.
  - Copy number variation analysis for the human genome can pinpoint the location, number, gene content, frequency, and approximate breakpoints of numerous copy number variations in healthy population.
- **Loss of heterozygosity (LOH) in hereditary nonpolyposis colon cancer (HNPCC) and lung carcinoma:** Loss of heterozygosity (LOH) is a common genetic event in HNPCC and lung carcinoma, whereby one allele is lost, leading to part of the genome appearing homozygous in the malignant tumor, where heterozygous in matching DNA. Loss of one allele of a genetic locus can have multiple possible functional effects including loss of gene expression, **haploinsufficiency** (insufficient gene product), and being the 'second hit' that unmasks a recessive tumor suppressor gene.
  - Loss of heterozygosity (LOH) can be caused by mitotic errors, chromothripsis, gene conversion and inappropriate DNA repair breaks. LOH is associated with specific cancer (e.g. colorectal carcinoma and lung carcinoma), and significant in individuals, who have inherited a predisposition for cancer.
  - The molecular diagnostic methods for detection of LOH include microsatellite analysis, genome-wide assays, single-nucleotide polymorphism arrays and massively parallel DNA sequencing.
- **Microsatellite instability (MSI) analysis of mutations in mismatch repair genes:** Microsatellites (also called short tandem repeats (STRs) or simple sequence repeats (SSRs) are stretches of DNA with repetitive sequence of 1–6 nucleotides (e.g. AAAAA or CGCGCG) located near the coding and noncoding regions (introns), that are particularly susceptible to acquire errors when mismatch repair gene function is impaired. Microsatellite generation occurs due to DNA slippage in the process of DNA replication. The distribution of microsatellite differs from small satellite DNA consisting of 15–65 nucleotides tandem repeats present near the ends of chromosomes.
  - Normal DNA repair system, called mismatch repair system, can correct the errors during DNA replication. Cancer arising in cells with defective MMR gene's function exhibit an inconsistent number of microsatellite nucleotide base pair repeats when compared to normal tissue, a finding referred to microsatellite instability (MSI) with the possibility of gene mutation. It has been observed that microsatellite instability is an important factor in the occurrence and development of human malignant tumors.
  - Microsatellite instability (**MSI**) is caused by one or more mutations in DNA mismatch repair genes (e.g. MLH1, MSH2, MSH3, MSH6 PMS2) in microsatellite sequences due to the deletion or insertion of one or more microsatellite repeats that are associated with cancer.
  - The presence of microsatellite instability is a sign of mutations in DNA mismatch repair genes, that can be inherited caused by germline mutation in the MSH2 gene in hereditary nonpolyposis colon cancer (**HNPCC**) syndrome or sporadic colorectal cancer due to hypermethylation of the MLH1 promoter.
  - Microsatellite instability (MSI) is also demonstrated in endometrial carcinoma. MSI testing is performed to detect tumors caused by defective MMR genes by comparing the number of nucleotide base pair repeats in a panel of microsatellite markers (BAT25, BAT26, D2S123, D5S346 and D17S250) in the malignant tumor tissue, and normal tissue from the same individual. In the line with frequency of MSI, it can be MSI-high, MSI-low, and microsatellite-stable.

- Microsatellite instability is present if the same number of nucleotide base pair repeats are present in each marker in both malignant tumor and normal tissue. MSI is present if the number of nucleotide base pair repeats in the tumor and normal tissue differs.
- Molecular testing for MSI is most often performed using polymerase chain reaction (PCR) amplification of selected microsatellite repeats followed by capillary gel electrophoresis. Typically, DNA isolated from normal tissue and malignant tumor tissue is separately amplified by PCR technique with fluorescent-labeled primers and automated detection platforms. The gel electrophoretic patterns of PCR products from the normal tissue and malignant tumor are compared to identify insertions or deletions of repetitive units in the malignant tumor tissue sample. High-frequency microsatellite instability is defined as an instability in two or more of the five markers. Low-frequency microsatellite instability is defined as an instability in one of the five markers.

- Target mutation analysis is highly specific, sensitive, quick-turn around and cost effective, making it ideal diagnostic tool for detection of more than two mutations in germline and somatic malignancies and done in blood, saliva, and tissue samples.

Polymerase Chain Reaction Technique

Polymerase chain reaction technique is the backbone of molecular mutation analysis. PCR is crucial to amplify specific DNA sequences of nucleic acid of interest to generate thousands to millions of copies of a particular DNA sequence using pairs of short sequence specific oligonucleotide primers.

- Polymerase chain reaction (PCR) technique requires five basic reagents, e.g. DNA template, forward and reverse primers, thermostable DNA polymerase (i.e. Taq polymerase), deoxynucleotide triphosphates (dNTPs) and suitable buffer.
- Genomic DNA, forward and reverse primers, thermostable DNA polymerase (i.e. Taq polymerase), and deoxynucleotide triphosphates (dNTPs) are combined in a suitable buffer, and subjected to repeated cycles of alternating temperatures which denature the DNA template, allow for primer annealing to the template and finally extend the primer to create a new strand of DNA (DNA amplicons).
- The process of PCR begins by denaturation of the DNA template by heating sample at 94°C, which breaks the hydrogen bonds between the nucleotide base pairs of double-stranded DNA, and produces single-stranded DNA. Primers specific to the region of interest then anneal to the single-stranded DNA in the sample at specific temperature of 55–65°C.
- The temperature is then raised to 72°C, which allows DNA polymerase to extend the primers into full length product, from the forward primer to the reverse primer sequence thereby copying the sequence of interest.
- The primer extension occurs with DNA polymerase (i.e. Taq polymerase) incorporating dNTPs, and produces a final elongated double-stranded DNA product. This process is repeated 25–35 times to generate millions of copies of genetic material for downstream analysis by gel electrophoresis, sequenced, or cloned into vectors for further analysis.
- Flexible platforms permit detection of conserved specific hotspot mutations (i.e. nucleotide base pair substitutions, small length mutations—deletions, insertions) and chromosomal translocations. Polymerase chain reaction technique is highly sensitive to detect minimal residual disease (MRD).

Quantitative Real-time Polymerase Chain Reaction Technique

Quantitative real-time polymerase chain reaction (RT-PCR) is highly sensitive method, that allows amplification and quantification of a specific targeted nucleic acid sequences by incorporating a fluorescent probe.

- RT-PCR plays key roles in detection of minimal residual disease (MRD), i.e. circulating tumor cells (CTCs) in patients with hematolymphoid malignancies such as acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), and non-Hodgkin's lymphoma associated with poor prognosis.
- Similarly, quantitative RT-PCR has been used to quantify target chromosomal translocation fusion transcripts, i.e. BCR-ABL fusion protein with enhanced receptor tyrosine kinase activity in chronic myelogenous leukemia (CML).
  - Philadelphia chromosome is created by the tumor specific chromosomal translocation t(9;22) in CML.
  - Serial quantitative RT-PCR assays are typically used during targeted therapy of CML with a tyrosine kinase inhibitor (e.g. imatinib) to monitor tumor response.
- Quantitative real-time PCR has performed to detect mutations such as K-RAS, BRAF, and EGFR mutations in solid malignant tumors, and KIT D816V mutation in acute myelogenous leukemia (AML). Real-time PCR can also characterize CSCs burden, i.e. minimal residual disease (MRD) from blood samples of patients with malignant epithelial tumors.

Reverse Transcription Polymerase Chain Reaction Technique

Reverse transcription polymerase chain reaction (RT-PCR) technique is a modified PCR-based exponential amplification of specific DNA fragments, that uses ribonucleic acid (RNA), rather than deoxyribonucleic acid (DNA), as a template. This process uses **viral transcriptase** to catalyze the conversion of RNA into complimentary DNA (cDNA) that provides the possibility to assess gene transcription in several body fluids and tissues.

- Reverse transcription polymerase chain reaction (RT-PCR) technique can identify aberrant oncogenic fusion products produced by chromosomal translocations associated with follicular lymphoma and acute promyelocytic leukemia (APL).
- Follicular lymphoma is caused by chromosomal translocation t(14;18) that juxtaposes the BCL-2 with immunoglobulin locus leading to oncogenic IGH-BCL-2 fusion product.
- Acute promyelocytic leukemia (APL) results in oncogenic APL-RARA fusion gene product in the blood, bone marrow, or lymph node.
- Achieving molecular remission in follicular lymphoma and acute promyelocytic leukemia is a superior measure of prognosis over clinical assessments of remission.

Allele-specific Polymerase Chain Reaction

Allele-specific polymerase chain reaction is considered targeted mutation analysis for detection of a specific single nucleotide variants (SNVs). It is sensitive method that can detect mutant DNA if present even 1–5%.

- Real-time polymerase chain reaction (RT-PCR) with fluorescent reporter probes in which reporter probes for one wild type of nucleic acid, and another primer with homology to the mutant allele are added to the reaction mixture.
  - Following hybridization to the genomic DNA, polymerase chain reaction extends probes in a complimentary fashion, releasing the reporter molecule for detection.
  - Subsequently, polymerase chain reaction cycles using the reporter probes result in amplified signals, allowing for precise analysis of one or both alleles of interest.
- Alternately, routine fluorescent polymerase chain reaction is coupled with capillary electrophoresis for detection of allele-specific mutation. Similarly, the 3′ end of the mutant-specific primer-template pair will prevent DNA polymerase extension.

DNA Fragment Analysis Technique

DNA fragment analysis technique is a powerful research tool that provided relative quantitation, sizing and genotyping information and enables a wide array of genetic analysis applications.

- DNA fragment analysis workup consists of four general steps: (a) deoxyribonucleic acid (DNA) extraction and purification, (b) fluorescent dyes attached to the primers and DNA fragments, and PCR amplification, (c) capillary electrophoresis to detect fluorescent-labeled primers by a laser/camera system, and (d) data analysis.
- DNA fragment analysis technique can be used to analyze the DNA damage induced by chemotherapeutic agents. This technique has been used to analyze DNA methylation in the BRCA1 and RAD51C genes in pancreatic cancers.

Sanger Dideoxy Sequencing Technique

Sanger dideoxy sequencing technique is used to detect specific mutations, including single nucleotide variants (SNVs) and small duplications, insertions, and deletions. Sanger dideoxy sequencing is performed on polymerase chain reaction (PCR) products.

- Sequencing primers are hybridized to the PCR product and extended by using DNA polymerase, four dideoxynucleotide triphosphates (dNTPs), and a mixture of fluorescently labeled dNTPs.
- Each of four dye-labeled, chain-terminating dNTPs results in termination of strands at each location along the sequence. The reaction primers can be extended by up to about 1000 nucleotides.
- Capillary electrophoresis separates the strands by depending on size, and the terminating nucleotides are analyzed using fluorescence spectroscopy. In a clinical laboratory, both the forward and reverse strands are sequenced.

Pyrosequencing Technique

Pyrosequencing is a replication-based DNA sequencing technique used to detect specific mutations in a small targeted region of gene in cancer patients to help oncologists understand if a disease is progressing or treatment is efficacious in clinical oncology. It is quick and sensitive detection of mutant DNA at a level of 5%.

- In pyrosequencing technique, DNA strand of interest is split into smaller fragments of about 100 nucleotide base pairs, which are amplified using PCR technique and placed into wells; and fragments will not be mixed within the same well.
- Each well of pyrosequencing technique also receives ATP sulfurylase, luciferase, apyrase, adenosine 5′-phosphosulfate and luciferin. Next, one of the four

possible nucleotide base pairs is added to the wells. As DNA polymerase incorporates the nucleotides into the DNA strand, pyrophosphate is generated to be converted into ATP by the ATP sulfurylase.
- ATP helps to covert luciferin into oxyluciferin and light is released. The sequence can be determined from the order of the dNTP additions, and the intensity of the light produced with each addition and captured by camera.

DNA Methylation of CpG Island Analysis Technique

In mammalian genome, DNA methylation of CpG dinucleotides (CpG island) is an epigenetic mechanism involving the transfer of a methyl group onto the C5 position of the cytosine to form 5-methylcytosine.
- Unmethylated CpG islands are regions of DNA with a minimum of 200 nucleotide base pairs to roughly 1000–2000 nucleotide base pairs along with a high concentration of phosphate-linked cytosine-guanine nucleotide pairs present near promoters of gene.
- Recently, these unmethylated stretches of CpG dinucleotides (CpG islands) are detected by clustering methods.
- DNA methylation occurs almost exclusively in CpG dinucleotides. DNA methylation usually inhibits the transcription of genes, particularly when it occurs in the vicinity of the promoter region. DNA methylation plays important role in the silencing of tissue-specific genes to prevent them from being expressed in the wrong tissue.
- In molecular oncology, DNA methylation of CpG island of tissue DNA has emerged as a clinically useful diagnostic tool for malignant tumor detection, outcome prediction, and treatment selection, as well as for assessing efficacy of treatment with use of demethylation agents, and monitoring for tumor recurrence.

WHOLE GENOME SEQUENCING ANALYSIS

Chromosomes are the thread-like structures inside the nucleus of the cells that contain the genetic information from parents to offsprings or from one generation to another. Humans have 22 pairs of autosomes and one pair of sex chromosomes, i.e. XX in eggs of females and XY in sperms of males.
- Chromosomes are made up of DNA, and certain stretches of DNA make up genes. Hereditary (germline) mutations occur in the germ cells (eggs and sperm). Somatic (acquired) mutations occur in somatic cells.
- Deoxyribonucleic acid (DNA) is the molecule in chromosomes that contains the genetic information that is passed on from one generation to the next. DNA is made of two linked strands that wind round each other to resemble a twisted ladder shape known as a double helix. DNA molecule is three meters long and stable in pH range of 4–9.
  - Individual nucleotide base pairs form the rings of the ladder. DNA has four types of nucleotide base pairs: adenine (A) compliments with thymine (T) and contains two hydrogen bonds, guanine (G) compliments with cytosine (C) and contains three hydrogen bonds. Nucleotide base pairs are hydrophobic. Deoxyribose sugar and phosphate are hydrophilic in DNA. Nucleotide base pairs absorb maximum ultraviolet light at 260 nm.
  - Two DNA strands that can bind because they contain nucleotide base pairs throughout their entire length are said to be complementary. For example, the nucleotide base sequence of AAAGA is complementary to the nucleotide base sequence of TTTCT.
  - Each nucleotide base pair in DNA binds with only one nucleotide base pair: adenine (A) pairs with thymine (T), and guanine (G) pairs with cytosine (C). These nucleotide base pairs are essential for the DNA's double helix structure, which resembles a twisted ladder.
- Central dogma of molecular biology includes main steps: replication, transcription, and translation, in which genetic information flows only in one direction: DNA → RNA → specific protein.
  - DNA replication is semiconservative unidirectional (5′-3′) process by which the genome's DNA is copied in cells.
  - Transcription is the process of synthesis of RNA from DNA. During transcription process, only one strand of DNA is usually copied to form template strand, and the RNA molecules produced are single-stranded messenger RNAs (mRNAs). Different post-transcriptional modifications include capping, polyadenylation, and splicing. Transcription occurs when there is a requirement from a particular gene product at a specific time in specific tissue.
  - The process of synthesizing a polypeptide chain from mRNA codons is known as **translation**.
- Chromosomes are made up of DNA, and certain stretches of DNA make up genes.
  - Gene expression is the process by which a gene gets 'turned on' in a cell to make ribonucleic acid (RNA) and proteins. Proteins are biochemicals made up of individual amino acids.
  - Alterations in genes encoding cellular signaling molecules, especially receptor tyrosine kinases, can result in malignant tumors.

- Drugs targeting mutant receptor tyrosine kinases are efficacious in cancer patients. The sensitivity of such targeted drugs is related to the genetic makeup of individual cancers.
- Thus, mutation profiles of malignant tumor DNA help prioritize anti-cancer therapy in the management of cancer patients.

- Most cancer-causing DNA changes occur in genes, which are sections of DNA, that carry the instructions to make proteins resulting from acquired (somatic) mutations, germline mutations of oncogenes (mutated proto-oncogenes), inactivation/biallelic loss of tumor suppressor genes, and mutations in DNA repair genes.
  - **Single nucleotide variants:** Single nucleotide variants (SNVs), also known as point mutations, in which a nucleotide base pair may be missing or replaced by another nucleotide bae pair.
  - **Frameshift mutation:** A frameshift mutation is a genetic mutation caused by a deletion or insertion in a DNA sequence, that shifts the way the sequence is read. A DNA sequence is a chain of many smaller molecules called nucleotides.
  - **Changes in exon or gene copy number:** Changes in exon or gene copy number, resulting from deletions, duplications, or genomic rearrangements lead to loss or gain of genetic material regions, that might change of levels of gene expression included in the regions of variable copy number allowing transcription levels higher or lower than that can be achieved by control of transcription of single copies per haploid genome.
  - **Genomic structural variations in chromosomes:** Genomic structural variation in chromosomes consists of microscopic and submicroscopic variations such as deletions, duplication, copy number variants, insertions, inversions, and chromosome translocations. Fusion gene products, which can occur when parts of different chromosomal regions are joined, may drive the development of many malignant tumors. Genomic structural variation in chromosomes drives oncogenesis progression in both solid malignant tumors and hematopoietic malignancies.
- **Next-generation sequencing (NGS) technology** has enabled the detection of diverse patterns of genomic changes in somatic cells. Often, cancer-causing mutations cluster in '**hotspots**', where malignant tumors from different patients harbor the same recurrent mutation.
  - Some genomic structural variation in chromosomes may occur frequently such as BRAF V400E mutation occurs in 40% of all melanomas, while BRAF L697S mutation occurs in 1% of all melanomas. Determining the order of nucleotide base pairs is called 'whole genome sequencing'. Pathologist must ensure adequate malignant tumor content in the biopsy for reliable mutation detection.
  - Both malignant tumors and normal tissues are sequenced to distinguish germline from somatically acquired mutations in the malignant tumor. Sequence results are presented at a multidisciplinary malignant tumor board to determine clinical significance.
  - Scientists conduct whole genome sequencing by following four steps: (a) DNA shearing—DNA is cut into small pieces by using molecular scissors for the sequencing machine to analyze, (b) DNA bar coding—small pieces of DNA tags are coded to identify which pieces of sheared DNA belongs to which bacteria, (c) DNA sequencing—the bar-coded DNA from bacteria is combined and put in a DNA sequencer that identifies nitrogen bases: adenine, thymine, guanine, and cytosine, which make up each bacterial sequence, and (d) data analysis—scientists use computer analysis tools to compare sequences from multiple bacteria and identify differences.
- A targeted therapy is a medication or other substance that treats cancer by interfering with molecules that are specifically involved in cancer cell growth, spread, and progression.

Next-Generation Sequencing Technique

Next-generation sequencing (NGS) is a powerful molecular diagnostic tool used in genomics research NGS can sequence millions of DNA fragments at once to identify whole genome, genetic variations, entire gene or group of genes activity, and alterations in gene behavior.

- Genomic studies using **NGS** analyze DNA using various approaches such as whole genome sequencing, whole exome sequencing, and targeted sequencing.
- Targeted genome sequencing analysis focuses on several targeted regions of genes of interest such as K-RAS, TP53, BRAF and EGFR genes are screened in various solid malignant tumors.
- Germline and somatic mutations can be tested using targeted next-generation panels, which work on a simple approach of enrichment by amplification using pools of region-specific oligonucleotide primers. Specific libraries that are produced, then sequenced and analyzed bioinformatically.
- Examples of next-generation sequencing in oncology include small targeted panels (3–50 genes) for non-small cell lung carcinoma, colon carcinoma, melanoma, and acute myelogenous leukemia.
- Recently, the number of somatic mutations per megabase of next-generation sequencing, also known as malignant tumor mutation burden, is being evaluated

as a potential biomarker of response to immunotherapy in patients with various malignancies.

Next-Generation Sequencing: Whole Genome Sequencing Analysis Technique

Whole genome sequencing (WGS) is the process to determine complete DNA sequence in an individual's whole genome at a single time. Humans have a unique genetic code, or genome, that is composed of nucleotide base pairs: adenine (A), thymine (T), guanine (G), and cytosine (C).

- Knowing the sequence in nucleotide base pairs, one can identify DNA fingerprint, or pattern. Whole genome sequencing is a laboratory procedure that determines the order of nucleotide base pairs in the whole genome.
- Whole genome sequencing (WGS) can identify genetic variations, ranging from single-nucleotide polymorphisms (SNPs) to larger structural changes such as substitutions, deletions, insertions, gene copy number changes, and indels (insertion-deletion of nucleotide bases in whole genome), and chromosomal inversions and translocation across the entire genome. The information obtained through WGS offers a multitude of applications in various fields.

Next-Generation Sequencing: Whole Exome Sequencing Analysis Technique

Whole exome sequencing (WES) is widely used next-generation sequencing technique, that involves sequencing the protein-coding regions of genome to detect substitutions, duplications, insertions, deletions, and gene copy number changes.

- In humans, exome is the part of the genome composed of exons, that represents approximately 1–2% of the entire genome.
- Whole exome sequencing allows cancer researchers, which frequently contain mutations that affect malignant tumor progression.
- Whole exome sequencing can identify patient groups at higher risk for certain malignant tumors.
- Whole exome sequencing provides a clear picture of gene mutations that affect tumor progression such as microsatellite instability (MSI). Whole exome sequencing can also identify heritable mutations.

Next-Generation Sequencing Custom Panels and Amplicon Capture

Next-generation sequencing—custom panels, amplicon capture enables simultaneous detection of substitutions, duplications, insertions, deletions and indels (insertion-deletion of nucleotide base pairs in genome) in a single assay. Next-generation sequencing molecular tests are custom-designed to interrogate tissues for mutations of interest in specific genes, but unable to detect gene copy number changes.

Next-Generation Sequencing Custom Panels and Hybridization Capture Assay Technique

Next-generation sequencing—custom panels, hybridization capture enables simultaneous detection of substitutions, duplications, insertions, deletions, exon, gene copy number changes in many genes and select chromosomal translocation in a single assay.

- Hybridization captures the regions of interest, followed by next generation sequencing and specialized bioinformatics analysis.
- Genomic DNA fragments are hybridized in solution to sequence-specific capture probes corresponding to target regions of the genome.
- Probes can also be designed to capture select chromosomal translocation breakpoints in recurrently rearranged genes. Next-generation sequencing assays are sensitive to detect low abundant gene mutations. Compared to next-generation sequencing methods, custom panels can be cheaper or faster.

Genome Microarrays Analysis Technique

Among the many benefits of the '**Human Genome Project**', genome-wide hybridization is a powerful diagnostic technique known as **DNA microarray**, which determines mutation of BRCA1 and BRCA2 genes in individuals.

- The principal behind genomic microarrays is that complementary sequences will bind to each other. The unknown DNA molecules are cut into fragments by restriction endonucleases; and fluorescent markers are attached to these DNA fragments. These are then allowed to react with probes of the DNA microarray chip.
- DNA microarrays consist of known sequence specific long probes (i.e. PCR probes, cDNA clones or long oligonucleotides) covalently bonded to a microarray chip consisting of a small glass plate encased in plastic material.
  - The input nucleic acid sample is first fluorescently labelled and then hybridized to the microarray chip.
  - Probes that bind nucleic acid sample emit fluorescent and effectively genotype the nucleic acid for that sequence.

Array-based Comparative Genomic Hybridization Assay Technique

One of the most common clinical assays using microarrays is array-based 'comparative genomic hybridization (cCGH)' that allows for the widespread screening of DNA for copy number (gene dosage) differences between a control sample and a test sample.

- Structural variation in the genome (deletions and duplications, copy number variation, insertions, inversions, and chromosomal translocations) can be detected using array-based comparative genomic hybridization.
- Comparative genomic hybridization (CGH) method is used to detect copy number differences in sample of interest. CGH has multiple applications such as screening for deletions or insertions and gene amplifications.
- Advanced array-based approaches have enabled detection of the main forms of genomic variation: amplifications, deletions, insertions, rearrangement, and nucleotide base pair changes.
- A copy number neutral reference and a patient sample are fluorescently labeled with two different dyes and hybridized to the chip. Regions that are a 1:1 ratio of the two colors denote copy neutral regions. Regions that diverge from the 1:1 ratio of the two colors suggest deletions or duplication in the tested sample.

Gene Expression Panels Technology

Genomics is the study of multiple genes in genome (DNA), including interactions of those genes with each other and with the individual's environment. A major part of genomics is determining the sequence of molecules that make up the genomic deoxyribonucleic acid (DNA) content of an individual. The genome is the entire DNA content that is present within each cell of an organism.

- Genomics employs recombinant DNA, DNA sequencing methods and bioinformatics to sequence, assemble and analyze the structure and function of genome. It also involves the study of **intragenomic processes** (e.g. **epistasis, heterosis,** and **pleiotropy**) as well as the interactions between loci and alleles within the genome.
- Gene expression panels measure messenger RNA (mRNA), and microRNA transcriptome, which show the pattern of few hundred genes to whole expressed by a cell at the transcription level (transcription initiation, elongation, and termination), the post-translational level (RNA translocation, RNA splicing, RNA stability), and covalent modifications.
- Gene expression panels technology generates results from clinically relevant samples, such as formalin-fixed paraffin-embedded malignant tumor tissue to support biomarker discovery, drug targeting and diagnostics development.
- Panel gene names conform to the names curated in the **HUGO Gene Nomenclature**. Each gene expression panel consists of validated probes for over 1,000 genes.

Oncotype DX® 21 Genes Test for Breast Carcinoma

Oncotype DX® test is a tumor profiling genomic quantitative RT-PCR-based assay that measures the expression of **21 genes** in formalin-fixed paraffin-embedded tissue (FFPE) in breast carcinoma, and evaluation of its recurrence. Oncotype DX® test is also a part of breast carcinoma staging for some estrogen receptor positive, lymph node negative and HER2/neu negative tumors.

- Factors that affect prognosis and treatment of breast carcinoma include lymph node status, tumor size, tumor grade, histologic type, hormone receptor status, HER2/neu status, proliferative rate, Oncotype DX®, MammaPrint test score and breast carcinoma stage.
- In addition to Oncotype DX® tumor profiling genomic test, 'MammaPrint' and 'Prosigna PAM50' are also available in estrogen positive (ER positive), HER2/neu negative and lymph node negative breast cancers.
- Oncotype DX® test is designed for prediction of the potential benefit of chemotherapy in addition to hormone therapy and likelihood of distant metastasis and recurrence of breast carcinoma in women with lymph node negative, ER positive, HER2/neu negative and lymph node negative breast carcinoma.
- Oncotype DX® genomic testing scoring is done to manage breast carcinoma cases.
  - Oncotype DX® testing score <26 is associated with low-risk of metastasis and treated with only estrogen.
  - Oncotype DX® testing score ≥31 is associated with high-risk of metastasis and treated with both hormone and chemotherapy.

MammaPrint® Test and BluePrint® Test: Breast Carcinoma

The MammaPrint® test identifies the activity of 70 genes from a sample of the breast carcinoma tumor removed during a biopsy or mastectomy then calculate recurrence score that is low-risk or high-risk BluePrint® test identifies **80 genes** and then calculates a recurrence score that is either low-risk or high-risk.

PAM-50 (Prediction Analysis of Microarray 50) Test: Breast Carcinoma

PAM-50 test identifies the activity of **70 genes** from a sample of the breast carcinoma tumor removed during a biopsy or mastectomy then calculate recurrence score that is low-risk or high-risk.

Pathology Pearls: Gene Expression Panels: HTG Transcriptome Panel

- **HTG transcriptome panel:** Next-generation sequencing (NGS)-based HTG transcriptome panel (HTP) provides extensive coverage of most human mRNA transcripts and detect 19398 target genes using neoplastic and normal formalin-fixed paraffin-embedded (FFPE) extracted RNA. HTP provides sensitivity and dynamic range of next generation sequencing to generate reliable results suing limited sample amount.
- **HTG EdgeSeq microRNA whole transcriptome assay:** Next-generation sequencing (NGS)-based HTG EdgeSeq microRNA whole transcriptome assay analyzes the expression of 2083 human microRNA transcripts using next generation sequencing. HTG EdgeSeq microRNA whole transcriptome assay is compatible with formalin-fixed paraffin-embedded tissue samples, biofluids, and cell lines.
- **HTG EdgeSeq oncology biomarker panel:** Next-generation sequencing (NGS)-based HTG EdgeSeq oncology biomarker panel analyzes simultaneous, quantitative detection of 2083 human microRNA transcripts in plasma or serum and to identify therapeutic targets and drug response in cancers.
- **HTG EdgeSeq precision immuno-oncology panel:** Next-generation sequencing (NGS)-based HTG EdgeSeq precision immuno-oncology panel is designed to analyze immune response both inside malignant tumor and the surrounding microenvironment. This technique analyzes 1392 genes from a single section of formalin-fixed paraffin-embedded (FFPE) tissue extracted RNA and PAX gene sample.
- **HTG EdgeSeq immune response panel:** Next-generation sequencing (NGS)-based HTG EdgeSeq immune response panel analyzes 2002 immune response mRNA targets in a single RNA extraction-free panel. This technique detects genes from a single section of formalin-fixed paraffin-embedded (FFPE) tissue and PAX gene sample, implicated in the immune response to pathogens and many autoimmune diseases.
- **HTG EdgeSeq pan B cell lymphoma panel:** Next-generation sequencing (NGS)-based HTG EdgeSeq pan B cell lymphoma panel analyzes 298 genes expression of mRNA transcripts in a single analysis commonly associated with indolent and aggressive lymphomas to identify lymphoma subgroups to elucidate the transcription response of disease state and drug therapy.

RIBONUCLEIC ACID (RNA) BIOMARKERS

The '**Central Dogma of molecular biology**' suggests that primary role of RNA is to convert the genetic information stored in DNA into proteins. The messenger RNA (**mRNA**) carries genetic information out of the nucleus to the site of protein synthesis in the ribosomes present in cytoplasm. The transfer RNA (**tRNA**) transfers amino acids from the cytoplasm to the protein synthesizing machinery. Ribosomal RNA (**rRNA**) plays key role in the binding of messenger RNA to ribosomes and its translation. Presence of a reactive OH group present at 2'-position in the ribose makes RNA labile and easily degradable. RNAs are unstable at alkaline conditions.

- In the field of oncology, RNA biomarkers generally possess three types of relevance: clinical, diagnostic, and prognostic values.
  - Diagnostic values include early diagnosis of disease, determination of origin and classification of cancer subtypes.
  - Prognostic values include prediction of response to treatment.
- RNA biomarkers have more sensitivity and specificity. RNA has multiple copies in the cell, which delivers more information than DNA, RNA biomarkers provide dynamic insights into the cellular states and regulatory processes than DNA markers. Specific structure of some RNAs provides stability in the plasma and/or serum.
- RNA biomarkers are classified into coding (e.g. mRNA) and noncoding (e.g. incRNA, piwiRNA, snoRNA and circRNA). These can be analyzed on tumor tissue, blood (plasma, serum), saliva, bile juice, urine, and cerebrospinal fluid.
- RNA biomarkers are analyzed by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), RNA sequencing, microarray and microfluid card analysis. Pure RNA signature procurements can be analyzed by laser capture-based microarray in various stages and grades of cancer treatment. RNA expression comparative analysis is helpful in studying cell functional status, diagnosis, and prognosis of human cancers.
- Extracellular messenger RNA is unstable and easily degraded to small pieces by RNase in circulation. In some cases, circulating mRNA can form complexes with chaperones either protein or lipid binding partners, and persist in the circulation. Circulating mRNA can be a useful source of cancer biomarkers.
- Researchers have confirmed that mRNA remains at the same level for 24 hours after blood sampling. Unlike DNA, the RNA molecule can reflect events in the human body, which occurred within 24 hours leading to an early diagnosis of the disease. However, high degree of variability and turnover in circulating mRNA makes it a challenging candidate for precision molecular diagnostics.

Tissue-based RNA Biomarkers in Human Cancers

The first well-studied type of RNA as biomarker is the mRNA. Globally, perspective of the mRNA expression forms and deregulated pathways may provide a more exact cancer picture including its clinical outcome. Positive or negative expression of genes correlates with disease pathology. A panel of multiple genes is applied

as biomarkers to study the clinical outcome in various human cancer studies. Tissue-based RNA biomarkers in human cancers are discussed here.

Messenger RNA Biomarker Assay Technique

For example, PAM50 messenger RNA biomarker a **70-gene** panel has been used to analyze diagnosis and prognosis of breast carcinoma. A panel of 30-gene mRNA related to cell cycle progression is used to study prognosis, progression, and recurrence of prostatic carcinoma. Variable expression tissue-based mRNA profiling suggested to distinguish three histologic subtypes: renal cell carcinoma [e.g. clear cell, papillary (chromophil) and chromophobe variants].

MicroRNA Biomarkers Assay Technique

MicroRNAs are small noncoding RNAs, which can function as tumor promoters and tumor suppressors. Some microRNAs play key roles in cell proliferation, differentiation, and apoptosis. MicroRNA biomarkers analysis helps in classification of poorly differentiated cancers. Low expression of microRNA-21 indicates low hazard rate for pancreatic ductal carcinoma patients after adjuvant therapy. MicroRNA-21 has been reported as a potential therapy target.

- **IncRNA biomarkers:** Upregulation of incRNA biomarkers is used in diagnosis and prognosis many human cancers. Examples of incRNA biomarkers include: upregulation of PCA3 (prostatic carcinoma), HULC (pancreatic carcinoma), and HOTAIR (nasopharyngeal carcinoma).
- **Piwi-interacting RNA biomarker (piRNA):** Piwi-interacting RNA participates in transposon silencing via DNA methylation, cell proliferation and invasion. Low expression of piRNA-651 biomarker is linked to poor prognosis in lymphoma patients.
- **Small nucleolar RNA (snoRNA) biomarker:** Small nucleolar RNA regulates ribosomal maturation and its function. Upregulation of SORD33, SNORD66 and SNORD76 biomarkers is demonstrated in non-small cell lung carcinoma.
- **Circular RNA (circRNA) biomarker:** Non-coding circular RNA is generated from pre-mRNA with back-splice mechanism, that makes circular RNA more resistant to exonucleases. Hsa_circ_002059 biomarker is downregulated in gastric carcinoma associated with metastasis.

Extracellular Vesicle-derived RNAs Biomarkers Detected in Blood and Body Fluids in Cancers

In addition to RNAs present within tumor cells, many extracellular vesicle-derived RNAs released by cells (normal and tumor cells) have been detected in blood (plasma and serum), breast milk, saliva, cerebrospinal fluid, bile juice and urine.

- **Extracellular vesicle-derived RNAs** are released by cells in the form of microvesicles, exosomes, oncosomes and apoptotic bodies.
  - Microvesicles directly shed from the plasma membrane, whereby budding microdomains undergoing phosphatidylserine translocation and remodeling of the actin cytoskeleton.
  - The proto-oncogene SRC promotes the release of promigratory exosome by phosphorylating syndecans and syntenin.
  - Exosomes are endosomal membrane-derived vesicles released by the fusion of the multivesicular bodies with the cell membrane. Upon secretion into extracellular space, microvesicles and exosomes can bind to the recipient cell surface via ligand receptor and their interactions initiate cell signaling, transfer of functional messages resulting in cellular phenotype.
- Extracellular vesicle-mediated RNA in cancer plays important role in initiation, progression, metastasis, and cancer immunology. CSCs secreting extracellular vesicle-derived RNAs reach the adjacent cells, that alter local tumor microenvironment resulting in local invasion and multifocal tumor growth.
- Extracellular vesicle-derived RNAs from different cancer cell lines show distinct RNA profiles in coding mRNA and non-coding RNAs (e.g. tRNA, piwiRNA, IncRNA, SnoRNA and circRNA).
- Analysis of extracellular-vesicle derived RNAs are promising diagnostic and prognostic biomarkers including therapeutic targets for human cancers. Their analysis helps in the classification of tumors, when the solid tissue is not available.
- Tumor-promoting RNAs also disrupt vascular endothelial barriers and transform normal cells into niche cells in distant tissues/organs resulting in premetastatic niche generation and metastasis.
- Extracellular vesicle-derived miR-105 from breast CSCs disrupts vascular integrity by targeting tight junction protein ZO-1 resulting in enhanced vascular permeability and increased dissemination to lung and brain. Moreover, extracellular vesicle miR-122 from breast cell lines is delivered to premetastatic niches and inhibits glucose consumption in the lung fibroblasts and brain astrocytes by targeting PKM thereby resulting in metastasis to lungs and brain.
- Extracellular vesicle-derived miR-103 from hepatocellular (HCC) cells disrupts vascular endothelial barriers, promotes endothelial permeability, transendothelial invasion by targeting VE-cadherin, ZO-1

and p120, thereby resulting in cancer dissemination to liver and lungs.
- Extracellular vesicle-derived circRNA from pancreatic CSC lines promotes endothelial permeability and transendothelial transmigration of CSCs by targeting miR-122 leading to dissemination to distant organs.

PROTEINS AS BIOMARKERS

Proteins are biomolecules composed of individual amino acids. The body utilizes proteins to make up the body structures and perform cellular functions of life. Examples of body proteins include: immunoglobulins (e.g. IgG, IgA, IgM, IgD, IgE), enzymes, messenger proteins (e.g. hormones), structural component proteins and transport or storage proteins (e.g. amino acids and metal ions).
- Proteins are the main executioner biomolecules of cells, which affect the molecular pathways in normal and transformed cells. Protein biomarkers are commonly performed in clinical practice, which are more significant than RNA-based or DNA-based biomarkers.
- **Proteomics** encompasses the identification and quantitative analysis of differentially expressed proteins relative to healthy tissue counterparts of different stages of disease (e.g. transformation of normal cells to malignant cells). Proteomics of malignant tumors includes details on cellular processes that happen in CSCs, tumor microenvironment and cancer–host interaction. Protein markers are most common type of biomarkers analyzed by molecular diagnostics.
- Cancer stem cells (CSCs) can shed certain proteins, that enter the bloodstream. These proteins can then be analyzed by taking a blood sample and subjecting it to a molecular diagnostic test. Some of these proteins can finish up in the bloodstream and thus serve as potential serum biomarkers. Other proteins can be analyzed in malignant tumor tissue (e.g. estrogen receptors and HER2 proteins in breast carcinoma). Few crucial antigens of CSCs are analyzed for diagnostic and prognostic cancer biomarkers such as carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), α-fetoprotein (AFP), and cancer antigen 125 (CA 125).
- Classically, protein-dependent signatures have been developed from the polyacrylamide gel electrophoresis and two-dimensional fluorescence difference gel electrophoresis analysis. Other protein-based molecular diagnostics include mass spectroscopy, matrix associated laser absorption/desorption ionization time of flight and reverse phase microarray surface enhanced laser absorption ionization time of flight. Recently nanoparticles and quantum dots have improved technologies usable to evaluate potential as cancer biomarkers.

Carcinoembryonic Antigen Biomarker Test

Carcinoembryonic antigen (CEA) is a protein that typically is not present in healthy adults. Though CEA is produced by CSCs, it may also be elevated in liver disease and inflammatory bowel disease.
- Blood levels of CEA is a protein biomarker, that is not performed for diagnostic purpose of many human cancers, but to monitor the treatment response in patients with already diagnosed cancers (e.g. colorectal carcinoma, gastric carcinoma, hepatocellular carcinoma, pancreatic carcinoma, ovarian carcinoma, breast carcinoma).
- Blood CEA can perform to determine the prognosis and staging of the cancer. Blood CEA molecular testing is performed along with other biomarkers to analyze whether metastasis has occurred or not.

Cancer Antigen 125 Serum Biomarker Test

Cancer antigen 125 (CA 125) test measures the amount of CA 125 protein in a patient's blood. CA 125 test may be used to monitor certain cancers during and after treatment.
- CA 125 is found in higher concentration in particularly in ovarian carcinoma. CA 125 concentration is high in cancers of endometrium, fallopian tube, pancreas, stomach, esophagus, colon, liver, breast, and lung.
- It is important to mention that conditions other than malignant tumors may cause higher levels CA 125, including uterine fibroids, endometriosis, lupus, liver diseases and pancreatitis.

Prostate-specific Antigen Serum Biomarker Test

Prostate-specific antigen (PSA) is a protein synthesized by normal cells and CSCs in the prostate gland. PSA is mostly found in the semen, but a small amount of PSA is also present in blood and expressed in units called nanograms per millimeter. There is no cut off point to diagnose prostatic carcinoma. PSA concentration may be high in prostatitis and benign nodular hyperplasia.

Flow Cytometry Technique

Flow cytometry is performed to quantify different types of cells according to their membrane antigen character and DNA content used in classification of **leukemias** and **lymphomas**, and to evaluate the risk of recurrence. This molecular test may be performed as a part of stem cell transplantation process. **Flow cytometry** measures the properties of size, granularity, and protein expression (immunophenotype) of individual cells in sample of blood, bone marrow and lymph nodes.

- There are four steps in most flow cytometry protocols: sample preparation, blocking, antibody specific to surface or intracellular proteins incubation and data collection.
  - Monoclonal antibodies are used to differentiate different hematopoietic cell lineages. Cells are made to flow in laminar flow in an isotonic fluid under pressure in front of a laser beam.
  - If monoclonal antibodies are attached to the cells, these emit light. Flow cytometry detects antigens on the surface of CSCs.
- Flow cytometry may also be performed to measure the amount of CSCs DNA. In cancer patients, CSCs are treated with special light-sensitive dyes that react with DNA. Presence of an abnormal amount of DNA indicates that patients have recurrence of malignant epithelial tumors of breast, lung, colorectal region, prostate, and urinary bladder. Comparison of immunohistochemistry and flow cytometry techniques is given in Table 6.124.

CHROMOSOMES ANALYSIS (KARYOTYPING TEST)

Chromosomes analysis, also known as karyotyping genetic test on peripheral blood sample may be clinically significant to diagnose genetic disorders and malignant tumors by looking structural and numerical chromosomal alterations at the metaphase stage of the cell cycle when chromatin is highly condensed, and the chromosome morphology is well-defined. Karyotyping that involves the pairing of homologous chromosomes.

- Chromosomes are thread-like structures made up of single molecular of deoxyribose nucleic acid (DNA) in the nucleus of each cell. Each chromosome contains several genes. **Gene** is defined as a segment of DNA that contains the instructions for making a particular protein.
- Humans have 23 pairs of chromosomes (i.e. 46 chromosomes in total). Twenty-two of these pairs, called autosomes, look the same in both males and females. The last 23rd chromosome pair is called the sex chromosome, that determines the genetic gender of the person. Females have two copies of the X chromosome (XX), while males have one X and one Y chromosome (XY). Chromosomal analysis test can provide information about structural and numerical abnormalities.

Cytogenetic Analysis

Cytogenetic analysis is the study of individual chromosomes, their structure, and inheritance patterns.

- Cytogenetic analysis can be utilized to diagnose solid malignant tumors and hematolymphoid malignancies including acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), chronic

Table 6.124 Comparison of immunohistochemistry and flow cytometry

Characteristics	Immunohistochemistry Technique	Flow Cytometry Technique
Definition	Microscopy-based technique that allows selective identification and localization of antigens in cells of a tissue	Laser-based technique that detects and measures the physical and chemical characteristics of a cell population
Cost of equipment needed	Less expansive equipment	Expensive equipment and additional technical staff
Specimen required	Formalin-fixed paraffin-embedded sections or frozen sections	Bone marrow, body fluids, fine needle aspirations, and fresh solid tissue
Technique utilizes	Monoclonal/polyclonal antibodies limited but gradually expanded	Abundant monoclonal antibodies
Instrument	Light microscopy or fluorescent microscope	Flow cytometer
Analysis (morphologic correlation)	Excellent morphologic correlation, viewed under a light microscope or fluorescence microscopy	No visual correlation, technique using a computer
Dual staining on same cells	Limited	5 to 6 color staining
Quantification	Estimation with imaging techniques	Accurate and reproducible
Distinction between surface and cytoplasmic staining	Difficult	Easy
Turnaround time	Four hours after hematoxylin and eosin-stained tissue section is examined	Three hours

Similarities between immunohistochemistry and flow cytometry are two techniques that use fluorescently-labeled antibodies to detect antigens on the cell surface or within cells to detect in cancers and infectious diseases.

myelogenous leukemia (CML) and lymphoma, determine appropriate therapy, and monitor the status of disease.

- After white blood cells or single-cell suspensions of CSCs are cultured in media, they are stimulated to divide with mitogens then arrested in cell metaphase. The chromosomes are released from the nucleus, fixed, and spread onto slides for staining and microscopic examination.
- In most banding methods, the individual chromosomes are identified by their size, chromosome short (p) and long (q) arms, the position of the centromere and patterns of transverse striations.
- Chromosomes are categorized based on their morphologic features, staining characteristics and placement of centromere: (a) acrocentric chromosomes have their centromere near one end of the chromosome and have satellite short arms encoding ribosomal RNA (rRNA), and (b) metacentric chromosomes have a centrally placed centromere.
- Chromosomal studies are indicated in cases for various reasons: developmental delay, dysmorphic features, malignant tumors, fertility-related issues, and pregnancy in women who are older than 35 years. Chromosome abnormalities include incorrect number of chromosomes (aneuploidy) and structural chromosome rearrangements.
- **Aneuploidy** refers to any number of chromosomes more or less than expected 26 (e.g. 47 in trisomy 21) and chromosome rearrangements occur when chromosomes break and constituent pieces reassemble abnormally (chromosome translocation). Balanced chromosome rearrangements may not be clinically evident because all the chromosomal material is intact. However, imbalanced chromosome translocations may result in aberrant expression of fusion genes leading to development of various lymphoid malignancies.

Chromosome Structural Alterations Analysis in Cancers

Chromosome structural alterations can be biomarkers for human cancers. Usually, chromosome structural alterations are not only biomarkers but also the underlying cause of the human cancer. Chromosomal translocation and exchange of sister chromatids give rise to structural abnormalities that can be easily marked by employing various banding techniques. Further, homogenously stained regions (**HSRs**) and double minute chromosomes (small fragments of extrachromosomal DNA) are frequently associated with gene amplification of a variety of human cancers (e.g. breast carcinoma, lung carcinoma, ovarian carcinoma, colon carcinoma and neuroblastoma), which can serve as biomarkers.

- **Cytogenetic analysis in chronic myelogenous leukemia (CML):** Philadelphia chromosome contains a BCR-ABL fusion gene that consists the amino portion of BCR on chromosome 22 and the carboxyl portion of ABL on chromosome 9. BCR-ABL fusion gene cannot '**turn off**' encodes abnormal protein, that results in cell growth, proliferation, and progression in unregulated manner.
  - BCR-ABL fusion gene is demonstrated in most patients with chronic myelogenous leukemia (CML) and some patients with acute lymphoblastic leukemia (ALL). Philadelphia chromosome can be detected by cytogenetic analysis.
  - Polymerase chain reaction (PCR) is performed to analyze DNA sequence that can find BCR-ABL fusion gene. PCR testing may be used to monitor a patient's clinical course. CML patients are treated by chemotherapeutic agent 'imatinib', that inhibits the activity of BCR-ABL fusion protein, thus prevents unrestricted leukemic cell proliferation and progression.
- **Cytogenetic analysis in acute myelogenous leukemia (AML):** Cytogenetic analysis plays a crucial role in the diagnosis on the bone marrow, classification, prognosis, and management of acute myelogenous leukemia (AML) patients.
  - Moreover, cytogenetic analysis assists in determining treatment response and overall prognosis. The t(8;21) is one of the most frequent chromosomal alterations associated with AML.
  - The chromosomal translocation which involves the AML1 gene (also called RUNX1) on chromosome 21 and the ETO gene (also called RUNX1T1 gene) on chromosome 8 generates AML1-ETO fusion gene that encodes leukemic inducing transcription factor leading to unrestricted cell proliferation, differentiation, and the viability of leukemia cells.
  - The disease can be diagnosed by cytogenetic analysis of AML blasts by G-banding technique and fluorescence *in situ* hybridization (FISH). Detection of AML1/ETO fusion gene can be detected by reverse transcriptase polymerase chain reaction (RT-PCR).
- **Cytogenetic analysis in acute promyelocytic leukemia (APL):** The PML-RARA fusion gene is the most critical event in the pathogenesis of acute promyelocytic leukemia (APL). Although APL is one of the most characterized forms of acute myelogenous leukemia (AML).
  - APL is defined by the PML-RARA rearrangement as a consequence of the translocation t(15;17) (q24;q21) resulting in the **PML RARA fusion gene**, that encodes an oncoprotein responsible for disruption

of myeloid distinction and abnormal proliferation of promyelocytes, life-threatening coagulopathy.
  - Rapid molecular testing is performed for fluorescence *in situ* hybridization (FISH) to detect RARA-PML fusion gene. APL patients are treated by all-*trans*-retinoic acid (ATRA) and arsenic trioxide (ATO) therapy.
- **Cytogenetic analysis in Burkitt's lymphoma:** The oncogenic transcription factor Myc is pathologically activated in many human cancers. A paradigm of Myc dysregulation is offered by Burkitt's lymphoma characterized by a high turnover rate of malignant B cells.
  - In Burkitt's lymphoma, reciprocal chromosomal translocation t(8;14) (q24;q32) involves the c-Myc gene (8q24) and immunoglobulin heavy-chain (IGH) locus (14q32).
  - Cytogenetic analysis performed on bone marrow and blood samples, most Burkitt's lymphoma cases show t(8;14) (q24;q32) (Myc-IGH), and less commonly t(8;22) (q24;q11) or t(2;8)(p12;24). The Myc breakpoints are diverse and distributed over a 2 Mb region. High quality metaphases are required to detect t(8;14) and t(8;22). Fluorescence *in situ* hybridization cannot detect all Myc rearrangements.
- **Cytogenetic analysis in Ewing sarcoma:** Reciprocal recurrent balanced chromosomal translocation between chromosome 11 and 22 results in fusion of the amino terminus of EWSR1 gene to the FLT1 gene and forming EWSR1/FLT1 fusion gene, which encodes a nuclear transcription factor resulting in cellular proliferation in about 85% of patients with Ewing sarcoma. **Diagnostic criteria of Ewing sarcoma**/primitive neuroectodermal tumor (PNET) includes (≥2 major features of neural differentiation): Homer Wright rosettes, MIC2 expression, vimentin expression, neuron-specific enolase (NSE), synaptophysin, and chromogranin. EWSR1/FLT1 fusion gene is detected by cytogenetic analysis.
- **Cytogenetic analysis in hematolymphoid malignancies:** The IGH/CCND1 gene has been reported in many B cell-derived hematolymphoid malignancies such as mantle cell lymphoma, chronic lymphocytic leukemia (CLL), prolymphocytic lymphoma, multiple myeloma, splenic lymphoma with villous morphology, and plasma cell leukemia. The chromosomal translocation juxtaposes the CCND1 gene located on chromosome 11q13 with IGH gene mapped on chromosome 14q32 results in formation of IGH-CCND1 fusion gene. Immunohistochemistry, cytogenetic analysis, fluorescence *in situ* hybridization (FISH), and flow cytometry play pivotal role in diagnosis and monitoring B cell-derived hematolymphoid malignancies.
- **Cytogenetic analysis in pediatric acute lymphoblastic leukemia (ALL):** The t(12;21) translocation generates the ETV6/RUNX1 (TEL-AML1) fusion gene is the most common chromosomal rearrangement in 25% pediatric acute lymphoblastic leukemia (ALL) between the ages of 2 and 10 years, with a median of 4 years, and is exclusively associated with B cell precursor acute lymphoblastic leukemia (BCP-ALL). **Real-time polymerase chain reaction (RT-PCR)** technique detects t(12;21) of the ETV6/RUNX1 (also called TEL/AML1) chimeric fusion transcript. Recently, fluorescence *in situ* hybridization (FISH) is also employed to detect t(12;21) of the ETV6/RUNX1 (also called TEL/AML1) chimeric fusion transcript.
- **Cytogenetic analysis in acute myelomonocytic leukemia (AMML)-AML-M4:** Researcher reported a related chromosomal 16 inversion in acute myelomonocytic leukemia (AMML) that shows evidence of both myeloid and monocytic differentiation.
  - **Bone marrow** contains myeloblasts and monoblasts constitute 20%. Monoblasts must be <80% of total nucleated cells.
  - **Cytogenetic analysis** detects chromosome 16 inversion. Flow cytometry will confirm the diagnosis.
- Monoblasts and promonocytes are CD45 bright and SSC slightly higher than myeloblasts; and these cells express CD11b, CD11c, CD13, CD14, CD64 and HLA-DR.

Chromosome Numerical Alterations Analysis in Cancers

Chromosomes are thread-like structures in which DNA is tightly packaged many times around associated histone proteins within the nucleus, that carries genetic information to the next generation. Human beings have 23 pairs of chromosomes for a total of 46 overall. Each cell contains 22 pairs chromosomes called autosomes and one pair of sex chromosomes: female has XX pattern and male has XY pattern.

- **Haploid cells versus diploid cells:** Somatic cells in human beings are diploid cells, which contain 23 pairs of chromosomes (46 total chromosomes) in each diploid cells, i.e. half come from the mother; and half come from the father.
  - The total number of chromosomes in diploid cells is described (2n), which is twice the number of chromosomes in a haploid cell (n). Diploid cells are produced by mitosis. Haploid cells contain only one set of chromosomes (n).

- Gametes are the most common types of haploid cells, which are produced by meiosis and are genetically diverse. When the haploid cells from male and female fertilize fuse together during fertilization, it forms a diploid cell. Diploid cells contain two sets of homologous (similar) copies of each chromosome.

- **Aneuploidy:** Chromosomal alterations involving addition or loss of chromosome in cell leading to unbalanced chromosome complement is termed aneuploidy. For example, human cell has 47 or 45 chromosomes instead of 46.
  - Trisomy and monosomy are examples of aneuploidy. Specific aneuploidy has been observed in various non-cancer genetic orders: Down syndrome with trisomy chromosome 21, Patau syndrome with trisomy 13, Edwards' syndrome with trisomy 18, Klinefelter syndrome with an extra X chromosome to normal male karyotype (XXY aneuploidy), and Turner syndrome with the absence of one X chromosome.
  - While, clonal aneuploidy is detected in some human cancers such as chronic lymphocytic leukemia (CLL) with trisomy 12 and acute myelogenous leukemia (AML) with trisomy 8.
  - Complete loss of chromosome 17 (monosomy of chromosome 17) has been detected in breast carcinoma.
  - Complete or partial loss of chromosome 7 (monosomy of chromosome 7) is associated with a variety of myeloid disorders including pediatric acute myelogenous leukemia (AML).

Fluorescence *in situ* Hybridization (FISH) Technique

Fluorescence *in situ* hybridization (FISH) is a cytogenetic diagnostic technique that uses fluorescent probes to hybridize with chromosomal DNA sequence fixed on slides to analyze gene amplification, gene fusions, and copy number of DNA segments and routine breast cancer studies (e.g. HER2/neu gene amplification, mitotic index with Ki-67, ER and PR) by FISH labeled with fluorescence dyes and fluorescence microscopy.

- The fluorescent staining pattern is then viewed at the appropriate wavelength of light to localize specific genes or chromosomes (e.g. to highlight red or green labels). Interphase fluorescence *in situ* hybridization uses a probe while cells are in the interphase, allowing for large number of nuclei to tested. Metaphase fluorescence *in situ* hybridization allows for specific localization of the probe-binding region within a specific region within a specific chromosome.
- Each probe is inserted to detect a unique sequence (e.g. HER2/neu) or specific portion of a chromosome. Sequence specific DNA probes permit unique DNA sequences to be analyzed for DNA insertions, deletions, and chromosome translocations.
  - Chromosomal paints are DNA probes for a mixed DNA from all or part of chromosome to visually inspect the chromosome for translocated fragments.
  - Multiple chromosomal paints may be used simultaneously to identify each metaphase chromosome with a unique color in a piece known as spectral karyotyping.

Pathology Pearls: DNA Probe Analysis

- Probes are copies of complementary DNA (cDNA). Two probes are prepared: one from the tumor and another from normal tissue. The tumor (cDNA) is now tagged with red color fluorochrome. Normal DNA probe is tagged with green color fluorochrome.
- DNA of the cells is digested by one or more restriction enzymes (bacterial enzymes). Nucleotide base pair of DNAs is identified and cut.
- DNA fragments are separated by gel electrophoresis, then denatured and transferred to nitrocellulose membrane and then DNA probe is applied resulting in hybridization.

CHAPTER 7

Nutritional and Infectious Diseases

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LEARNING OBJECTIVES

NUTRITIONAL DISORDERS

- Protein-calorie malnutrition
  - Marasmus
  - Kwashiorkor (edematous malnutrition)
- Obesity
  - Pathogenesis
  - Obesity-associated health problems

VITAMINS DEFICIENCY

- Fat-soluble vitamins
  - Vitamins A, D, E and K
- Water-soluble vitamins
  - Vitamin C (ascorbic acid)
  - Vitamin B-complex
    - Thiamine (vitamin B_1)
    - Riboflavin (vitamin B_2)
    - Niacin (vitamin B_3)
    - Pantothenic acid (vitamin B_5)
    - Pyridoxine (vitamin B_6)
    - Biotin
    - Folate (folic acid)
    - Cyanocobalamin (vitamin B_{12})

BACTERIAL INFECTIONS

- Gram-positive bacterial infections
  - Gram-positive cocci
    - *Staphylococcus aureus*
    - *Staphylococcus epidermidis*
    - *Staphylococcus saprophyticus*
    - *Streptococcus pneumoniae*
    - *Streptococcus viridans*
    - *Streptococcus pyogenes*
    - Enterococci
  - Gram-positive bacilli
    - Anthrax (*Bacillus anthracis*)
    - Diphtheria (*Corynebacterium diphtheriae*)
    - Listeriosis (*Listeria monocytogenes*)
    - Nocardia
    - Erysipelothriosis (*Erysipelothrix rhusiopathiae*)
    - Actinomycosis (*Actinomyces israelii*)
- Gram-negative bacterial infections
  - *Neisseria meningitidis*
  - *Bordetella pertussis* (whooping cough)
  - Plague (*Yersinia pestis*)
  - Granuloma inguinale (*Klebsiella granulomatis*)
  - Chancroid (*Haemophilus ducreyi*)
  - *Salmonella typhi* infection
- *Mycobacterium tuberculosis*
- Mycobacterium leprosy (Hansen's disease)
- Spirochetes
  - Syphilis (*Treponema pallidum*)
  - Lyme disease
- Anaerobic bacteria
  - Anaerobic bacterial abscesses
  - Clostridial infections
    - Tetanus (*Clostridium tetani*)
    - Botulism (*Clostridium botulinum*)
    - Gas gangrene (*Clostridium perfringens*)
- Obligate intracellular bacteria
  - Chlamydial infection (*Chlamydia trachomatis*)
  - Rickettsial infections

VIRAL INFECTIONS

- Viral infections: overview
  - Pathogenesis
  - Protection of cells against viruses
- RNA viruses
  - Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) for COVID-19 pandemic
  - Poliovirus infection
  - Coxsackievirus infection
  - Hepatitis A virus infection
  - Norovirus (Norwalk virus) infection
  - Yellow fever virus infection
  - Rubella virus infection
  - Ebola virus and Marburg virus infection
  - Mumps virus (paramyxovirus family) infection
  - Measles virus (paramyxovirus family) infection
  - Rabies virus infection
  - Hepatitis C virus infection
  - Human T cell leukemia virus type 1 infection
  - Human rotavirus infection
- DNA viruses
  - Variola (smallpox) infection
  - Varicella-zoster virus (chickenpox) infection
  - Molluscum contagiosum virus 1 and 2 (MCV-1/MCV-2) infection
  - Herpes simplex virus (HSV-1/HSV-2) infection
  - Human papillomavirus infection
  - John Cunningham virus (JC virus) infection
  - Hepatitis B virus (HBV or Dane particle) infection
  - Parvovirus B19 infection
  - Human adenoviruses infection

FUNGAL DISEASES

- Ascomycota
  - Dermatophytes (*Epidermophyton flocossum, Microsporum canis, Trichophyton interdigitale, Trichophyton rubrum*)
  - Molds (*Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger*)
  - Yeast (*Candida albicans, Candida glabrata, Candida krusei*)
  - Dimorphic fungi (*Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis*)
- Basidiomycota
  - *Cryptococcus neoformans*
  - *Malassezia furfur*
- Zygomycota
  - *Absidia corymbifera*
  - *Rhizomucor pusillus*
  - *Rhizopus oryzae*
- Dematiaceous fungi
  - Mycetoma (madura foot)
  - Phaeohyphomycosis
  - Chromoblastomycosis
- Other fungi causing skin and subcutaneous tissue infections
  - *Sporothrix schenckii*
  - Cladosporium

PARASITIC INFESTATIONS

- Protozoa
  - *Entamoeba histolytica, Giardia lamblia, Trichomonas vaginalis,* malarial parasite, Leishmaniasis, babesiosis, African trypanosomiasis, Chagas disease
- Metazoa (Helminths)
  - Strongyloidiasis, lymphatic filariasis, Cysticercosis—tapeworm (cestode), Hydatid cyst—tapeworm (cestode), *Ancylostoma duodenale* (nematode), *Enterobius vermicularis* (pinworm) nematode, trichinosis, schistosomiasis, onchocerciasis

NUTRITIONAL DISORDERS

Nutritional disorders occur when a person's dietary intake lacks the right amount of nutrients for normal functioning, or when a person is unable to absorb nutrients from food. Nutritional disorders can be caused by undernutrition, overnutrition leading to obesity or incorrect balance of nutrients.

- Nutrient deficiencies can occur due to poor dietary nutrient intake, acute or chronic diseases, medications, altered nutrient metabolism or a combination of these factors leading to alterations in energy metabolism, immune system function, growth and development, if the nutrient deficiency is present during fetal development and early childhood.
- Globally, both undernutrition and obesity are important public health problems. Interventions in the treatment of nutritional disorders may involve the use of therapeutic diets and the administration of dietary supplements.

PROTEIN-CALORIE MALNUTRITION

Protein-calorie malnutrition (PCM), or protein-energy malnutrition (PEM) refers to a form of malnutrition where there is inadequate calorie or protein intake or both. Protein-calorie malnutrition is accompanied by varying degrees of micronutrient deficiency such as vitamin A, vitamin E, folate, vitamin B_6, iron, copper, zinc and selenium. Protein-calorie malnutrition occurs in two forms: marasmus and kwashiorkor.

- Marasmus results from low calorie intake, which is characterized by growth failure and wasting.
- Kwashiorkor occurs due to protein deficiency, which is characterized by tissue edema and tissue damage. Protein-calorie malnutrition increases the risk of mortality from pneumonia, measles or chickenpox.
- Protein-calorie malnutrition is diagnosed by measurement of body weight for given height with standard tables (weight <80% of normal), evaluation of fat stores (thickness of skin folds), muscle mass (mid-arm circumference), serum proteins (albumin), decreased mid-arm circumference (marasmus) and decreased serum proteins (kwashiorkor). Comparison of kwashiorkor and marasmus is given in Table 7.1.

MARASMUS

Marasmus is caused by widespread deficiency of almost all nutrients, notably protein and calories, which often coexists with vitamin deficiencies. It typically occurs in children younger than 1 year of age who are not breast-fed and do not have an adequate intake of substitute nutrients. Chronic diarrhea is also underlying cause of marasmus. Weight for age is <60% of expected normal in marasmus.

Table 7.1 Comparison of kwashiorkor and marasmus

Characteristics	Kwashiorkor	Marasmus
Age group	2–5 years	Less than one year
Deficiency of nutrients	Protein deficiency is marked	Protein and calories deficiency
Loss of protein in compartment	Depletion of liver proteins stores	Depletion of protein stores in somatic tissues
Etiology	Weaning of child at early age without protein supplement	Weaning of child at early age without protein as well as carbohydrates, and chronic diarrhea
Skeletal muscle	Relatively spared	Loss of skeletal mass due to catabolism
Serum protein levels	Markedly reduced	Normal or slightly decreased
Immune status	Immunodeficiency with secondary infection	Immunodeficiency with secondary infection
Subcutaneous fat	Spared	Mobilized for energy purpose
Extremities	Edematous due to decreased serum albumin level	Emaciated
Skin folds and mid-arm circumference	Relative spared	Reduced skin fold, and decreased mid-arm circumference
Growth retardation	Present but less	Present but more severe
Skin lesions	▪ Skin-flaky paint appearance ▪ Hair loss of color, alternating bands of pale and dark hair, loss of scalp attachment	▪ No characteristics skin and hair changes ▪ No characteristics skin and hair changes

Contd...

Table 7.1 Comparison of kwashiorkor and marasmus (*Contd...*)

Characteristics	Kwashiorkor	Marasmus
Hepatomegaly	Fatty change present	Fatty change absent
Small bowel changes	Mucosa is atrophic, loss of villi, and decreased mitotic index in crypts of glands	No such change
Bone marrow	Hypoplastic with decreased erythropoiesis	Hypoplastic with decreased erythropoiesis
Thymic and lymphoid atrophy	More marked	Less marked
Brain changes	Usually, absent	Brain-cerebral atrophy, reduced number of neurons, impaired myelination of white matter
Associated nutritional deficiency	Present	Present

Clinical Features

Patient of marasmus presents with retarded growth due to depletion of protein stores in tissues and emaciated extremities. Loss of subcutaneous fat ('wasting away') due to mobilization of fat for energy purpose. Child is prone to recurrent infections due to immunosuppression. On clinical examination, patient does not show signs of edema. Schematic representation of marasmus child is shown in Fig. 7.1.

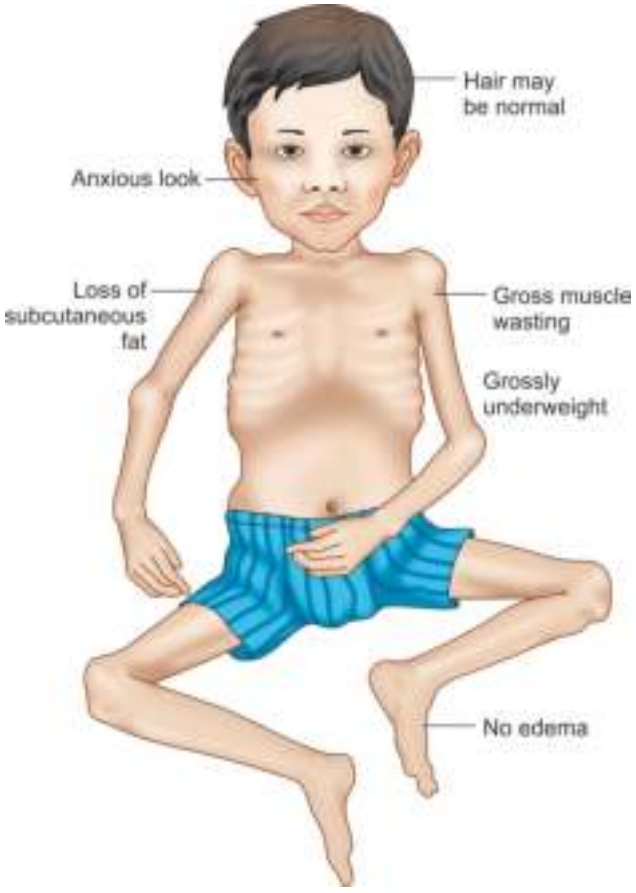


Fig. 7.1: Schematic representation of marasmus child. Marasmus is caused by lack of proteins and calories resulting in emaciated extremities, loss of subcutaneous fat, muscle wasting, retarded growth but no edema.

KWASHIORKOR (EDEMATOUS MALNUTRITION)

Kwashiorkor is caused by protein deficiency but with adequate caloric intake, which usually affects children older than 1 year of age who are no longer breastfed and receive a starch-rich and protein-poor diet. Serum proteins are markedly reduced. There is suppression of bone marrow and thymus. Child is underweight with edema having electrolyte abnormalities.

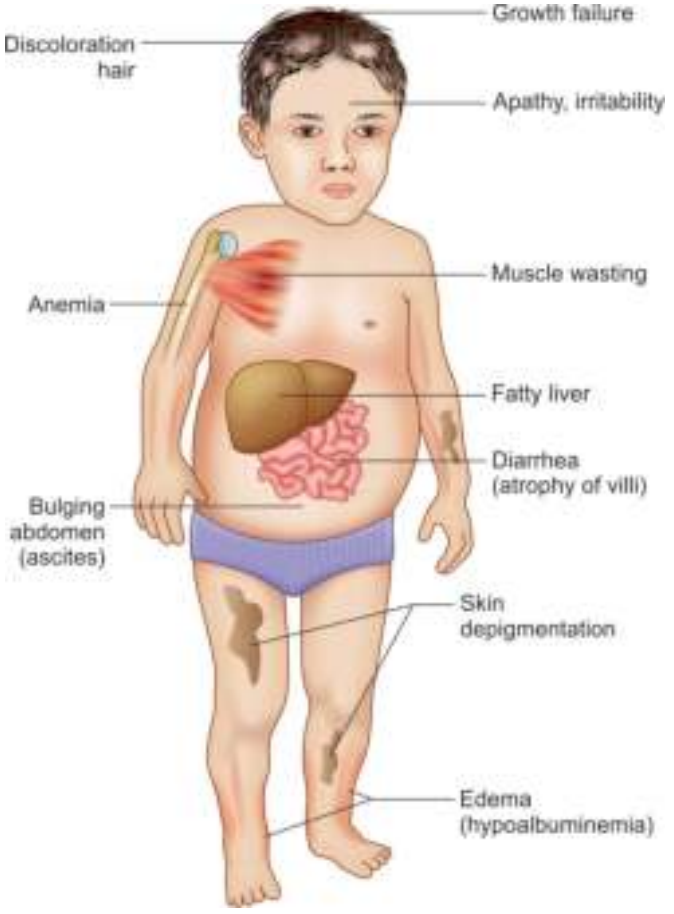


Fig. 7.2: Schematic representation of kwashiorkor in a child. It is caused by lack of proteins resulting in edema, ascites, anemia, skin pigmentation, muscle wasting, fatty liver and growth failure.

Clinical Features

Patient presents with retarded growth and muscle wasting due to inadequate protein intake, but with preservation of subcutaneous fat. Kwashiorkor is distinguished from marasmus by the presence of the following abnormalities: fatty liver, severe edema due to protein deficiency and decreased oncotic pressure, anemia, malabsorption as a result of atrophy of the small intestinal villi, depigmented bands with pale streaking in the hair or skin. Schematic representation of kwashiorkor in a child is shown in Fig. 7.2.

OBESITY

Obesity is defined as abnormal or excessive accumulation of fat that may impair health, which is independent risk factor for development of venous thromboembolic disease.

- Adipose tissue has endocrine role in synthesis of adipokines, which have been implicated in the development of inflammation, atherosclerosis, cancer and thrombosis. Obesity is also associated with increased risk of type 2 diabetes mellitus, hypertension, gallbladder stones, and osteoarthritis.
- As specific diagnostic tools such as magnetic resonance imaging or dual energy X-ray absorptiometry for assessment of obesity are not easily available, hence body mass index (**BMI**) is the most accepted definition of obesity, which is calculated by weight in kilograms divided by the square of the height in meters (kg/m^2).
- For adults, WHO defines overweight (BMI 25) and obesity (BMI 30). For children, age needs to be considered when defining overweight and obesity.
- For children under 5 years of age, WHO defines overweight as weight-for-height ≥2 standard deviations above WHO defined child growth standards median, while obesity is weight-for-height ≥3 deviations above WHO defined child growth standards.
- For children between 5 and 19 years of age, WHO defines overweight as BMI-for-age ≥1 standard deviation above WHO defined child growth standards median, while obesity is ≥2 standard deviation above WHO defined child growth standards median.
- World Health Organization classification system for BMI defines obesity as a BMI >30 with further subcategories given in Table 7.2.

Table 7.2 World Health Organization classification of obesity

BMI (kg/m^2)	Alternative Commonly used Terminology	WHO Classification
<18.50	Underweight	Underweight
18.50–24.99	Normal weight	Normal weight
25–29.99	Overweight	Overweight
≥30.00–34.99	Obese	Obese class I
35.00–29.99	Obese	Obese class II
≥40.00	Morbid obesity	Obese class III
≥50.00	Super-obesity	Obese class IV
≥60.00	Super-super-obesity	Obese class V

PATHOGENESIS

Obesity usually results from combination of causes and contributing factors such as family inheritance, lifestyle (e.g. unhealthy diet, physical inactivity), medical illnesses (e.g. Prader-Willi syndrome, Cushing's syndrome), medications (e.g. some antidepressants, antiepileptic medications, diabetes mellitus medications, antipsychotic medications, corticosteroids and β-blockers), pregnancy, lack of sleep, stress and microbiome. Obesity pathogenesis involves two related distinct processes: (a) sustained positive energy balance, i.e. energy intake > energy expenditure; and (b) resetting the body weight 'set point' at an increased value through changes of diet and/or lifestyle (Fig. 7.3).

Leptin Synthesis and Functions

Leptin is the adipocyte peptide hormone, which circulates at concentrations proportion to body weight, which regulates food intake, body mass and reproductive function. Leptin plays a key role in energy homeostasis, fetal growth, proinflammatory immune responses, angiogenesis and lipolysis. Leptin deficiency is linked to hyperphagia and obesity.

Leptin Structure and its Regulation

Leptin peptide hormone is the product of the '**obese (Ob) gene**'. Leptin molecule comprises 167 amino acids including a 21-amino acid sequence. Leptin exhibits the tertiary structure of globular protein.

- **Insulin regulates leptin:** Insulin is primary regulator of leptin production. Persistent hyperinsulinemia enhances plasma concentration of leptin via glucose metabolism, while short-term hyperinsulinemia does not affect plasma concentration of leptin.
  - The blockade of glucose transport or glycolysis in the presence of hyperinsulinemia suppresses the expression and secretion of leptin from adipocytes.
  - Leptin flows from adipocytes into the bloodstream passes through blood–brain barrier and reaches in regions of hypothalamus involved in regulation of energy balance.
- **Catecholamines regulate leptin:** Unlike insulin, catecholamines bind to β_2- and β_3-adrenergic receptors and suppress leptin synthesis from adipocytes, indicating a link between neuroendocrine and

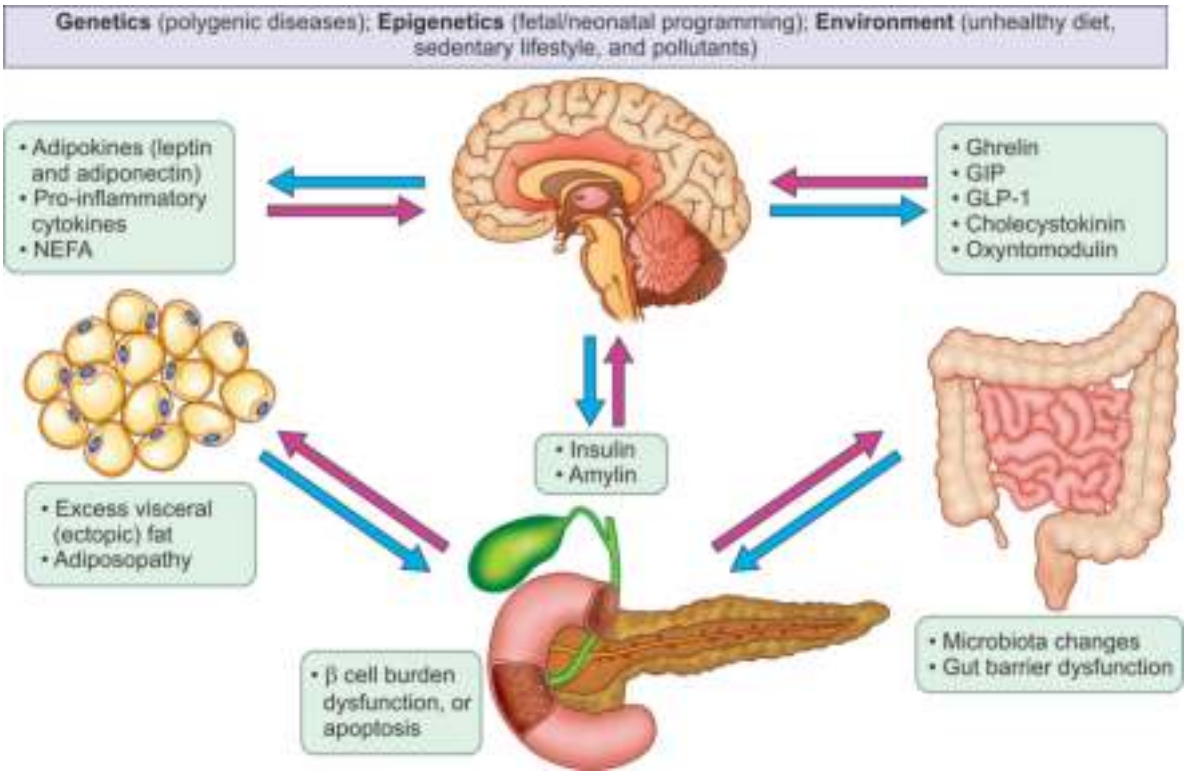


Fig. 7.3: Pathogenesis of obesity. Leptin gene is most often defective in obesity. Mutation of leptin gene inhibits leptin release by adiposities. Obesity is related to synthesis of leptin, an antiobesity hormone produced by adipocytes. Neuropeptide Y, pro-obesity is synthesized by the hypothalamus in response to leptin deficiency.

sympathetic regulation of adipose tissue endocrine function through negative feedback between brain and adipose tissue.

- **Corticosteroids and thyroid hormones regulate leptin:** Corticosteroids and tumor necrosis factor-α (**TNF-α**) enhance leptin synthesis, while thyroid hormones are likely to suppress its synthesis from adipocytes. Increased adipose tissue store enhances leptin synthesis. Leptin decreases food intake via hypothalamus and stimulates energy expenditure.
- **Adipose tissue store regulates leptin:** On the contrary, decreased adipose tissue store suppresses leptin synthesis from adipocytes, which increases food intake via hypothalamus and suppresses energy expenditure.

Leptin and its Cognate Receptors

Leptin receptor (LEP-R) exists in several alternatively spliced variants labeled as LEP-Ra, LEP-Rb, LEP-Rc, LEP-Rd, LEP-Re and LEP-Rf. Following leptin synthesis, and secretion from adipocytes in white adipose tissue binds to its cognate transmembrane leptin receptor (LEP-R).

- LEP-R distribution facilitates leptin's pleotropic effects by regulating body mass via a negative feedback mechanism between white adipose tissue and the hypothalamus.
- LEP-R exhibits structural similarity to the class I family of cytokine receptors, which include receptors for interleukins, colony stimulating factor 3 (CSF-3), growth hormone, prolactin, erythropoietin and leukemia inhibitory factor.

Leptin and Energy Homeostasis

Brain has dual-center: (a) satiety center in the ventromedial hypothalamic nucleus (VMH); and (b) hunger center in the lateral hypothalamic nuclei (LH). These centers in brain maintain energy input and energy expenditure.

- Leptin regulates appetite and metabolism by inhibiting the synthesis and its release of pro-obesity neuropeptide Y (**NPY**) in the arcuate nucleus (**ARC**) in brain leading to obesity.
- Subsequently, researchers discovered the LEP-Rb isoform in the ventromedial hypothalamic nucleus (**VMH**), arcuate nucleus (**ARC**), lateral hypothalamic nuclei (**LH),** and dorsomedial hypothalamic nucleus (**DMH**), which play an important role in the regulation of energy balance and body mass.

Leptin Hormone and Obesity

Obese gene mutation inhibits release of **antiobesity leptin hormone** synthesis from adipocytes.

- **Leptin expression in obesity:** Severe early obesity develops due to obese (ob) gene mutation as a result of either hereditary leptin deficiency or leptin resistance that affects leptin signaling pathways.
- **Leptin resistance in obesity:** Leptin resistance is characterized by reduced satiety, overconsumption of nutrients, and increased total body mass. Leptin resistance leads to obesity, which reduces the effectiveness of using exogenous leptin as a therapeutic agent. Thus, combining leptin therapies with leptin sensitization may assist to overcome leptin resistance and consequently obesity.

OBESITY-ASSOCIATED HEALTH PROBLEMS

Persons with obesity are more likely to develop a number of potentially serious health problems including heart diseases, cerebral stroke, type 2 diabetes mellitus, certain cancers, digestive problems, osteoarthritis, gynecological and sexual disorders. Schematic representation of obesity associated health problems is shown in Fig. 7.4.

Obesity-related Respiratory System Disorders

Obesity causes obstructive sleep apnea, obesity hypoventilation syndrome, bronchial asthma, cor pulmonale (pulmonary hypertension and right ventricular hypertrophy).

- **Obstructive sleep apnea:** Obstructive sleep apnea refers to recurrent episodes of apnea (interrupted airflow) due to obstruction of the upper airway during sleep followed by transient awakening to restore airway patency, which occurs due to accumulation of fat in neck region. Recurrent obstructive sleep apneas cause chronic alveolar hypoxia resulting in

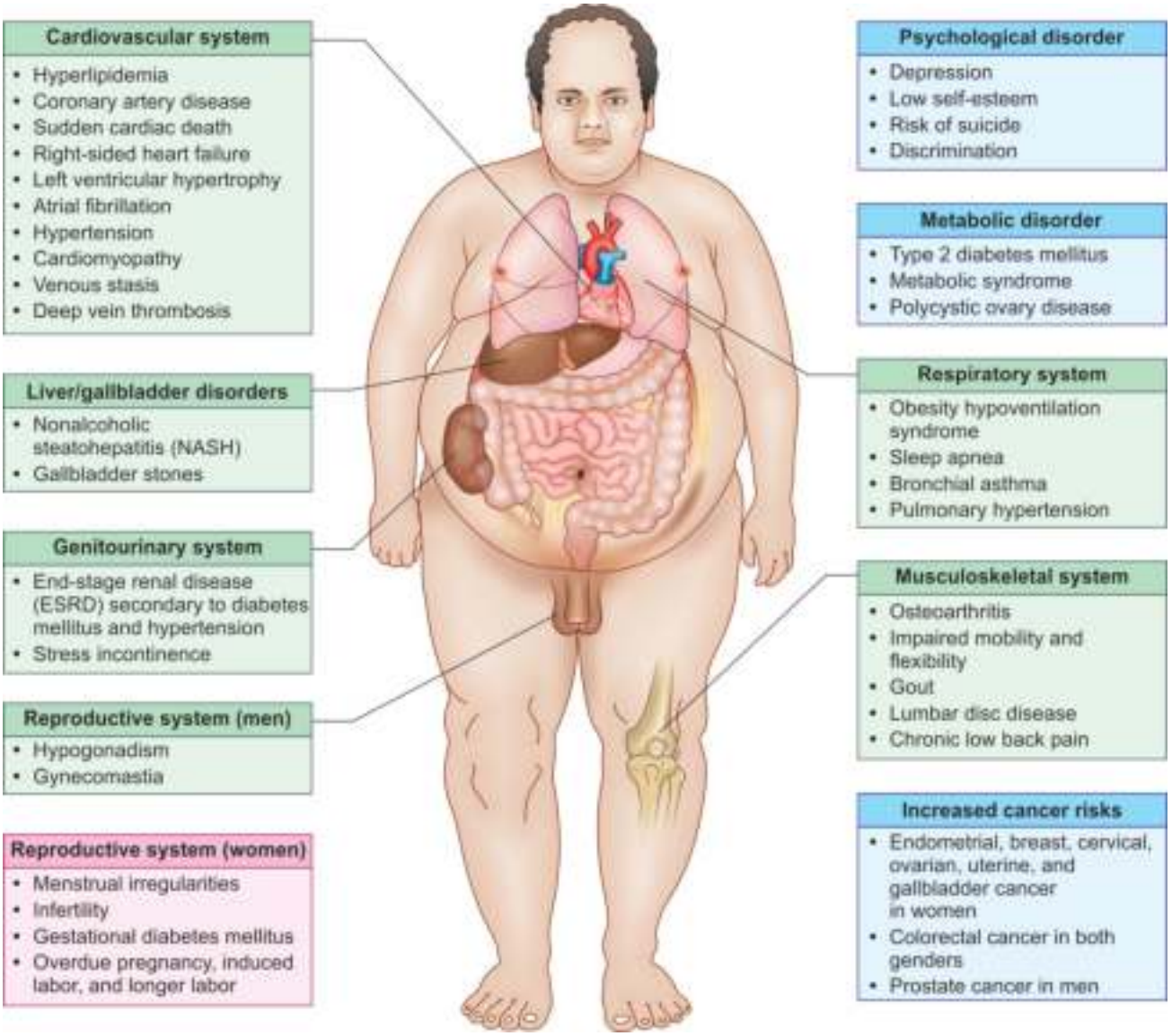


Fig. 7.4: Schematic representation of obesity-associated health problems.

pulmonary hypertension and cor pulmonale, which is confirmed by polysomnography. Obstructive sleep apnea is exacerbated by tobacco smoking, sedative drugs and alcohol consumption. Obstructive sleep apnea is independent risk factor for congestive heart failure, hypertension, myocardial infarction, cardiac arrhythmias and sudden cardiac death.

- **Obstructive hypoventilation syndrome:** Obstructive hypoventilation syndrome is also known as 'Pickwickian syndrome' comprising hypoventilation, daytime hypercapnia and hypoxemia ($paCO_2$ >45 mm Hg and paO_2 <10 mm Hg). The diagnostic test for obstructive hypoventilation syndrome is daytime arterial blood gas analysis. This disease has a high mortality rate due to leptin resistance resulting in central hypoventilation and impaired compensatory response to acute hypercapnia.

Obesity-related Cardiovascular System Disorders

Obesity contributes directly to cardiovascular system disorders related risk factors, including dyslipidemia, hypertension, type 2 diabetes mellitus and sleep disorders. Obesity also leads to the development of cardiovascular disease and cardiovascular disease mortality independently of other cardiovascular system risk factors.

- **Cardiomyopathy:** Obesity is directly responsible for alterations in the structure and function of the heart leading to cardiomyopathy, which is multifactorial involving metabolic disturbances (insulin resistance, increased free fatty acid levels, increased levels of adipokines), activities of angiotensin-aldosterone system, sympathetic nervous system, myocardial remodeling and small vessel disease. Increased plasma insulin increases sodium retention and expansion of plasma volume leading to cardiomyopathy. Thickness of left ventricle wall is >10 mm, which should be differentiated from a genetic disorder dilated cardiomyopathy with left ventricle wall thickness <10 mm.
- **Deep vein thrombosis:** Obesity is independent risk factor for development of deep vein thrombosis. Pathogenesis of thromboembolic phenomenon in obese is complex potentially involving a number of hormones, cytokines, growth factors and leptin synthesized by adipose tissue.
- **Coronary artery disease:** Obesity is independent risk factor for development of coronary artery disease. Proinflammatory state is caused by excess of deposition of fat in obese persons. Coronary artery disease occurs due to increased synthesis of **adipokines** such as **adiponectin** associated with inflammation and insulin resistance resulting in atherosclerosis.
- **Sudden cardiac death:** Obese persons have increased risk of development of cardiac arrhythmias and sudden cardiac death even in the absence of obvious cardiac pathology. There is prolonged QT interval in obese persons.

Obesity-related Hepatobiliary System Disorders

Obesity is associated with an increased risk of non-alcoholic fatty liver disease (**NAFLD**) and cholelithiasis.

Nonalcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is characterized by an increase in intrahepatic triglyceride content (steatosis) with or without inflammation and fibrosis (i.e. steatohepatitis). NAFLD is so named because it closely resembles alcoholic fatty liver.

- The hallmark of nonalcoholic fatty liver disease (NAFLD) occurs when the rate of hepatic fatty acid uptake from plasm and *de novo* fatty acid synthesis is greater than the rate of fatty acid oxidation and export as triglyceride within very-low-density lipoproteins. Therefore, there is increased deposition of triglyceride within hepatocytes leading to adverse alterations in glucose, fatty acid and lipoprotein metabolism.
- There is increased risk of nonalcoholic steatohepatitis (NASH) in obese persons, which represents a spectrum of liver injuries that initially display steatosis, with or without hepatitis.
- Steatohepatitis may progress to bridging fibrosis and cirrhosis of the liver, which is consistently associated with metabolic syndrome (central obesity, type 2 diabetes mellitus, dyslipidemia, and microalbuminuria).

Cholelithiasis

Obese person is more to develop gallbladder stones as a result of supersaturation of fat. Obesity, particularly abdominal or centripetal obesity, is a well-established risk factor for gallbladder stone disease, which is associated with an increased activity of the rate-limiting step in cholesterol synthesis, the hepatic enzyme, 3-hydroxyl-3-methyl-glutaryl coenzyme A (**HMG-CoA**) reductase, leading to increased cholesterol synthesis in the liver, its secretion into bile and storage in the gallbladder.

Obesity-related Metabolic Disorders

According to WHO definition, obesity-related metabolic syndrome occurs due to insulin resistance, impaired glucose tolerance or diabetes mellitus together with at least two of the following: (a) hypertension, (b) obesity, (c) high triglycerides and/or low-high-density lipoproteins, and (d) macroglobulinemia.

Obesity-related metabolic syndrome increases risk for cardiovascular disease.

- Increased adipose tissue in the body downregulates insulin receptor synthesis resulting in increased concentration of serum insulin and thus diabetes mellitus. Weight reduction increases insulin receptor synthesis.
- Uncontrolled diabetes mellitus is associated with increased risk for life-threatening complications, i.e. macrovascular diseases (atherosclerosis) such as coronary artery disease, cerebral vascular disease, peripheral vascular disease (gangrene) and microvascular diseases like retinopathy, nephropathy and neuropathy.

Obesity-related Cancers' Risk

Obesity is associated with increased risk for development of several cancers of breast, endometrium, esophagus, gastric, colon, rectum, pancreas, liver, biliary tract, kidney, prostate and skin (melanoma). Insulin resistance, increased insulin level, increased insulin-like growth factor (IGF), increased steroid level as a result of aromatization of androgens to estrogens in adipose tissue, increased peptide level and systemic inflammation as a result of due to adipokine (leptin) and tumor necrosis factor α (TNF-α) participate in the development of malignant tumors in obese persons.

Obesity-related Reproductive System Disorders

Obese men present with gynecomastia and hypogonadism. Obese women are at risk for menstrual irregularities, infertility, gestational diabetes, overdue child birth, induced labor and prolonged labor.

Obesity-related Genitourinary System Disorders

Obese persons are more prone to develop end-stage renal disease as a result of hypertension and diabetes mellitus. End-stage renal disease refers to GFR <5% of normal, which is the terminal stage with uremic manifestations. Glomeruli are markedly sclerosed, tubular atrophy with thyroidization, intestinal fibrosis and vascular thickening.

Obesity-related Musculoskeletal System Disorders

Obese persons are at higher of osteoarthritis, impaired mobility, gout, prolapse lumbar intervertebral discs and chronic low back pain. Osteoarthritis is a slowly progressive destruction of the articular cartilage. Articular cartilage loses its elasticity results to fragmentation, which floats into synovial fluid. Floating cartilage erodes bone, which exhibits polished and ivory-like appearance (eburnation), which is accompanied by new bone formation (osteophytes) in subchondral region and at the margins of the affected joint.

VITAMINS DEFICIENCY

Vitamins are group of organic compounds essential for normal growth and functions. These are required in small quantities in the diet because they cannot be synthesized by the body. Vitamins are categorized into two groups: (a) fat-soluble vitamins include A, D, E and K; and water-soluble vitamins include thiamine (vitamin B_1), riboflavin (vitamin B_2), niacin (vitamin B_3), pantothenic acid (vitamin B_5), pyridoxine (vitamin B_6), biotin, folate/folic acid, cyanocobalamin (vitamin B_{12}) and vitamin C. Major functions of vitamins and deficiency syndrome are given in Table 7.3.

FAT-SOLUBLE VITAMINS

Vitamins A, D, E and K are called the fat-soluble vitamins, because these are soluble in organic solvents and insoluble in water. Fat-soluble vitamins are absorbed and transported in a manner similar to that of lipids.

VITAMIN A

Vitamin A is essential for the maintenance of normal mucous membranes, cell growth, reproduction, enhancement of immune system and for normal vision. Vitamin A is found in foods of animal origin, such as liver, fish, butter, whole milk and egg yolk. However, body converts certain carotenoids derived from plants especially β-carotene to vitamin A.

- Vitamin A occurs in two forms: (a) retinol is an active form of vitamin A found in animal liver, whole milk and some fortified foods; and (b) carotenoids are dark-colored pigments found in plant foods that can turn into active form of vitamin A. There are more than 500 known carotenoids. One such carotenoid is β-carotene that acts as an oxidant.
- β-Carotene is converted to retinol (steroid hormone essential for growth and development). Retinol is converted to retinal (Wald's visual cycle) and retinyl phosphate (glycoprotein synthesis).

Table 7.3 Major functions of vitamins and deficiency syndrome

Nutrient	Functions	Deficiency Syndrome
Fat-soluble vitamins		
Vitamin A	Visual pigment regulating cell growth	Nyctalopia (night blindness), xerophthalmia (conjunctival xerosis), Bitot's spots, keratomalacia, corneal scarring, infertility, delayed growth, metaplasia of columnar epithelium in ducts. Immune deficiency and recurrent infections
Vitamin D	Vitamin D facilitates calcium and phosphate absorption from small intestine, and maintains plasma calcium and phosphate levels	Rickets in children, osteomalacia in adults and hypocalcemic tetany
Vitamin E	Antioxidant maintaining nervous system	Spinocerebellar syndrome
Vitamin K	Acts as cofactor for hepatic carboxylation of prothrombin; VII, IX and X, protein C and protein S	Bleeding diathesis
Water-soluble vitamins		
Thiamine (B_1)	Thiamine pyrophosphate functions as a coenzyme essential for maintaining nervous system	Wet beriberi, dry beriberi and Wernicke-Korsakoff syndrome
Riboflavin (B_2)	Acts as cofactor for enzyme including FMN and FAD	Ariboflavinosis, cheilosis, glossitis, dermatitis, keratitis
Niacin (B_3)	Component of coenzyme NAD and NADP	Pellagra, dementia, diarrhea and dermatitis
Pantothenic acid (B_5)	Component of coenzyme A and acyl carrier proteins	Recognized only under experimental conditions: constitutional and gastrointestinal symptoms, paresthesia, cramps, impaired coordination
Pyridoxine (B_6)	Forms pyridoxal-5-phosphate, a coenzyme in many reactions	Cheilosis, glossitis, dermatitis and peripheral neuropathy
Biotin	Acting as cofactor in several carboxylation reactions	Deficiencies extremely rare. Patient presenting with anorexia, dermatitis, atrophic glossitis, myalgia, ECG changes, hypothermia and mild anemia
Folate (folic acid)	Acting as coenzyme in transfer and utilization of 1-carbon units, essential step in nuclei acid synthesis.	Megaloblastic anemia
Cyanocobalamin (B_{12})	▪ Participates in utilization of folate in nucleic acid synthesis ▪ Essential for maintenance of nervous system	▪ Megaloblastic anemia ▪ Subacute combined degeneration of spinal cord in midthoracic region
Vitamin C (ascorbic acid)	Acting as cofactor in hydroxylation and amination reactions	Scurvy

- Carotenoids are present in yellow and green, leafy vegetables and fruits (carrots, sweet potatoes, pumpkins, mango, spinach), which can turn into active form of vitamin A in the body. Biochemical functions of vitamin A are given in Table 7.4.

Vitamin A and Normal Vision

George Wald described the biochemical function of vitamin A in the process of vision, known as Wald's visual cycle or rhodopsin cycle. Two types of light receptors, rods and cones, exist in the retina of the eye. Rods are present in the periphery and are involved in dim light vision (e.g. cats and owls). **Cones** are present at the center are responsible for bright light vision and color vision in humans.

Wald's Visual Cycle

Retinol is type of retinoid also called vitamin A_1, which is produced from vitamin A. Oxidation of retinol leads to formation of aldehyde, retinal and retinoic acid compounds. Retinyl esters are storage form of vitamin A. Schematic representation of Wald's visual cycle is shown in Fig. 7.5.

- Retinol is a component of the photoprotective visual pigment rhodopsin present on cones, which is sensitive in reduced light. Iodopsins in cones are responsive in bright light. Retinol is transported to retina via circulation, which moves into retinal pigment epithelial cells, where it is esterified to form retinyl esters and then stored.

Table 7.4 Biochemical functions of vitamin A

Vitamin A is essential for maintenance of healthy epithelial tissue and normal vision
Vitamin A maintains proper immune system to fight against infections
Vitamin A is required for synthesis of cholesterol and glucocorticoids
Retinol and retinoic acid function almost like steroid hormones and regulate protein synthesis essential for cell growth and differentiation
Retinol and retinoic acid function in the synthesis of transferring the iron transport protein
Retinyl phosphate derived from retinol participates in synthesis of glycoproteins and mucopolysaccharide compounds, which are essential for cell growth and mucus secretion
Carotenoids (most important β-carotene) possess antioxidant property that reduce synthesis of oxygen-derived free radicals and thus reduce the risk for cancers

- When needed, retinyl esters are hydrolyzed and isomerized to form 11-*cis*-retinol, which shuttles to rod cells of retina, where it binds to protein called opsin to form visual pigment rhodopsin, also known as 'visual purple'. Rhodopsin is a conjugated protein in rods, which contains 11-*cis*-retinal and a protein opsin.
- The primary process in Wald's visual cycle is that on exposure to light, the isomerization of 11-*cis* retinal to 'all-*trans*-retinal' takes place, which results in conformational change in opsin responsible for the generation of electrical signal to optic nerves. The nerve impulse generated by optic nerve is transmitted to the brain, where it is interpreted.
- The all-*trans*-retinal is immediately isomerized by retinal isomerase to 11-*cis*-retinal, which combines with opsin to generate rhodopsin and completes the Wald's visual cycle.

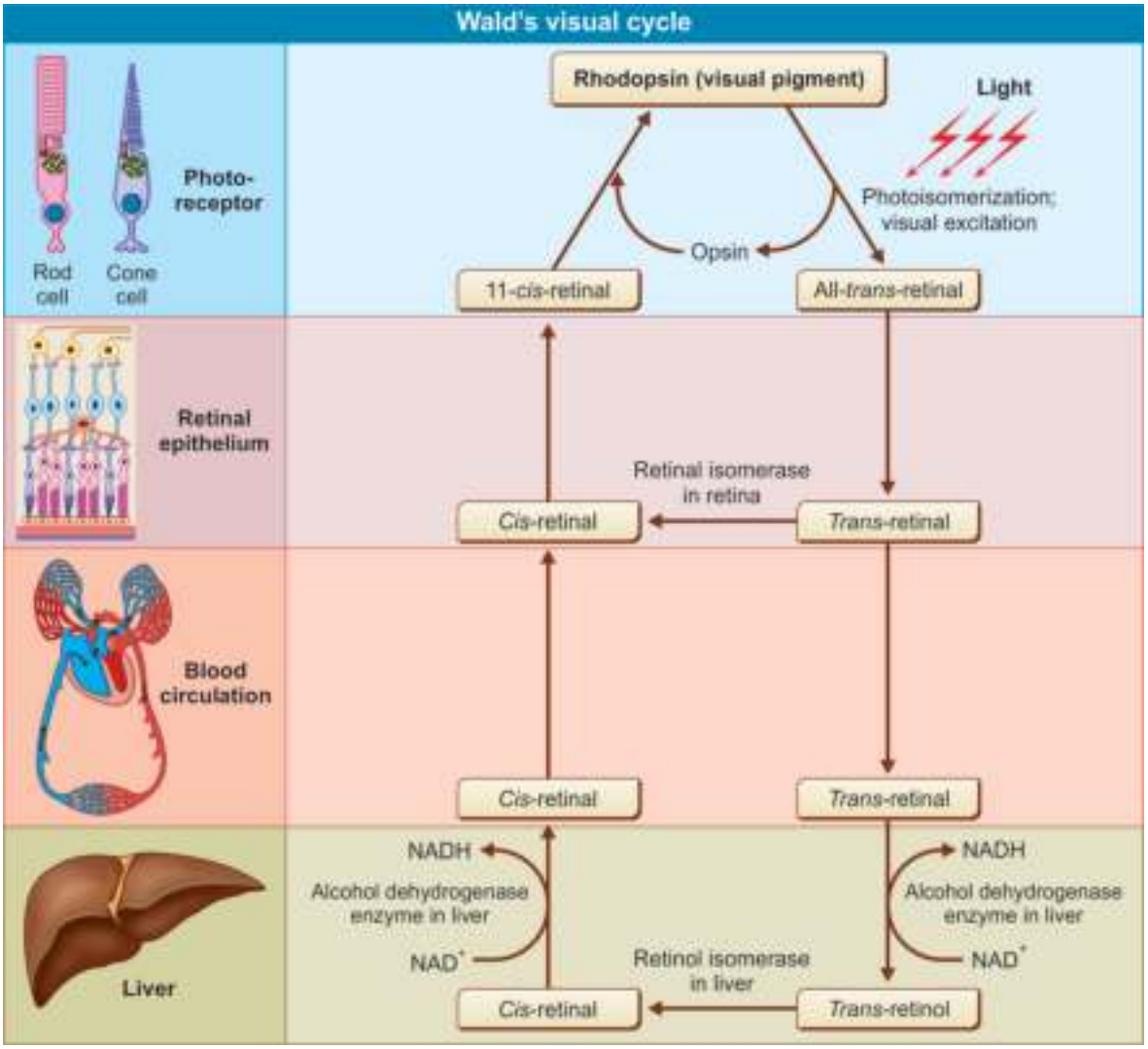


Fig. 7.5: Schematic representation of Wald's visual cycle. Oxidation of retinol leads to formation of aldehyde, retinal and retinoic acid compounds. A derivative, retinol, is a component of the photoprotective visual pigment rhodopsin. This visual pigment rhodopsin present on cones is sensitive in reduced light. Iodopsins in cones are responsive in bright light.

- However, the conversion of all-*trans*-retinal to 11-*cis* retinal is incomplete. Therefore, most of the 'all-*trans*-retinal' is transported to the liver and converted to 'all-*trans*-retinol' by alcohol dehydrogenase enzyme, which undergoes isomerization to '11-*cis*-retinol' which is then oxidized to 'all-*trans*-retinol' to participate in the Wald's visual cycle.

Wald's Visual Cycle and Color Vision

Cones are specialized for bright and color vision. Wald's visual cycle occurs in rods and also in cones. The color vision is governed by color sensitive pigments such as porphyropsin (red), idopsin (green) and cyanopsin (blue). All these pigments are retinal opsin complexes.

- When bright light strikes the retina, one of the color sensitive pigments is bleached depending on the particular color of light. When exposed to light, the color of rhodopsin changes from red to yellow by bleaching process. Bleaching occurs within milliseconds and many unstable intermediate products are formed during this process (Rhodopsin Prelumirhodopsin → Lumirhodopsin → Metarhodopsin I → Metarhodopsin II → All-*trans*-retinal + opsin).
- The color sensitive pigments dissociate to all-*trans*-retinal and opsin, as in case of rhodopsin. The bleaching process transmits nerve impulse to brain as a specific color red when porphyropsin splits, green when iodopsin splits, or blue for cyanopsin. The perception of different color in the brain occurs due to splitting of color sensitive pigments in different proportions (Fig. 7.6).

Clinical Features

Vitamin A deficiency can result from inadequate dietary intake, fat malabsorption or liver disorders.

- Night blindness (nyctalopia) is the earlier clinical symptom of vitamin A deficiency. Patient has difficulty to see in dim light since dark adaptation time is increased.
- Severe deficiency of vitamin A leads to xerophthalmia, which is characterized by dryness of conjunctiva and cornea; Bitot's spots and keratomalacia. Night blindness is prevented in children under five years of age by vitamin A supplementation. Clinical photograph of keratomalacia in vitamin A deficiency is shown in Fig. 7.7.
- Vitamin A deficiency results in growth retardation, keratinization of epithelial cells in gastrointestinal tract, respiratory tract, glandular ducts and urinary tract. Impairment of immune system results in infections in persons with vitamin A deficiency.
- Reproduction system is adversely affected due to vitamin A deficiency. Patients with vitamin A deficiency may develop gonadal dysgenesis. Clinical manifestations of vitamin A are given in Table 7.5.

Hypervitaminosis A

Both acute and chronic types of hypervitaminosis A are responsible for dermatitis, headache, hepatomegaly, weight loss, alopecia, bone changes and joint pains.

- Person with acute hypervitaminosis A may experience one or more of the symptoms such as irritability, drowsiness, nausea, vomiting and abdominal pain.

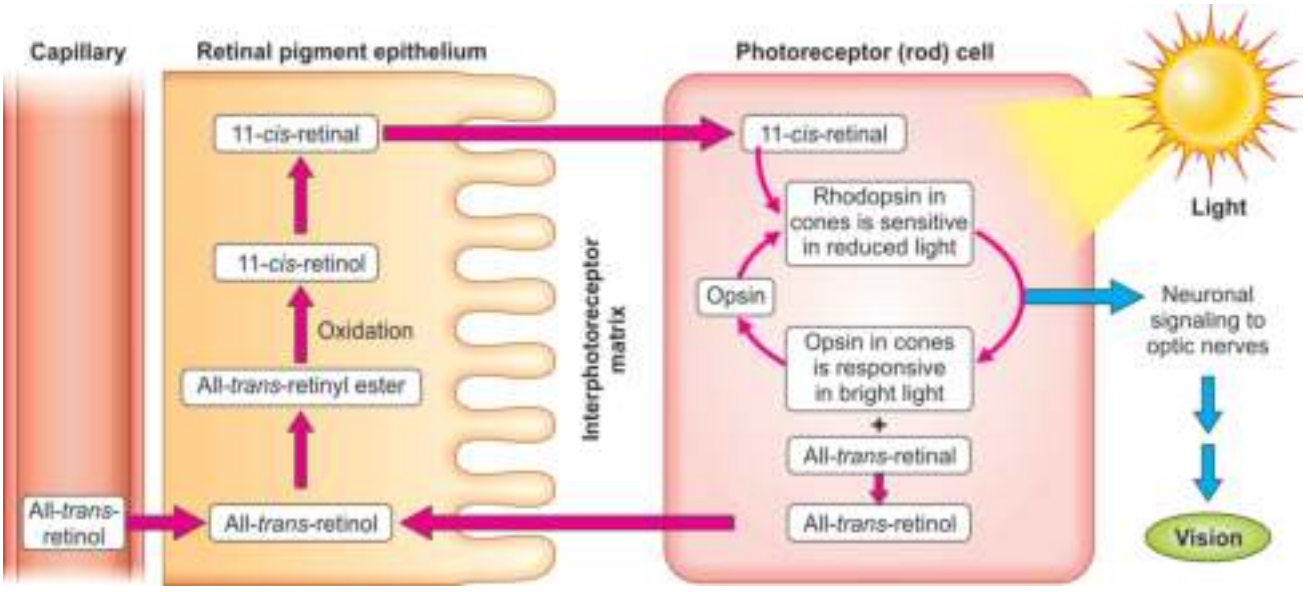


Fig. 7.6: Schematic representation of Wald's visual cycle. Oxidation of retinol leads to formation of aldehyde, retinal and retinoic acid compounds. A derivative, retinol, is a component of the photoprotective visual pigment rhodopsin. This visual pigment rhodopsin present on cones is sensitive in reduced light. Iodopsins in cones are responsive in bright light.

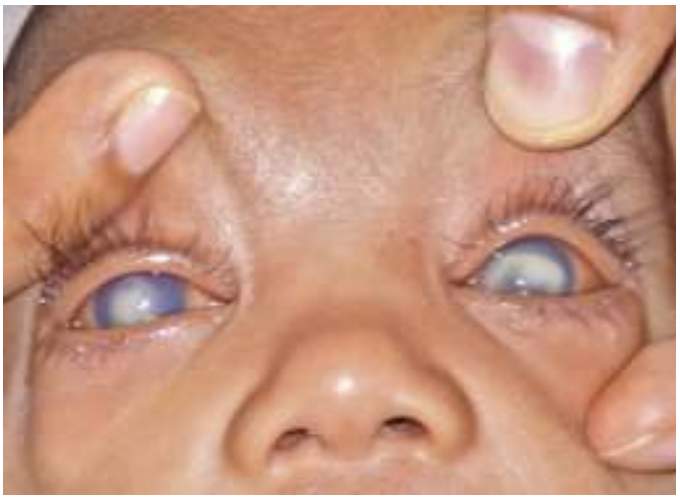


Fig. 7.7: Clinical photograph of keratomalacia in vitamin A deficiency. Vitamin A deficiency causes night blindness, Bitot's spots, corneal ulcer, keratomalacia and squamous metaplasia of epithelium lining bronchi, urinary system and glandular ducts. (*Courtesy:* Dr. JL Goyal, Director Professor, Department of Ophthalmology, Maulana Azad Medical College and Associated Guru Nanak Eye Centre, New Delhi).

- Person with chronic hypervitaminosis A may have symptoms such as mouth ulcers, swelling of the bones, cracked fingernails, anorexia, bone pain, cheilosis, blurred vision, dizziness, nausea, vomiting, sensitivity to light and rough, dry itchy skin and alopecia; jaundice, confusion and respiratory tract infections.

VITAMIN D

Vitamin D (also referred to as calciferol) is a fat-soluble vitamin derived from dietary supplement and endogenously when ultraviolet rays from sun triggering vitamin D. Schematic representation of vitamin D metabolism is shown in Fig. 7.8.

- Dietary vitamin D exist in two forms: (a) vitamin D_2 (ergocalciferol) found in plants such as trout, fortified cereals, broccoli, apple, banana, whole wheat, and sunflower seeds and mushrooms, and (b) vitamin D_3 (cholecalciferol) found in cod liver oil, milk products, tuna fish, salmon fish and egg yolk.
- Vitamins obtained from dietary and sun ultraviolet rays is biologically inert and must undergo two hydroxylations in the activation. Vitamin D which enters blood and reaches liver and kidneys.
  - First hydroxylation occurs in the liver converts vitamin D to 25-hydroxyvitamin D[25(OH)] by D-25 hydroxylase. The 25-hydroxyvitamin D[25(OH)] is also known as calcidiol.
  - Second hydroxylation occurs primarily in the kidney and forms physiologically active 1,25-dihydroxyvitamin D[1,25(OH)2D] by α_1-hydroxylase, also known as calcitriol.

Vitamin D Functions

Vitamin 1, 25$(OH)_2$ D acts on gastrointestinal tract and promotes absorption and maintains normal plasma levels of calcium and phosphorus. Calcium and phosphorus participate in bone mineralization.

- Vitamin D collaborates with parathormone in mobilization of calcium from bone, which facilitates parathormone dependent reabsorption of calcium in the distal renal tubules. Vitamin D is requisite for normal mineralization of cartilage and bone.
- Vitamin D plays other roles in the body, including reduction of inflammation as well as modulation of cellular processes such as cell growth, neuromuscular and immune function including glucose metabolism.
- Serum concentration of 25(OH)D is currently the main indicator of vitamin D status, which has a half-life of 15 days.

Table 7.5 Clinical manifestations of vitamin A deficiency

Organ Involvement	Clinical Manifestations
Ocular changes	▪ Nyctalopia (night blindness) ▪ Xerophthalmia (conjunctival xerosis) ▪ Bitot's spots ▪ Keratomalacia ▪ Corneal ulcer ▪ Xerophthalmic scarring ▪ Xerophthalmic fundus (white spotted fundus due to changes in outer segment of rods)
Growth	Delayed growth
Skin changes	Follicular hyperkeratosis
Bone changes	Failure of bone remodeling
Gonadal changes	Gonadal dysfunction (infertility and troubled conception)
Keratinization of lining epithelium	Metaplasia of columnar epithelium lining gastrointestinal tract, respiratory tract, respiratory tracts and ducts
Immune system	Immune deficiency and recurrent infections
Tissue healing	Poor wound healing

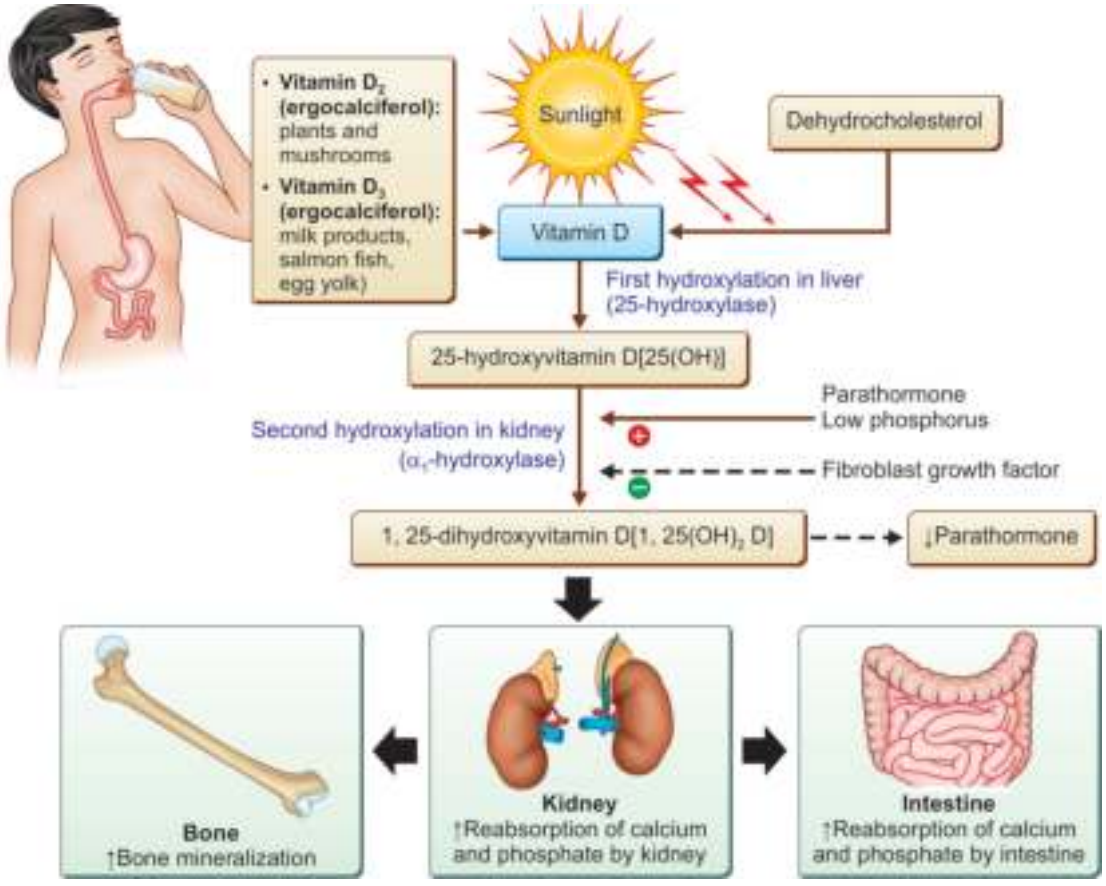


Fig. 7.8: Schematic representation of vitamin D metabolism. Vitamin D is synthesized from 7-dehydrocholesterol in the skin. Vitamin D is also derived from dietary source (vitamins D_2 and D_3). Vitamin D is metabolized by hydroxylation in the liver and kidney. The first hydroxylation, which occurs in the **liver** converts vitamin D to 25-hydroxyvitamin D[25(OH)] by vitamin D 25-hydroxylase. The 25-hydroxyvitamin D[25(OH)] is also known as '**calcidiol**. The second hydroxylation occurs primarily in the kidney and forms physiologically active 1,25-dihydroxyvitamin D[1,25$(OH)_2$D] by α_1-hydroxylase, also known as calcitriol.

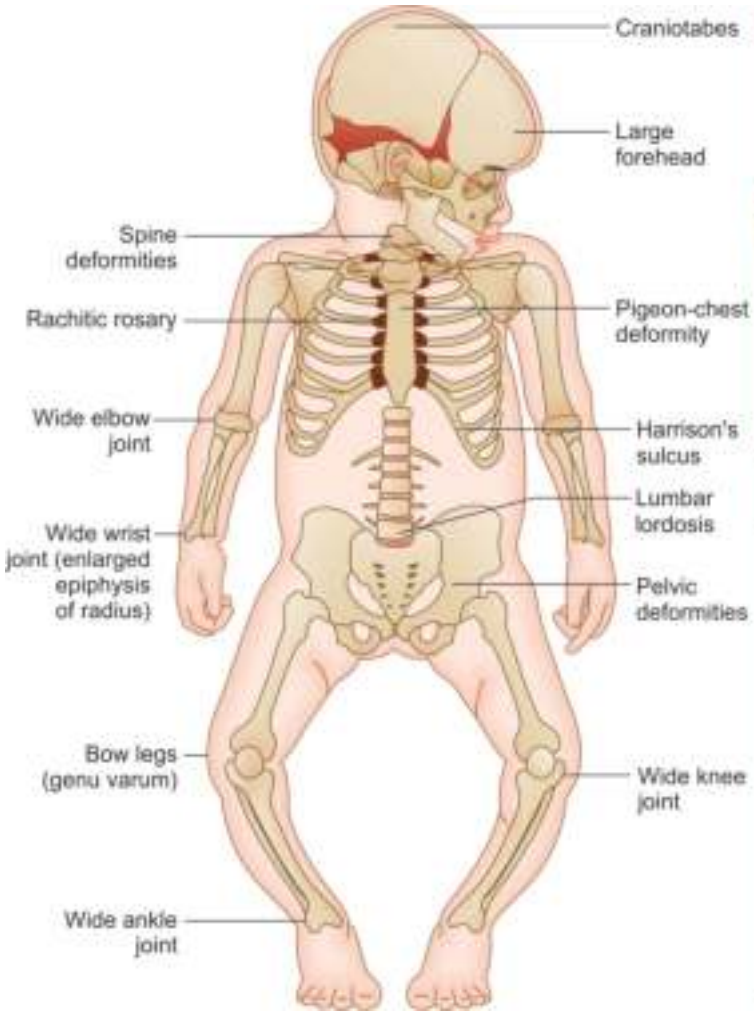
Vitamin D Deficiency-related Disorders

Persons can develop vitamin D deficiency when usual dietary intakes are lower over time that recommended, or exposure to sunlight is limited, the kidneys cannot convert 25(OH)D to its active form, or inadequate vitamin D absorption in gastrointestinal tract. Dietary vitamin D deficiency is more common in vegetarians and persons with milk allergy and glucose intolerance. Vitamin D deficiency leads to rickets in children, osteomalacia in adults and hypocalcemic tetany.

Rickets

Prolonged exclusive breastfeeding without vitamin D supplementation can cause rickets in infants. In children, vitamin D deficiency results in rickets, which is characterized by a failure of bone tissue to become properly mineralized, resulting in soft bones and skeletal abnormalities. Schematic representation of rickets is shown in Fig. 7.9.

- During infancy, head and chest sustain the greatest stresses. Child develops rickets and presents with craniotabes, frontal bossing, rachitic rosary, Harrison's groove, pigeon-shaped chest, deformities of pelvic bones and spine as a result of inadequate calcification of cartilage and replacement of cartilage by osteoid matrix on inadequately mineralized cartilaginous remnants. It leads to projection of distorted, irregular masses of cartilage into marrow cavity and enlargement and lateral expansion of the osteochondral junction.
- Radiograph of the wrists in rickets demonstrates irregular fraying and capping of metaphysis of the distal ends of radius and ulna with widening of growth plate. Further, there is evidence of a transverse lucency in the bilateral distal end of radius and ulna with irregular sclerotic margins suggestive pseudofractures (looser zones). All ossification centers expected for age have not appeared with looser zones. Radiograph of wrists in rickets is shown in Fig. 7.10.
- Fortification of milk and other staples, such as breakfast cereals and margarine, with vitamin D along with cod liver oil help in prevention of rickets.



Vitamin D sources

- Vitamin D synthesis in skin on exposure to sunlight
- Vitamin D_2 (ergocalciferol found in plants and mushrooms)
- Vitamin D_3 (ergocalciferol found in milk products, cod liver oil, salmon fish, tuna fish, egg yolk)

Vitamin D functions

- Regulation of calcium and phosphorus homeostasis
- Regulation of growth and bone mineralization
- Regulation of immune system
- Regulation of insulin secretion
- Regulation of cell proliferation and differentiation
- Regulation of calcium transport in muscles

Vitamin D deficiency: Clinical features of rickets in children

- **Craniotabes:** Thinning and softening of occipital and parietal bones occurs and late closure of fontanelles.
- **Frontal bossing:** Frontal bone is squared due to increased formation of osteoid tissue.
- **Rachitic rosary:** Thickening of costochondral junction occurs due to overgrowth of cartilage and osteoid tissue results in a string-of-beads-like appearance known as rachitic rosary.
- **Harrison's sulcus:** Depression along the line of insertion of the diaphragm into the rib cage occurs due to inward pull of diaphragm.
- **Pigeon-shaped chest:** Anterior protrusion of sternum occurs due to inward pull of metaphyseal areas of ribs by respiratory muscles
- **Pelvis bones:** Deformity of pelvic bones occurs
- **Spine deformity:** Lumbar lordosis occurs in ambulatory child.

Fig. 7.9: Schematic representation of rickets. Rickets child shows craniotabes, frontal bossing, rachitic rosary, Harrison's groove, pigeon-shaped chest, deformities of pelvic bones and spine as a result of inadequate calcification of cartilage and replacement of cartilage by osteoid matrix on inadequately mineralized cartilaginous remnants.

Clinical Pearls: Clinical Features of Rickets in Children

- **Craniotabes:** Thinning and softening of occipital and parietal bones occurs and late closure of fontanelles.
- **Frontal bossing:** Frontal bone is squared due to increased formation of osteoid tissue.
- **Rachitic rosary:** Thickening of costochondral junction occurs due to overgrowth of cartilage and osteoid tissue results in a string-of-beads-like appearance known as rachitic rosary.
- **Harrison's sulcus:** Depression along the line of insertion of the diaphragm into the rib cage occurs due to inward pull of diaphragm.
- **Pigeon-shaped chest:** Anterior protrusion of sternum occurs due to inward pull of metaphyseal areas of ribs by respiratory muscles.
- **Pelvis bones:** Deformity of pelvic bones occurs.
- **Spine deformity:** Lumbar lordosis occurs in ambulatory child.

Osteomalacia

In adults and adolescents, vitamin D deficiency can lead to osteomalacia, which means loss of skeletal mass or too little bone (osteopenia) leading to softening of bones.

- Osteomalacia is characterized by inadequate mineralization of osteoid matrix, excess of unmineralized osteoid resulting in weak bones vulnerable to fractures (vertebrae, hips, wrists and ribs) in 25% cases.
- Patient may develop kyphoscoliosis, apparent loss of bone density and cortical thickness, visualized on radiographs.

VITAMIN E

Vitamin E is a fat-soluble vitamin with several forms (tocopherol α, β, γ and δ). α-Tocopherol is the active form of vitamin E used by human body. Vitamin E is found in plant-based oils, nuts, seeds, fruits and vegetables.

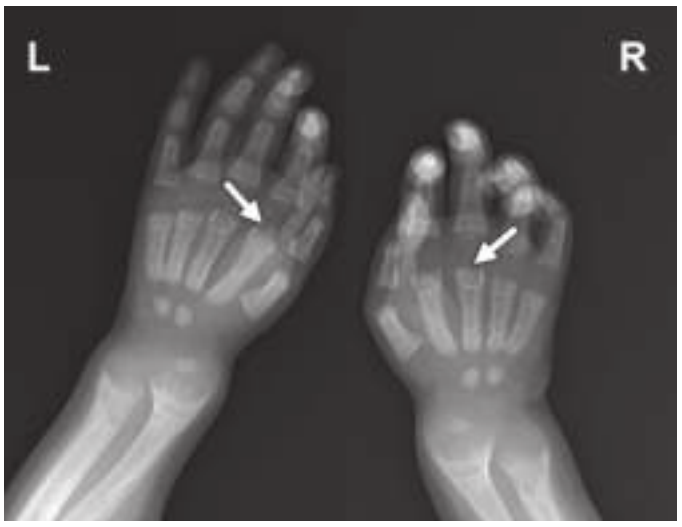


Fig. 7.10: Radiograph of wrists in rickets. Radiograph demonstrates irregular fraying and capping of metaphysis of the distal ends of radius and ulna with widening of growth plate. These findings are suggestive of rickets. Further, there is evidence of a transverse lucency in the bilateral distal end of radius and ulna with irregular sclerotic margins suggestive pseudofractures (looser zones). All ossification centers expected for age have not appeared with looser zones. These findings are suggestive of rickets with looser zones. (*Courtesy*: Dr. Vigyat, Dr. DY Patil Medical College, Pune.)

Vitamin E sources include wheat germ oil, sunflower oil, safflower oil, soybean oil, almonds, peanuts, peanut butter, beat greens, spinach, pumpkin, red bell pepper, mango, asparagus and avocado.

Vitamin E Functions

Vitamin E main function is to act as an antioxidant, scavenging loose electrons—so-called 'oxygen-derived free radicals'—that can cause damage to cells.

- Vitamin E intercepts oxygen-derived free radicals and prevents destruction of cell membrane by modulation of lipid peroxidation in certain situation. Vitamin E participates in maintenance of nervous system.
- Vitamin E protects the lipid in the LDL molecule from oxidation and thus prevents atheromatous plaque formation in large elastic and medium-sized arteries. Vitamin E inhibits platelets aggregation, but enhances vasodilatation.
- Vitamin E inhibits the activity of protein kinase C, which is involved in the function of other proteins through phosphorylation of hydroxyl groups of serine and threonine amino acid residues on proteins.

Clinical Features

Because vitamin E is found in a variety of foods and supplements. Deficiency of vitamin E occurs as a result of gastrointestinal disorders such as pancreatitis, cystic fibrosis and celiac disease.

- Patient with vitamin E deficiency develops retinopathy, peripheral neuropathy, spinocerebellar syndrome and impaired immune system.
  - Spinocerebellar syndrome is characterized by neurological symptoms (impaired coordination and muscle weakness due to degenerations of axons in posterior columns). There is loss in sensory nuclei of trigeminal, auditory and vagus nerves.
  - There is increased risk of cardiovascular diseases in adults, and hemolytic anemia (premature destruction of red blood cells) in children.
  - Vitamin E is administered to prevent of cardiovascular disease, diabetes mellitus, cancer, and dementia, and to boost immunity.

Vitamin E Hypervitaminosis

Hypervitaminosis E manifests primarily as an anticoagulant state by inhibiting vitamin K-derived coagulation factors (2, 7, 9 and 10), that may cause impaired blood clotting leading to increased risk for bleeding in some persons. This is due to its antagonism of vitamin K resulting in prolonged prothrombin time and therefore, increased international normalized ratio (INR). Prothrombin time–INR is used to monitor persons who are treated with anticoagulant therapy. It is recommended that vitamin E supplements to be stopped one month before elective surgery.

VITAMIN K

Vitamin K is one of the fat-soluble vitamins, that is divided into two main groups: vitamin K_1 (phylloquinone derived from plants) and vitamin K_2 (menaquinone derived from animal-based and fermented foods).

- Vitamin K is found in green leafy vegetables (spinach, broccoli, lettuce, parsley), vegetable oils, almonds, Avocado cereal grains. Small amounts can also be found in meat and dairy products. Bacteria in large intestine produce vitamin K_2 and 40–50% of human body. Vitamin K is absorbed in the small intestine and stored in adipose tissue and the liver.
- Vitamin K is essential for normal blood clotting and bone homeostasis. Vitamin K is a cofactor (coenzyme) for hepatic glutamyl carboxylation required for the synthesis of γ-carboxyglutamyl residues of active serine proteases (e.g. clotting factors II, VII, IX, and X); protein C and protein S.

Clinical Features

Vitamin K deficiency occurs due to cystic fibrosis, celiac disease, common bile duct obstruction and diabetes mellitus, that can cause bleeding diathesis. It is uncommon in adults, except in those with severe liver disease and on oral anticoagulants. Deficiency of vitamin K exclusively occurs in breastfed and premature

babies, because human milk is low in vitamin K, and their gut is not yet colonized with bacteria. Hemorrhagic disease of the newborn is a serious threat to life. Routine vitamin K administration is administered prophylactically.

WATER-SOLUBLE VITAMINS

The water-soluble vitamins include ascorbic acid (vitamin C), thiamine (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin, folate/folic acid, and cyanocobalamin (B_{12}).

- B complex vitamins (except vitamin B_{12}) are present in whole grain cereals, green leafy vegetables, fish, meat, and dairy foods. Vitamin B_{12} is derived from foods of animal origin only. Folic acid is derived from leafy vegetables, cereals, fruits, and a number of animal products. Vitamin C is derived from citrus fruits, tomatoes, vegetables, milk and meat. Biotin and pantothenic acid deficiencies are extremely rare, which are found in numerous foods and also are synthesized by intestinal bacteria. Biotin deficiency may occur with prolonged antibiotic therapy and ingestion of raw eggs.
- Water-soluble vitamins participate in the release and storage of energy especially in tissues with active metabolism.
- Water-soluble vitamins are not stored in the body except for vitamin B_{12}. That is the reason that regular intake of water-soluble vitamins is essential. Vitamin B_{12} is stored in the liver in large quantities.
- Toxicity from excessive intake is of vitamin B-complex rare, because excess of these vitamins is excreted in the urine. In general, deficiencies of vitamin B complex are often marked by glossitis, dermatitis, and diarrhea.

VITAMIN C (ASCORBIC ACID)

Ascorbic acid, also known as vitamin C, occurs naturally in foods such as citrus fruit, tomatoes, potatoes and leafy vegetables. It is not synthesized endogenously. It is stored in the body (normal range 1.5–4.0 g). Vitamin C is essential for the growth and development and repair of the body tissues.

Vitamin C Functions

Vitamin C plays important role in the synthesis of collagen and reticulin fibers from mucopolysaccharide ground substance in wound healing, absorption of iron needed for red blood cell production, the proper functioning of the immune system, and the maintenance of cartilages, bones and teeth.

- Vitamin C participates in the synthesis of collagen and reticulin fibrils from mucopolysaccharide ground substance in wound healing.
- Vitamin C is required for hydroxylation of proline and lysine, essential for collagen synthesis in coats of blood vessels. It is required for hydroxylation of dopamine in synthesis of norepinephrine.
- Vitamin C enhances maintenance of reduced state of other metabolic products such as iron and FH4 (activated tetrahydrofolate).
- L-ascorbic acid is converted to dehydroascorbic acid (DHA) that participates in oxidation and reduction reactions. Vitamin C accumulates in mitochondria where oxygen-derived free radicals are produced by entering dehydroascorbic acid (oxidized form) through glucose transporters, GLUT-10.
- Ascorbic acid protects the mitochondrial genome and membrane. Vitamin C has antioxidant property by scavenging oxygen-derived free radicals. Vitamin C acts indirectly by regenerating the antioxidant form of vitamin E.

Clinical Features

The most common causes of ascorbic acid (vitamin C) deficiency are poor diet, anorexia, alcoholism, tobacco smoking and hemodialysis/peritoneal dialysis.

- Ascorbic acid deficiency results in scurvy. While symptoms of severe vitamin C deficiency can take months to develop scurvy.
- Scurvy is characterized by defective formation of collagen fibers and osteoid matrix. Patient presents with bleeding gums, subperiosteal hemorrhage; and perifollicular petechial hemorrhages, defective wound healing; hemorrhagic phenomena. Hemorrhages and healing defects occur in both children and adults. Schematic representation of manifestations in scurvy is shown in Fig. 7.11.

Bleeding (Hemorrhages)

Defective connective tissue also leads to fragile capillaries, resulting in abnormal bleeding. Infants and children present with hemorrhages in mucocutaneous and skeletal muscles along fascial planes at mechanical stress points due to loosened vascular endothelial cells from capillaries especially in nail beds, subperiosteal hematomas, bleeding into joint spaces, retrobulbar region and subarachnoid, and intracerebral regions. In adults, ulceration and hemorrhage in gums occur due to loosening of teeth.

Skeletal Manifestations

Bone changes in scurvy are secondary to defective osteoid matrix formation. Insufficient production of osteoid matrix results in cartilaginous overgrowth, widening of epiphysis, bowing of the long bones and

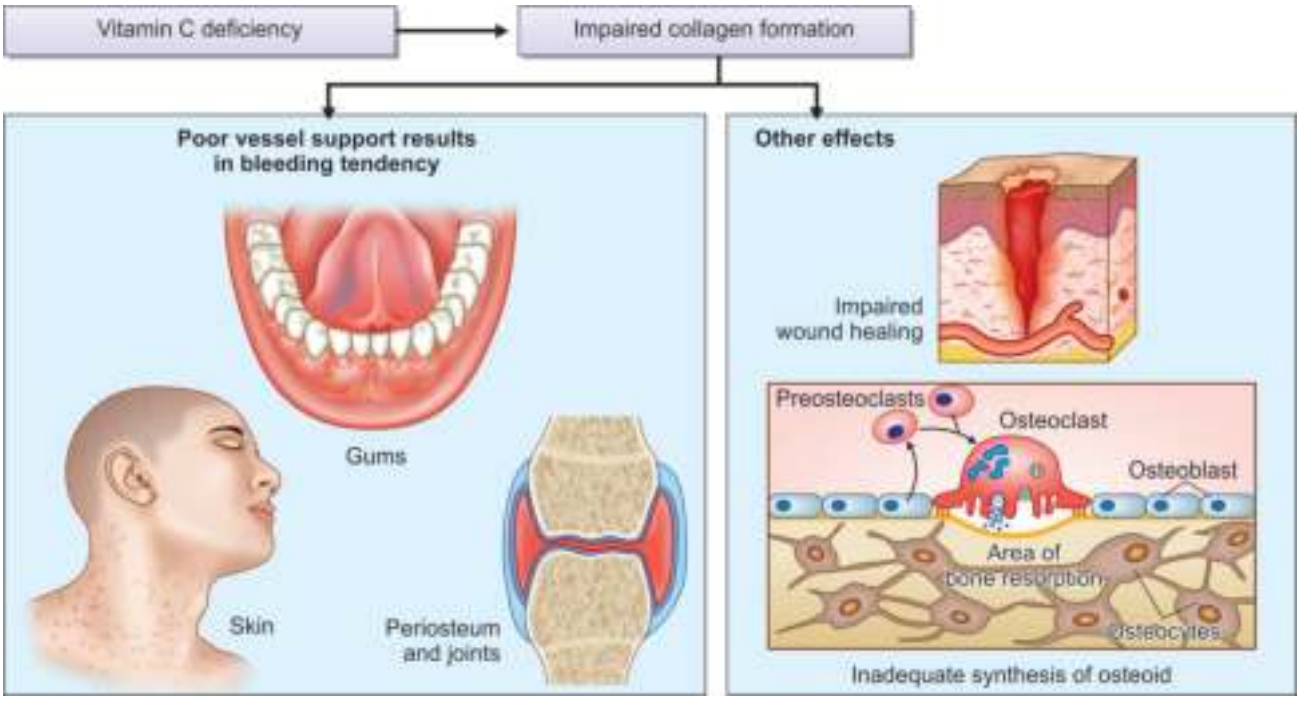


Fig. 7.11: Schematic representation of manifestations in scurvy. Vitamin C deficiency causes impaired synthesis of collagen fibers leading to bleeding gums, hemorrhages in mucocutaneous, muscles along fascial planes, retrobulbar, subarachnoid, and intracerebral regions and bone changes.

chest deformity. Osteoporosis is seen especially at the metaphyseal ends of bone.

Other Manifestations

Patient presents with gingival swelling and periodontal infection, impaired wound healing, impaired localization of focal infections and anemia.

VITAMIN B-COMPLEX

Vitamin B-complex includes thiamine (vitamin B_1), riboflavin (vitamin B_2), niacin (vitamin B_3), pantothenic acid (vitamin B_5), pyridoxine (vitamin B_6), biotin, folate (folic acid) and cyanocobalamin (vitamin B_{12}).

Thiamine (Vitamin B_1)

Thiamine is a water-soluble vitamin, also known as vitamin B_1. It is found in many foods, including cereals, whole grains, beans, nuts, yeast, pea and meat, which enables the body to use carbohydrates as energy. Dietary requirement of thiamine is proportional to the caloric intake of the diet and ranges from 1.0 to 1.5 mg/day for normal adults.

Thiamine Functions

Thiamine is essential for glucose metabolism, which plays a key role in nerve, skeletal muscle and cardiac function.

- Thiamine is rapidly converted into thiamine pyrophosphate (active form) in the brain and liver by thiamine diphosphotransferase enzyme.
- Thiamine pyrophosphate is essential as cofactor for the reactions of the pentose phosphate pathway for maintaining nervous system.

Thiamine Deficiency

Thiamine deficiency is most often associated with severe malnutrition and alcoholism. Other causes of thiamine deficiency include increased demand in disorders (malaria and AIDS) and excessive loss of thiamine (hemodialysis and diuretics). Anti-thiamine factors in diet such as tea and coffee retard thiamine absorption. Thiaminases are found in raw fish, raw shellfish and silkworms.

Clinical Features

Thiamine is used for treatment of congestive heart failure and Alzheimer's disease as well as in cancer prevention. Deficiency of thiamine causes dry beriberi, wet beriberi and Wernicke-Korsakoff syndrome.

- **Dry beriberi:** Patient presents with peripheral neuropathy with resultant atrophy of the skeletal muscles of the extremities. Peripheral neuropathy occurs due to fragmentation of myelin sheath, axons of extremities. There is also involvement of vagus nerve.
- **Wet beriberi:** It is characterized by congestive heart failure associated with dilated cardiomyopathy. Congestive heart failure occurs due to peripheral dilation of arterioles and capillaries.
- **Wernicke-Korsakoff syndrome:** It is characterized by impairment of symmetric motor and sensory reflexes,

involvement of extraocular muscles (ophthalmoplegia), confusion, ataxia and marked memory loss. Degenerative changes occur in the brainstem and diencephalon, with hemorrhagic lesions of cortical and bilateral paramedian masses of grey matter and the mammillary bodies.

Riboflavin (Vitamin B_2)

Riboflavin is water-soluble vitamin derived from milk, meat, eggs, nuts, enriched flour, green vegetables, which works with other B complex vitamins. Riboflavin acts as a cofactor in many cellular processes essential for normal growth and functions and also aids in the release of energy from metabolism of carbohydrates, proteins and lipids.

Riboflavin Functions

- In biochemistry, riboflavin synthesizes flavin adenine dinucleotide (**FAD**) and two molecules of ATP.
- Riboflavin is phosphorylated by ATP to give riboflavin 5′-phosphate (also called flavin mononucleotide—**FMN**).
- Flavin adenine dinucleotide acts as redox-active coenzyme associated with various proteins.
- Both FAD and FMN are essential for various cellular enzymatic oxidation-reduction reactions in metabolism.

Clinical Features

Deficiency of riboflavin occurs in alcoholic persons and newborn especially born to vegetarian mother. The signs and symptoms of riboflavin deficiency include glossitis, angular stomatitis, cheilosis (skin fissures, crust at angle of mouth), seborrheic dermatitis (nasolabial folds, scrotum, or vulva), keratitis (vascularization of cornea, photophobia), hair loss, reproductive system problems and pure red cell aplasia (PRCA).

Niacin (Vitamin B_3)

Niacin is one of the water-soluble vitamins. Niacin is a generic name for nicotinic acid (pyridine-3-carboxylic acid), nicotinamide (pyridine-3-carboxamide) and related derivative such as nicotinamide riboside.

- Niacin is naturally found in many foods such as yeast, rice, fortified cereal grains, green vegetables, beans, liver meat, eggs, chicken, tuna fish, salmon fish anchovies and beef.
- Niacin (nicotinic acid) can be synthesized from the essential amino acid tryptophan with the help of pyridoxine, which is a component of the nicotinamide adenine dinucleotides (NAD and NADP). Niacin is essential to glycolysis, the citric acid cycle, and other oxidative metabolic processes.
- Niacin deficiency occurs in alcoholic persons, Hartnup disease, malignant carcinoid syndrome and isoniazid therapy in tuberculosis.
- Excessive administration of niacin lowers plasma cholesterol levels, and elevates blood glucose and blood uric acid levels. So, it is not recommended in patients with diabetes mellitus and gout.

Clinical Features

Severe niacin deficiency results in pellagra and cause symptoms related to the skin, gastrointestinal tract and nervous system.

- Pellagra is characterized by the 'three **Ds**': dementia, dermatitis (keratotic scaly pigmented rashes on skin exposed to sunlight such as face, neck, dorsum of hands and feet), bright red tongue, and diarrhea.
- There is presence of red ulcerated oral mucosa, thickening of colon wall with atrophy of crypts and demyelination of posterior and lateral columns of spinal cord as well as cerebrum.

Pantothenic Acid (Vitamin B_5)

Pantothenic acid is one of the water-soluble vitamins, which is naturally present in some foods such as seafoods, organ meats, eggs, milk products, mushrooms, avocados, potatoes and broccoli, whole grains such as wheat, rice and oats, peanuts and sunflower seeds.

- The main function of pantothenic acid is in the synthesis of coenzyme A and acyl carrier protein.
- Pantothenic acid helps the body to break food and utilize its metabolic products for cell growth.

Clinical Features

Patient presents with fatigue, headache, irritability, insomnia, nausea, vomiting, numbness in hands or feet and muscle cramps.

Pyridoxine (Vitamin B_6)

Pyridoxine is one of the water-soluble vitamins, which forms pyridoxal-5-phosphate, which acts an essential cofactor for many series of reactions involved in amino acid and protein metabolism such as transamination, deamination, decarboxylation, phosphorylation, racemization and cleavage of cystathionine to cysteine in the methionine pathway. Pyridoxine also converts glutamic acid to γ-aminobutyric acid.

Pyridoxine Sources

Pyridoxine is naturally present in peanuts, soybeans, wheat grains, oat, banana, chicken, some fish and pork. Daily requirement of pyridoxine is 0.5–2.0 mg.

Pyridoxine Deficiency

Pyridoxine deficiency can occur in various disorders such as malnutrition, chronic alcoholism, sickle cell disease, celiac disease, rheumatoid arthritis, hepatitis, extrahepatic biliary obstruction and hepatocellular carcinoma. Pyridoxine deficiency can occur due to therapeutic administration of isoniazid and phenylamine. Isoniazid reacts as competitive inhibitor for pyridoxine binding sites.

Clinical Features

Patient presents with glossitis, cheilosis, dermatitis, pyridoxine-responsive microcytic anemia due to reduced heme synthesis, neonatal seizures, peripheral neuropathy and homocystinuria. Neonatal convulsions occur due to decreased activity of pyridoxal-dependent glutamate decarboxylase, which leads to deficient production of γ-aminobutyric acid (**GABA**), a neurotransmitter. Clinical manifestations are similar to those of vitamin B_2 (riboflavin) deficiency.

Biotin

Biotin is a water-soluble vitamin, which acts as a cofactor in several carboxylation reactions. Biotin is naturally present in whole grain bread, mushrooms, cauliflower, bananas, raspberries, avocados, eggs, sardines stored fish and salmon fish.

- In general, healthy diet provides sufficient amounts of biotin. Biotin deficiency is extremely rare. Biotin is used for hair loss and brittle nails.
- Patient with biotin deficiency may develop lassitude, anorexia, dermatitis, atrophic glossitis, myalgia, ECG changes, hypothermia and mild anemia.

Folate (Folic Acid)

Folate is one of the water-soluble vitamins. Folic acid is the synthetic form of folate. Folate is obtained from yeast, leafy vegetables and animal liver. Animals cannot synthesize folate; thus, it must be obtained from diet.

- Folate acts as a coenzyme in transfer and utilization of 1-carbon unit, an essential step in nucleic acid synthesis and cell division, which is also important for prenatal health. Folate deficiency during pregnancy can lead to neural tube defects such as spina bifida and anencephaly in the newborns. Folate is used for treatment of chronic hemolytic anemia.
- Deficiency of folate occurs due to inadequate intake, intestinal malabsorption, alcoholism, increased demand during pregnancy, hemolytic anemia, and chemotherapeutic agents containing folic acid antagonists.
- Megaloblastic anemia occurs due to deficiency of vitamin B_{12} or folate resulting in defective DNA synthesis, which is characterized by macrocytic anemia, leukopenia, hypersegmented neutrophils and thrombocytopenia in peripheral blood and megaloblastic erythropoiesis.

Clinical Features

Folate deficiency causes megaloblastic anemia that can cause a wide range of symptoms, which usually develop gradually, but can worsen if the condition goes untreated.

- Patient presents with fatigue, lethargy, breathlessness, headache and palpitations. Folic acid deficiency does not cause neurologic changes (in contrast to vitamin B_{12} deficiency). Folate deficiency causes megaloblastic anemia and neural tube defects *in utero*.
- Vitamin B_{12} and folate nutritional aspects are given in Table 7.6. Major functions of vitamin B_{12} and folate nutritional aspect are given in Table 7.7. Comparison between vitamin B_{12} and folate deficiency inducing megaloblastic anemia is given in Table 7.8.

Cyanocobalamin (Vitamin B_{12})

Cyanocobalamin (vitamin B_{12}) is an essential water-soluble vitamin derived from animal source and absorbed by small intestine by two-step processes. First hydrochloric acid in the stomach separates vitamin B_{12} from the protein that is attached to it. Second, the freed vitamin B_{12} then combines with a protein synthesized by the gastric parietal cells, called intrinsic factor, and the body absorbs them together.

Vitamin B_{12} Functions

Vitamin B_{12} acts as cofactor for enzymes required for the catabolism of fatty acids, and the conversion of homocysteine to methionine. High homocysteine in blood is a risk for ischemic heart disease and cerebral stroke. Vitamin B_{12} plays a key role in utilization of folate in nucleic acid synthesis, which is essential for maintenance of nervous system.

Vitamin B_{12} Deficiency

Vitamin B_{12} deficiency is common in strict vegetarians, pernicious anemia, Crohn's disease, blind loop syndrome, *Diphyllobothrium latum* (giant fish tapeworm) infestation and prolonged antibiotic treatment.

Clinical Features

Vitamin B_{12} deficiency causes megaloblastic anemia and demyelination of peripheral nerves leading to sensorimotor disturbances. Subacute combined degeneration of spinal cord occurs in midthoracic region.

- **Physiologic state:** Normally, methylcobalamin converts homocysteine to methionine, which acts as a donor in the synthesis of choline containing

Table 7.6 Vitamin B_{12} and folate nutritional aspects

Parameters	Vitamin B_{12} (Cobalamin)	Folate
Normal daily dietary intake	7–30 μg	200–250 μg
Minimal adult daily requirement	1–2 μg	100–150 μg
Main dietary foods	Animal (dairy) products	Liver, green raw leafy vegetables, yeast, fresh fruits, whole grain cereals
Cooking effect	Little effect	Easily destroyed
Absorption site	Ileum	Duodenum and jejunum
Mechanism of absorption	Vitamin B_{12} combines with intrinsic factor synthesized by gastric parietal cells	Folate converted to methyltetrahydrofolate
Limit of absorption	2–3 μg	50–80% of dietary content
Enterohepatic circulation	5–10 μg	80 μg
Transport in plasm	Most bound to haptocorrin (transcobalamin 1) essential for cell uptake	Folate weakly bound to albumin
Major intracellular physiological forms	Methyl carbylamine, deoxyadenosylcobalamin	Reduced polyglutamate derivatives
Storage sites	Liver (main site), kidney, heart and brain	Liver acts as a coenzyme in transfer and utilization of 1-carbon unit, an essential step in synthesis of nucleic acid
Functions	Vitamin B_{12} participates in utilization of folate in synthesis of nucleic acid; and essential for maintenance of nervous system	Folate acts as a coenzyme involving 1-carbon unit transfer resulting in the synthesis of nucleic acid
Usual therapeutic form	Hydroxocobalamin	Folic (pteroylglutamic) acid
Serum assays	Serum vitamin B_{12} (160–925 ng/L)	Serum folate (3–15 μg/L)

Table 7.7 Major functions of vitamin B_{12} and folate nutritional aspect

Vitamin B_{12} (Cyanocobalamin)
Vitamin B_{12} participates in utilization of folate in synthesis of nucleic acid and maintenance of nervous system. Its deficiency causes megaloblastic anemia and subacute combined degeneration of spinal cord in midthoracic region
Metformin blocks vitamin B_{12}. Hence, reticulocyte count is low in vitamin B_{12} deficiency
Vitamin B_{12} deficiency raises LDH and indirect bilirubin by destroying red blood cells early, as they come out of the bone marrow. This phenomenon is called 'ineffective erythropoiesis', that is why the reticulocyte count is low
After vitamin B_{12}, reticulocyte count improves first and neurological abnormalities improve last
Folate (Folic Acid)
Folate acts as a coenzyme in transfer and utilization of 1-carbon unit, an essential step in the synthesis of nucleic acid
Folic acid deficiency causes megaloblastic anemia without central nervous system involvement

phospholipids an important component of myelin. Normally, deoxyadenosylcobalamin converts methylmalonyl-CoA into succinyl-CoA. Deficiency of methylcobalamin results in decreased synthesis of choline required for myelin synthesis.

- **Pathologic state:** Vitamin B_{12} deficiency results in accumulation of methylmalonyl-CoA, which is converted into methylmalonate and propionate, which leads to increased production of abnormal fatty acids, which incorporate into neuronal lipids. Myelin sheath breakdown occurs. Posterior and lateral white columns of mid-thoracic spinal cord involved. Patient develops total paralysis of lower legs. Subacute combined degeneration of spinal cord occurs in mid-thoracic and cervical region.
  - **Morphology of spinal cord:** Spinal cord shows pale areas of demyelination in the posterior columns and the lateral corticospinal tracts. Spinal cord shows swollen myelin sheath with disintegration and axonal degeneration.
  - **Clinical features:** Patient develops spastic paraparesis, sensory ataxia and marked paresthesia leading to total paralysis of trunk and lower limbs. Associated lesions include optic atrophy and axonal peripheral neuropathy.

Table 7.8 Comparison between vitamin B_{12} and folate deficiency inducing megaloblastic anemia

Parameters	Vitamin B_{12} Deficiency	Folate Deficiency
Etiology	▪ Decreased dietary intake in vegetarians, impaired absorption, increased requirement and impaired utilization of vitamin B_{12} ▪ Failure to synthesize intrinsic factor by gastric parietal cells results in pernicious anemia	▪ Decreased dietary intake, impaired absorption, increased requirement and impaired utilization of folate ▪ Synthesis of intrinsic factor by gastric parietal cells is normal
Pathological findings	▪ Demyelization of the posterior and lateral columns of spinal cord in the midthoracic region ▪ Peripheral blood smear examination shows pancytopenia, macrocytes, hypersegmented neutrophils ▪ Bone marrow shows megaloblastic erythropoiesis ▪ Schilling test abnormal in pernicious anemia	▪ Spinal cord demyelization absent ▪ Peripheral blood smear examination shows pancytopenia, macrocytes, hypersegmented neutrophils ▪ Bone marrow shows megaloblastic erythropoiesis ▪ Schilling test normal
Clinical features	Neurological manifestations present, e.g. ataxia, impaired proprioception and vibrating sensations; anemia and glossitis	Neurological manifestations absent; but anemia and glossitis present
Serum assays	▪ Serum vitamin B_{12} assay decreased ▪ Anti-intrinsic factor autoantibodies demonstrated in pernicious anemia	▪ Red blood folate decreased ▪ Anti-intrinsic factor autoantibodies absent
Treatment	Vitamin B_{12} supplement and intrinsic factor administration in pernicious anemia caused by autoimmune gastritis	Folate supplement

Laboratory Diagnosis: Megaloblastic Anemia

Vitamin B_{12} and folic acid are essential for maturation of nuclei developing precursors in bone marrow. Deficiency of either of the two results in megaloblastic erythropoiesis due to decreased DNA synthesis. Abnormalities will be observed in all the developing cells in the bone marrow. Investigations that may be needed in patients with macrocytosis.

- Serum vitamin B_{12} assay
- Serum and red blood cell folate assay
- Liver and thyroid function tests
- Serum protein electrophoresis
- Reticulocytosis
- Vitamin B_{12} deficiency investigations: serum parietal cell and intrinsic factor antibodies, radioactive vitamin B_{12} adsorption with and without intrinsic factor (Schilling test), possibly serum gastrin concentration.
- Folic acid deficiency investigations: serum antigliadin, antiendomysial and antireticulin antibodies.
- Endoscopy: gastric biopsy (vitamin B_{12} deficiency) and duodenal biopsy (folate deficiency).
- Peripheral blood smear examination shows macrocytes, leukopenia, thrombocytopenia and hypersegmented neutrophils.
- Bone marrow examination shows megaloblastic erythropoiesis.

Peripheral Blood Smear

- Red blood cells show anisocytosis, poikilocytosis, macrocytes (macrocytes are oval with MCV >100 femtoliters in megaloblastic anemia, while round in shape in normoblastic anemias in hemolytic anemia and posthemorrhagic anemia). Macrocytosis and increased MCV may be seen in alcoholic patients.
- Reticulocytosis results in polychromasia. Presence of tear drop cells, Howell-Jolly bodies, basophilic stippling and Cabot's rings indicate dyserythropoiesis.
- White blood cells show leukopenia and hypersegmented neutrophils 8–9 lobes (>5/100 neutrophils).
- Moderate thrombocytopenia with giant platelets is present. Giemsa-stained peripheral blood smear in megaloblastic anemia is shown in Fig. 7.12.

Reticulocyte Count

Reticulocyte count is decreased as a result of dyserythropoiesis in megaloblastic anemia. Post-therapeutic response should be assessed by demonstration of increased reticulocyte count on the sixth day. Reticulocytes are round with polychromasia in peripheral blood smear.

Bone Marrow Smear Examination

- Bone marrow smear examination shows megaloblastic erythropoiesis. Bone marrow changes at all stages of RBCs development. Maturation of the nuclei of erythroid, myeloid and megakaryocytes lags behind and thus chromatin of these cells remains 'open sieve-like'.
- Bone marrow becomes hypercellular with reversal of myeloid to erythroid ratio (M:E ratio 1:1) due to erythroid hyperplasia. Normal myeloid to erythroid ratio is 4:1. Ineffective erythropoiesis leads to intramedullary hemolysis of erythroid precursors. Bone marrow iron store is increased due to dyserythropoiesis.
- Megaloblasts are extremely large erythroid precursors. Chromatin remains open and arranged in a fine reticular fashion to give stippled appearance. Although some of the

megaloblasts are well hemoglobinized, the nucleus is still present suggesting nuclear/cytoplasmic developmental asynchrony.

- Presence of giant band form of neutrophil and metamyelocytes in bone marrow give a clue of megaloblastic anemia.
- Megakaryocytes demonstrate nuclear hypersegmentation with open sieve-like nuclear chromatin. Maturation of the cytoplasm of these cells is normal. The size of these cells remains large. Most of these cells die within bone marrow.

Giemsa-stained bone marrow smear in megaloblastic anemia is shown in Fig. 7.13.

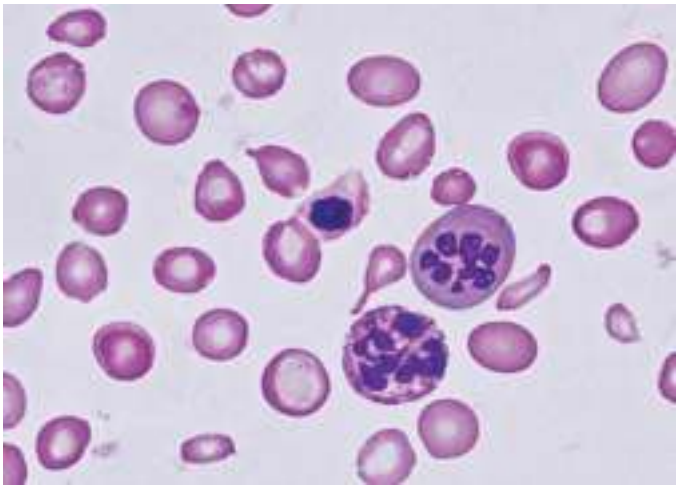


Fig. 7.12: Megaloblastic anemia: Giemsa-stained peripheral blood smear in megaloblastic anemia. It is showing anisocytosis with numerous macrocytosis and scattered tear drop cells (1000X).

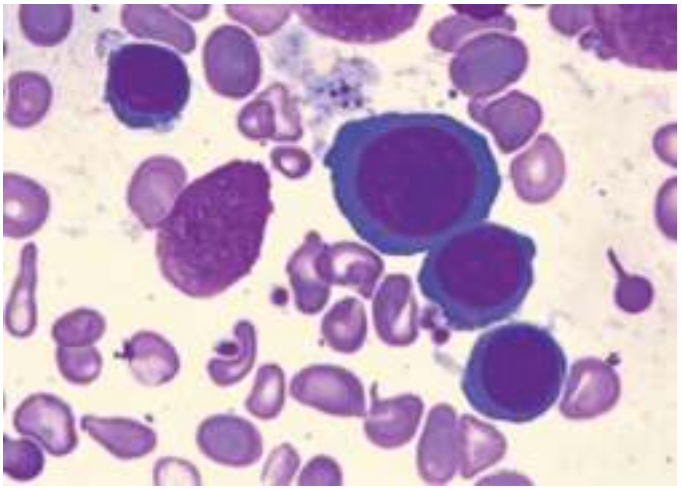


Fig. 7.13: Megaloblastic anemia: Giemsa-stained bone marrow smear in megaloblastic anemia. Bone marrow becomes hypercellular with reversal of myeloid to erythroid ratio (M:E ratio 1:1) due to erythroid hyperplasia. There is preponderance of early and intermediate normoblasts showing stippled nuclear chromatin (open sieve-like) on bone marrow smear examination (1000X).

BACTERIAL INFECTIONS

BACTERIAL INFECTIONS: OVERVIEW

A bacterial infection is a proliferation of a harmful strain of bacteria on body surface or inside the body. Bacteria are transmitted in human beings through direct contact, airborne, droplets and living vectors. Bacteria have circular double-stranded DNA (except in Mycoplasma) and cell walls. Examples of virulence factors produced by pathogenic bacteria are given in Table 7.9.

- Preventive measures such as personal hygiene, safe water intake, immunization and safe sexual practice have impact on morbidity and mortality. Examples of diseases caused by harmful bacteria are pneumonia, meningitis and food poisoning.

Table 7.9 Examples of virulence factors produced by pathogenic bacteria

Pathogenic Bacteria	Virulence Factor	Effect on Host
Vibrio cholerae	Cholera toxin (exotoxin)	Secondary diarrhea
Corynebacterium diphtheriae	Diphtheria toxin (exotoxin)	Inhibits protein synthesis
Clostridium botulinum	Botulinum toxin (exotoxin)	Neuroparalysis, inhibits acetylcholine release
Streptococcus pneumoniae	Pneumolysin (exotoxin)	Inhibition of respiratory ciliated and phagocytic cell function
Many gram-negative bacteria	Liposaccharide (endotoxin)	Fever, hypotension, shock
Staphylococcus aureus	Toxic shock toxin (enterotoxin)	Skin rash, vomiting, diarrhea, hepatitis
Neisseria gonorrhoeae	Pili (adherence)	Induces infection
Haemophilus influenzae	IgA protease	Inactivates antibody
Pseudomonas aeruginosa	Collagenase (invasive)	Penetration of tissue
Clostridium perfringens	Phospholipase (invasive)	Penetration of tissue

- Bacteria occur in three basic shapes: spherical cocci, rod-shaped (bacilli), or helical (spirilla). In clinical practice, bacteria can be broadly classified as gram-positive or gram-negative and anaerobes, mycobacteria, spirochetes, ureaplasma and obligate intracellular organisms and other organisms included in miscellaneous group. Gram-positive bacteria possess a thick cell wall while gram-negative bacteria lack a thick cell wall.
- Gram staining, bacterial culture with antibiotic sensitivity determination, chemical tests, and genetic analysis are used to identify bacterial strains and help to determine the appropriate course of treatment.
- Certain bacteria are encapsulated. The capsule inhibits phagocytosis of *Haemophilus influenzae*, pneumococcus and meningococcus. Bacteria such as Clostridium and Bacillus sporulate under unfavorable growth conditions. Antibacterial drugs are administered to destroy bacterial wall or inhibit their growth at their cellular processes such as protein systhesis.

GRAM-POSITIVE BACTERIAL INFECTIONS

Gram-positive bacteria are classified based on their color demonstrated in the gram staining method developed by Christian Gram in 1884. Gram staining method uses crystal violet dye, which is retained by the thick peptidoglycan cell wall found in gram-positive bacteria, which give a blue color when viewed under light microscope.

- It is worth mentioning that gram-negative organisms have a thin outer membrane consisting of thin peptidoglycan layer, which does not hold the blue color dye used in the initial staining process.
- Other information used to differentiate bacteria is their shape. Gram-positive bacteria comprise cocci, bacilli or branching filaments.
- It is important to identify patients with sepsis and order echocardiogram to detect endocarditis, blood cultures and various laboratory investigations such as complete blood counts and fluid examination.

GRAM-POSITIVE COCCI

Gram-positive cocci cause certain infections by *Staphylococcus aureus*, streptococci, enterococci and pneumococci.

Staphylococcus Aureus

Staphylococcus aureus is transmitted by touching infected blood or body fluids and contaminated hands, which can cause skin and soft tissue infections such as boils (abscesses in hair follicle or sebaceous gland), furuncles, impetigo, cellulitis and Staphylococcus scalded syndrome. *Staphylococcus aureus* can disseminate via bloodstream and cause serious infections such as pneumonia, osteomyelitis and arthritis.

Staphylococcus Epidermidis

Staphylococcus epidermidis is a gram-positive, catalase positive, coagulase negative arranged in clusters, which commonly infects prosthetic devices and intravenous catheters. *Staphylococcus epidermidis* is novobiocin sensitive.

Staphylococcus Saprophyticus

Staphylococcus saprophyticus is a normal flora of the genital tract and perineum that is novobiocin resistant, which accounts for the second most common cause of uncomplicated urinary tract infection (UTI).

Streptococci

Streptococci are gram-positive aerobic organisms transmitted through cough and sneezing. There are different types of streptococci related infections varying in severity from mild sore throat (pharyngitis) to pneumonia and sepsis in both children and adults. Streptococcal infections are primarily treated by antibiotics. Streptococci are divided into two groups: α-hemolytic streptococci and β-hemolytic streptococci. α-Hemolytic streptococci include *Streptococcus pneumoniae*, *Streptococcus viridans*, and *Streptococcus pyogenes*.

- ***Streptococcus pneumoniae:*** The bacterium resides on the skin surface and inside throat, which is transmitted through cough and sneezing. It causes sinusitis, middle ear infections, necrotizing fasciitis, pneumonia, bacteremia, meningitis and brain abscess in newborns, elderly and immunocompromised persons.
- ***Streptococcus viridans:*** The bacterium most often lives in the mouth, gastrointestinal tract and genital region, which disseminates via bloodstream to cause endocarditis.
- ***Streptococcus pyogenes:*** The organism can cause pyogenic infections (e.g. pharyngitis, cellulitis, impetigo and erysipelas), toxigenic infections (e.g. scarlet fever and necrotizing fasciitis) and immunogenic infections (e.g. rheumatic fever and glomerulonephritis). ASO (antistreptococcal-O) detects *Streptococcus pyogenes* infection.

Enterococci

Enterococci are gram-positive cocci that often occur in pairs (diplococci) or short chains, which cause urinary tract infections, endocarditis, bacteremia,

catheter-related infections, wound infections, intra-abdominal infection and pelvic inflammatory disease. Many strains originate from the patient's intestinal flora. It is difficult to distinguish enterococci from Streptococci on physical characteristics alone.

GRAM-POSITIVE BACILLI

Gram-positive bacilli cause certain infections by anthrax, diphtheria, listeriosis, Nocardia and erysipelothricosis.

Anthrax

Anthrax is a serious infectious disease caused by spore forming gram-positive, rod-shaped bacteria known as *Bacillus anthracis*.

- Bacteria anthracis are found in soil that commonly affects domestic and wild animals across world.
- People can become infected through inhaled bacteria spores, contaminated water or food or skin wounds.
- Certain persons are at great risk for bacillus anthrax infection in farmers, military persons, researchers and laboratory workers, veterinarians and workers in wool mill, tannery and slaughterhouse.

Clinical Pearls: Anthrax—Three Forms

Anthrax can occur in three forms: cutaneous (95%), pulmonary and gastrointestinal tract. Antibiotics are the first-line of treatment for this potentially deadly infection.

Cutaneous Anthrax

- Cutaneous anthrax is the most common form of anthrax infection that develops within 1–7 days after exposure. When anthrax spores gain entrance through a skin cut or scraping leading to cutaneous anthrax.
- Patient presents with a group of small itchy blisters, swelling around a painless skin ulcer on the face, neck, arms or hands.

Pulmonary Anthrax

- Inhalation of anthrax spores cause pulmonary anthrax, which do not cause pneumonia, but do cause pulmonary edema, hemorrhagic pleural effusions and mediastinitis.
- Pulmonary anthrax is the most severe form of the disease associated with bacteremia, lymphadenopathy and death even with treatment.
- Patient presents with flu-like symptoms such as sore throat, mild fever, fatigue and skeletal muscle ache.

Gastrointestinal Tract Anthrax

- Ingestion of anthrax spores may cause gastrointestinal tract anthrax involving oral cavity to the rectum regions.
- Patient presents with fever and chills, swelling neck, sore throat, painful dysphagia, hoarseness of voice, nausea, vomiting, hematemesis and bloody diarrhea and headache.

Diphtheria

Diphtheria is caused by *Corynebacterium diphtheriae* that affects mucous membranes of nose and throat, which can lead to breathlessness, loud barking cough, bluish skin, discomfort, dysphagia, slurred speech, signs of shock (i.e. pale cold skin, sweating and tachycardia) heart failure, paralysis and even death. Although, bacteria are transmitted from one person to another, diphtheria can be prevented through the use of vaccines. Untreated cases develop severe damage to kidneys, nervous system and heart leading to death in 3% of cases.

Listeriosis

Listeriosis is a serious infection caused by consumption of contaminated refrigerated foods such as meat, seafood, unpasteurized milk and milk products with the gram-positive rod-shaped bacterium *Listeria monocytogenes*. Patient presents with fever, chills, skeletal muscle aches. Central nervous system infection causes headache, stiff neck, confusion, loss of balance and convulsions. Disease is treated by oral antibiotics. If septicemia or meningitis develop, patient should be administered intravenous antibiotics for six weeks.

Nocardia

Nocardiosis is a disease caused by weakly staining gram-positive, and catalase-positive bacillus *Nocardia asteroides*. The bacillus forms partially acid-fast branching filaments (acting as fungi, but being true bacteria). Nocardia is found in soil and water, and transmitted through open wounds and skin cuts in persons working in soil. The organism can affect the lungs, brain and skin in immunocompromised persons. Pulmonary manifestations include fever, weight loss, night sweats, cough, chest pain and pneumonia.

Erysipelothricosis-Erysipeloid

Erysipelothricosis-erysipeloid is caused by gram-positive bacillus *Erysipelothrix rhusiopathiae*. The most common manifestation is erysipeloid, an acute slowly evolving localized cellulitis.

- Patient presents with raised, purplish red, indurated, slowly, localized itchy skin rash on hands within one week of infection.
- Dissemination may occur in rare cases causing endocarditis, meningitis, osteomyelitis and intra-abdominal infection.
- Disease is diagnosed by performing culture of a biopsy specimen or occasionally polymerase chain reaction testing. Patients are treated by antibiotics.

Actinomyces Israelii

Actinomyces israelii is a gram-positive anaerobic filamentous bacterium no longer classified as a fungus. It is a normal colonizer of the vagina, colon, and mouth. It is an opportunistic pathogen. Infection is established first by a breach of the mucosal barrier during various procedures (dental, gastrointestinal tract), aspiration, or diverticulitis.

- Patient develops chronic abscess with sinus tract formation in cervicofacial, pulmonary, abdominal (colon and appendix) and skin. Lower genital tract infection may be associated with intrauterine device.
- Histologic examination of lesion shows exudates containing sulfur granules, yellow clumps of the organism.

GRAM-NEGATIVE BACTERIAL INFECTIONS

Gram-negative bacilli are responsible for numerous diseases. Some are commensal organisms present among normal intestinal flora.

NEISSERIA MENINGITIDIS

Meningococcal infections are caused by the aerobic or facultative anaerobe bacterium *Neisseria meningitidis*, which affects persons of all ages.

- *Neisseria meningitidis* resides in the nasopharynx of normal persons, which is transmitted through inhalation of infected aerosolized particles. The incubation period varies and ranges from 1 to 14 days.
- The organism possesses several virulent factors that aid in its invasion and infection of human beings: capsule, pili, meningococcal serine protease, opacity proteins, human factor H-binding protein and oligosaccharide.
- *Neisseria meningitidis* is associated with fulminant meningococcemia and meningococcal meningitis in children and adults, including pneumonia, septic arthritis, pericarditis and urethritis. Manifestations of meningococcal meningitis include sudden onset of fever, headache, nausea, vomiting, severe myalgia, nonspecific rash, sore throat and symptoms related to upper respiratory tract.

BORDETELLA PERTUSSIS (WHOOPING COUGH)

Bordetella pertussis (whooping cough) is highly contagious disease caused by the bacterium *Bordetella pertussis* that adversely affects respiratory tract. There are three stages to clinical course of pertussis: catarrhal, paroxysmal and convalescent.

- *Bordetella pertussis* (whooping cough) is characterized by uncontrollable violent coughing and breathlessness due to difficulty in expelling thick mucus from the tracheobronchial tree.
- Long inspiratory effort is accompanied by a high-pitched 'whoop' at the end of the paroxysms. Patient develops cyanosis, vomiting and exhaustion.
- Whooping cough is diagnosed by medical history, physical examination, throat swab, blood test and chest radiograph. Vaccines are available that can help prevent *Bordetella pertussis* infection.

PLAGUE

Plague is an infectious disease caused by bacterium *Yersinia pestis*, usually found in small mammals and their fleas. Bacterium is transmitted to human beings through direct contact with infected fleas either by bite of fleas or inhalation of organisms. Plague can be very severe disease in human beings especially in its bubonic, septicemic and pneumonic forms, which may have fatal outcome.

- **Bubonic plague:** Patient presents with sudden onset of fever, headache, chills and weakness and one or more swollen, tender and painful lymph nodes (known buboes).
- **Septicemic plague:** Patient presents with fever, chills, weakness, abdominal pain, shock and possibly bleeding into the skin and other organs.
- **Pneumonic plague:** Patient presents with fever, headache and rapidly developing pneumonia associated with breathlessness, chest pain, cough and sometimes bloody or water sputum. The pneumonia progresses to respiratory failure and shock within 2–4 days.

GRANULOMA INGUINALE (DONOVANOSIS)

Granuloma inguinale is sexually transmitted disease caused by bacterium *Klebsiella granulomatis* (formerly known as *Calymmatobacterium granulomatis*). The disease is more prevalent in tropical and subtropical regions such as southeast India, Guyana and New Guinea. Patient presents with painless red lump on or near genitals or anus, which progresses to become painful genital ulcers. The disease slowly spreads and destroys genital tissue.

CHANCROID (SOFT CHANCRE)

Chancroid is caused by gram-negative, facultative anaerobic bacillus *Haemophilus ducreyi* transmitted by sexual contact. Incubation period is 4–7 days. Patient develops painful genital ulcers associated with painful suppurative inguinal lymphadenopathy. Genital ulcer has undermined edges, and is covered by a gray-yellow exudate.

Surgical Pathology: Chancroid

Light Microscopy

Histologic examination shows three zones: surface zone, intermediate zone and deep zone. Diagnosis is based on histologic features and histochemical staining (giving railroad track or alignment of short rods).

- **Surface zone of ulcer:** It consists of exudate, fibrin and neutrophils with debris at the base of the ulcer.
- **Intermediate zone of ulcer:** It consists of wide regions with prominent vascular proliferation and thromboses with vascular ectasia.
- **Deep zone of ulcer:** It consists of dense infiltration by lymphocytes and plasma cells.

SALMONELLA TYPHI INFECTION (TYPHOID FEVER)

Typhoid fever is a systemic disease caused by gram-negative *Salmonella typhi* bacilli, which has three antigenic structures: O antigens, H antigens and Vi antigens.

- The organism is transmitted by faecal–oral route. There is involvement of mononuclear phagocytic system with nodule formation in the Peyer's patches of lower ileum.
- Patient may present with **stepladder** rise of **fever** during first week and continued during second and third stage, relative bradycardia, headache, nausea, vomiting, abdominal tenderness, rose spots (2–4 mm on trunk) and hepatosplenomegaly.

MYCOBACTERIUM TUBERCULOSIS

Tuberculosis is worldwide health problem caused by mycobacterium tubercle bacilli (*Mycobacterium hominis* strain or *Mycobacterium bovine* strain). It is transmitted by inhalation of droplets (most common), ingestion of infected milk and skin inoculation (handling infected specimens). It may involve multiple systems.

MYCOBACTERIUM TUBERCULOSIS: OVERVIEW

Mycobacterium tubercle bacillus is strict aerobe and acid-fast due to presence of mycolic acid in its cell wall and stained by Ziehl-Neelsen staining (decolorized by 20% H_2SO_4).

- Mycobacterium tubercle bacilli contain large quantity of hydrophobic waxes and fatty acids of high molecular weight, which prevents entry of Gram's stain.
- Virulence of bacillus depends on presence of cord factor (a sulphated glycolipid), which prevents fusion of phagosomes with lysosomes and thus favors intracellular revival of AFB in macrophages. If cord factor is extracted, AFB becomes avirulent.
- Mycobacterium tubercle bacillus is viable for months in dry or wet sputum. Single AFB is potentially infective in immunocompromised persons. Virulence and bacterial factors in Mycobacterium tubercle bacilli are given in Table 7.10.

Predisposing Factors

There is increased risk for tuberculosis in persons, who have been in close contact with a newly diagnosed tuberculosis case (sputum smear positive), history of previous tuberculosis exposure, immunocompromised individuals (AIDS and corticosteroids therapy). History of silicosis, diabetes mellitus, malnutrition, cancer, Hodgkin's disease, or leukemia, gastrectomy, chronic renal failure and alcoholism increase the risk for tuberculosis.

Mode of Transmission

Mycobacterium tubercle bacilli are transmitted by droplet infection, ingestion of infected milk products, skin inoculation and vertical transmission.

- **Droplet infection:** Infection is most common contracted by droplet infection from open case of pulmonary tuberculosis (cavities formation in lung).

Table 7.10 Virulence and bacterial factors in Mycobacterium tubercle bacilli

Name	Functions
Virulence factors	
*Cord factor composed of glycolipids, mycolic acid and trehalose 6,6'-dimycolate	▪ Cord factor preventing fusion of phagosomes with lysosomes thus favoring intracellular revival of AFB in macrophages ▪ Cord factor eliciting granulomas formation
Catalase-peroxidase and lipoarabinomannan (LAM)	Resisting the host cell oxidative response
Sulfatides and trehalose 6,6'-dimycolate	Triggering toxicity in animal models
Lipoarabinomannan (LAM)	Induction of cytokines and resist host oxidative stress
Bacterial factors	
Nonreplicating persistent (NRP1) state	Under aerophilic conditions with increased glycine dehydrogenase activity
Nonreplicating persistent (NRP2) state	Under anaerobic conditions with decreased glycine dehydrogenase activity

**If cord factor is extracted, AFB becomes avirulent.*

- **Ingestion of unpasteurized milk containing AFB:** Ingestion of unpasteurized milk containing AFB produces Ghon's focus in tonsils and ileocecal region. Acid-fast bacilli produce circumferential ulcers perpendicular to long axis of small intestine. Fibrosis in this region is responsible for 'stricture formation'.
- **Skin inoculation by AFB:** Primary cutaneous inoculation with AFB occurs in person handling infected specimens. Patient develops painless non-healing papule (Ghon's focus) in 2–4 weeks after inoculation, which form cold abscess resulting to discharging sinuses after several weeks.
- **Vertical transmission:** Placental transmission of tuberculosis from mother to fetus shows lesions in liver and portal lymph nodes seen at birth.

Tubercular Infection and Disease

- **Tubercular infection:** Tubercular infection refers to a positive tubercular skin test (Mantoux test or tuberculin test or purified protein derivative test) with no evidence of active disease. Purified protein derivative (PPD) is injected by intradermal route on forearm. Area of induration is demonstrated after 48–72 hours in immunocompetent individuals after exposure to tubercle bacilli after 2–4 weeks. Positive tuberculin test does not distinguish infection (inactive disease) from active disease.
- **Tuberculous disease:** Tuberculous disease refers to cases that have positive acid-fast bacilli on smear or culture for *Mycobacterium tuberculosis* or radiographic and clinical presentation of tuberculosis. Children below 2 years of age are more susceptible. Patients with silicosis and with immunosuppression are also more susceptible to tuberculosis.

Tissue Susceptibility

Lungs are the most common site of tuberculosis in children and adults. Most common organs involved due to Mycobacterium tubercle bacilli in children include lungs, lymph nodes, spleen and meninges. Lungs, adrenal glands, kidneys, liver, spleen, bone, meninges, serous membranes, fallopian tubes, epididymis and lymph nodes are involved in adults. Tissues rarely involved include cardiac muscle, skeletal muscle, stomach, thyroid gland, and pancreas.

Pulmonary and Extrapulmonary Tuberculosis

Tuberculosis is divided into two categories: **pulmonary tuberculosis** (85% cases) and **extrapulmonary tuberculosis** (15% cases). Active disease develops in 5–15% of those infected with *M. tuberculosis*. Pulmonary tuberculosis has two distinct phases, i.e. primary tuberculosis (children) and secondary tuberculosis (adults). Organs involved in primary, secondary and miliary tuberculosis are shown in Figs 7.14 and 7.15.

Primary Tuberculous (Ghon's Complex)

Primary tuberculosis is also known as 'Ghon's complex' caused by Mycobacterium tubercle bacillus. 'Ghon's complex' consists of lesion involving organ, lymphatics and draining lymph node, which is demonstrated in lungs (hilar region), tonsils (cervical region), gastrointestinal tract (ileocecal region) and skin (cutaneous region), which usually does not progress to clinically evident disease. Epithelioid cell granuloma in tuberculosis is known as tubercle. Histologic examnation of tubercular lesion reveals caseous necrosis, epithelioid cell granulomas, Langhans' giant cells.

Secondary Tuberculosis

Secondary tuberculosis usually occurs due to reactivation of old tubercular lesions or gradual progression of primary tuberculosis to chronic form of tuberculosis. However, recent evidence suggests that reinfection is responsible for some form of the secondary tuberculosis. The granulomatous inflammation is much more florid and widespread. Typically, the upper lobes of the lung are most commonly affected and cavitation can occur. Disseminated tuberculosis can occur in which Mycobacterium tubercle bacilli have spread from the lungs to extrapulmonary organs such as bone, brain, adrenal glands, genital system and other organs of the body through the hematogenous or lymphatic system within one week of infection.

Miliary Tuberculosis

Miliary tuberculosis is potentially a life-threatening type of tuberculosis that occurs when numerous Mycobacterium tubercle bacilli travel through blood circulation and disseminate throughout the body. Miliary tuberculosis is so named because the innumerable tiny spots that form in the lungs, which are of the size of millet.

Multidrug-resistant Tuberculosis

Patient develops drug resistance used to treat tuberculosis due to spontaneous gene mutations in acid-fast bacilli involving mycolic acid, catalase and peroxidase enzyme. Catalase enzyme is required to activate isoniazid.

Pathology Pearls: Pathogenesis of Tuberculosis

Mycobacterium tubercle bacillus antigens play an important role in cell-mediated immunity and type 4 hypersensitivity reaction. To study the virulence of Mycobacterium tubercle bacilli, virulence and bacterial factors have been devised, which are associated with intracellular survival, and genotype differences in the community prevalence of clinical strains.

Tuberculosis Pathogenesis <3 Weeks Duration

- Mycobacterium tubercle bacilli's mannose capped glycolipid (lipoarabinomannan) binds to mannose receptors expressed on alveolar macrophages leading to engulfment of AFB.
- Cord factor synthesized by Mycobacterium tubercle bacilli prevents fusion of phagosomes with lysosomes.
- Hence, unchecked proliferation of tubercle bacilli continues inside alveolar macrophages.

Tuberculosis Pathogenesis >3 Weeks Duration

Macrophages are antigen presenting cells, which transmit Mycobacterium tubercle bacilli antigen to CD4+ helper T cells. Macrophages synthesize IL-12, which takes part in differentiation of T-helper cells in lungs and lymph nodes.

- **Bactericidal activity of immune system:** CD4+ helper T cells synthesize interferon-γ (IFN-γ), which stimulates 'nitrogen synthase' enzyme resulting to synthesis of 'nitrogen intermediates' (NO, NO_2 and HNO_3) and oxygen-derived free radicals causing oxidative destruction of AFB.
- **Formation of epithelioid granulomas:** Macrophages synthesize tumor necrosis factor α (TNF-α) and chemokines participate in recruitment of monocytes and sensitization of T cells. This process leads to formation of epithelioid granulomas comprised of macrophages and surrounded by lymphocytes. Granulomas prevent spread of infection by confining bacteria within a compact collection of activated macrophages and lymphocytes. Schematic representation of mechanism of epithelioid cell granuloma formation in tuberculosis is shown in Fig. 7.16.
- **Caseous necrosis:** CD8+ cytotoxic T cells cause destruction of macrophages by 'Fas-independent' mechanism. On the other hand, CD4+ helper T cells cause destruction of macrophages laden with Mycobacterium tubercle bacilli 'Fas-dependent' mechanism resulting in caseous necrosis. Histopathologic examination of tubercular lesion shows epithelioid cell granulomas, caseous necrosis and Langhans' giant cells (Fig. 7.17). Langhans' giant cells are demonstrated in a case of tuberculosis as shown in Fig. 7.18.

PULMONARY TUBERCULOSIS

Pulmonary tuberculosis occurs when Mycobacterium tubercle bacilli attack the lung. However, it can spread to other organs. Pulmonary tuberculosis is curable with an early diagnosis and treatment. Summary of patterns of tuberculous infection of the lungs are given in Table 7.11.

Primary Pulmonary Tuberculosis of Ghon's Complex

Primary pulmonary tuberculosis of Ghon's complex consists of Ghon's focus involving lung, lymphatic channels and draining lymph nodes. Primary pulmonary tuberculosis of Ghon's complex is shown in Fig. 7.19A and B.

- Primary tuberculosis produces a small mid-zone subpleural focus of consolidation near with interlobar fissure, involvement of lymphatic channels and hilar lymph nodes. Approximately 5% of immunocompromised children develop symptomatic infection (cavities in lung, bronchopneumonia, pleural effusion or miliary tuberculosis).
- The tubercular lesion most often heals by fibrosis and dystrophic calcification in 95% cases. The calcified lesion known as 'Ranke's complex' is often visible on radiography. Because, acid-fast bacilli persisting in dormant form in old necrotic calcified lesions is still capable of initiating lesion, and thus a nidus for secondary tuberculosis.
- Lymphatic channels are laden with Mycobacterium tubercle bacilli without producing lesion. Hilar lymph nodes draining pulmonary Ghon's focus become enlarged. Histologic examination of tubercular lesion shows caseous necrosis, epithelioid cell granulomas and Langhans' giant cells. Lymph node lesions of tuberculosis take longer time to regress hence remain a potential source of re-infection.

Post-primary Pulmonary (Secondary) Tuberculosis

Secondary tuberculosis is also known as post-primary pulmonary tuberculosis or reactivation disease. Pulmonary lesion in secondary tuberculosis is known as 'Simon's focus', which develops during adult life especially in immunocompromised persons. Secondary tuberculosis occurs either due to inhalation of Mycobacterium tubercle bacilli or reactivation of dormant lymph node and old calcified healed parenchymal lesion, which causes extensive tissue destruction (caseous necrosis) by the action of cytokines synthesized by memory T cells.

Clinical Features

Patient presents with cough, expectoration, hemoptysis (blood-tinged sputum), night sweats, and evening rise of temperature, anorexia, loss of weight, lassitude and chest pain. Fever is probably caused by the absorption of toxic products from the site of infection and synthesis of cytokines (i.e. **TNF-α and IL-1**) by macrophages.

Clinical Examination

On clinical examination, percussion note is dull over the affected area. On auscultation, one can hear bronchial breath sounds, crepitant crackles and wheeze. Sound is heard through stethoscope, when the patient whispers, it is known as **'whispered pectoriloquy'**.

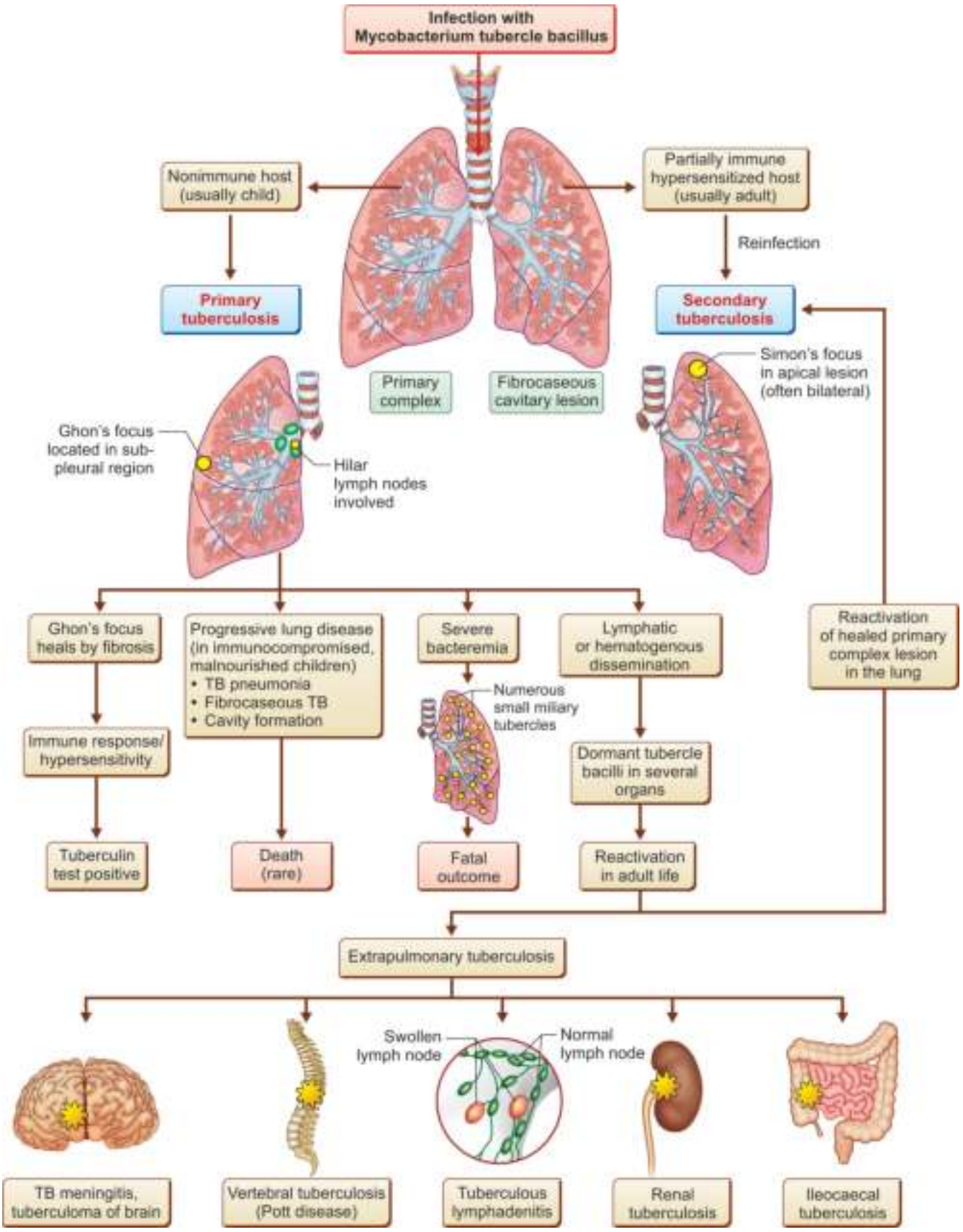


Fig. 7.14: Organs involved in primary, secondary and miliary tuberculosis.

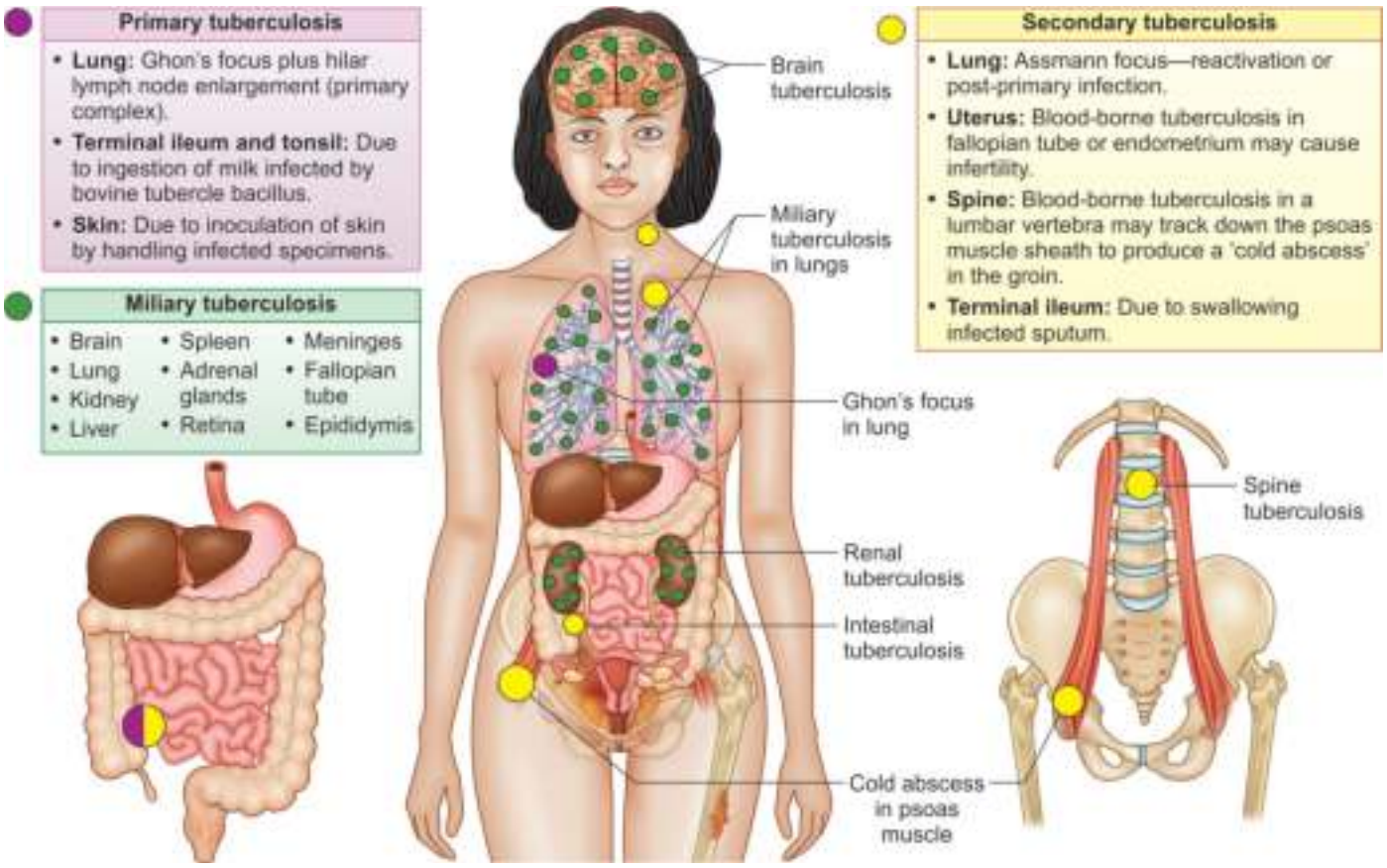


Fig. 7.15: Organs involved in primary, secondary and miliary tuberculosis. Primary tuberculosis produces a subpleural mid-zone lesion in lung. In secondary tuberculosis, lesion is located in apical region of lung. Miliary tuberculosis shows tubercles in various organs.

Pathology Pearls: Pulmonary Tuberculosis—Morphological Patterns

Morphological patterns in pulmonary tuberculosis are discussed below. Morphological patterns in a case of pulmonary tuberculosis are shown in Fig. 7.20A to F.

Primary Tuberculosis (Ghon's Complex)

Ghon's complex consists of three components: (a) midzonal subpleural tubercular focus in lung, (b) lymphatics involvement, (c) hilar lymph nodes involvement.

Simon's Focus in Lungs in Secondary Tuberculosis

- Initially, the tubercular lesion is 2 cm, diffuse solid, gray white to yellowish, with poorly defined margins and central areas of caseous necrosis.
- The lesion is located in the apical region of upper lobes of posterior segments, high oxygen tension and paucity of macrophages due to decreased blood supply.
- Growth of Mycobacterium tubercle bacilli is inhibited at pH <6.5. Some patients may develop tubercular cavities in lungs, which are highly contagious. Large cavities are associated with hemoptysis. Hilar lymphadenopathy is prominent. Pulmonary cavity lesion frequently ruptures into the bronchi.

Aspergilloma in Pulmonary Cavities in Tuberculosis

Aspergillus species may also grow in preexisting cavities caused by tuberculosis. They proliferate to form fungus balls, which are also referred to as aspergillomas.

Pulmonary Cavities in Tuberculosis

- Caseous material may liquefy resulting in cavity lesions is a characteristic of secondary, but not primary tuberculosis. The cavity is filled with yellow-grayish caseous material more or less surrounded by fibrous tissue.
- Acid-fast bacilli (AFB) can easily be demonstrated in open pulmonary cavities. Tubercular lesion may erode into airways.
- Patient may develop empyema and hemoptysis, and sputum becomes positive for AFB. Causes of hemoptysis are given in Table 7.12.

Pulmonary Consolidation in Tuberculosis

- Immunocompromised young children and elderly persons may develop tubercular bronchopneumonia or lobar pneumonia.
- These patients are very infectious. Patient presents with high fever and productive cough.

Tubercular Pleuritis and Pleural Effusion

- Tubercular pleuritis with effusion usually develops soon after initial infection. Tuberculous focus located at the edge of the

lung ruptures into the pleural space causing pleurisy and pleural effusion.
- Patient presents with dyspnea and sharp chest pain that worsens with a deep breath (pleurisy). Tuberculosis pleurisy generally resolves without treatment. Differences between primary and secondary pulmonary tuberculosis are given in Table 7.13. Differences between tuberculosis and sarcoidosis are given in Table 7.14.

Pathology Pearls: Pulmonary Tuberculosis—Consequences

Outcomes of pulmonary tuberculosis are lung fibrosis, lung cavitation, miliary tuberculosis and other lesions described as under.

Pulmonary Fibrosis

Healing of secondary pulmonary tuberculosis occurs by fibrosis in favorable conditions.

Pulmonary Lesions

In some patients, tubercular lesion continues to progress for months and years results in further pulmonary damage. It leads to cavity formation, lobar pneumonia, fibrocaseous tuberculosis and miliary tuberculosis. Lung cavity in a case of tuberculosis is shown in Fig. 7.21.

Airways Involvement

Endobronchial and endotracheal tuberculosis spread cause laryngeal (vocal cords) tuberculosis. Erosion of airways reveals AFB in sputum.

Miliary Tuberculosis

- Secondary tuberculosis may disseminate via hematogenous route resulting in multiple small innumerable small 'millet seed-like lesions' 'tubercular granulomas' known as miliary tuberculosis.
- Mycobacterium tubercle bacilli may disseminate via systemic circulation to seed distant organs such as bone marrow, liver, spleen, adrenal glands, retina, meninges, kidneys, fallopian tubes, and epididymis.
- Mycobacterium tubercle bacilli reach the lungs via pulmonary arteries. Chest radiograph reveals very small nodules throughout the lungs that look-like millet seeds. It occurs shortly after primary infection in immunocompromised persons. Miliary tuberculosis in lung is shown in Fig. 7.22.

Other Complications

Patient may develop tuberculous laryngitis, bronchopleural fistula, bronchiectasis, vertebral tuberculosis (Pott's disease), amyloidosis, scar carcinoma of the lung and granulomatous hepatitis.

TUBERCULOSIS IN HIV PATIENTS

Mycobacterium avium-intracellulare (MAC) complex is a progressive systemic disorder, often occurring in patients who have AIDS. Approximately 33% of all patients develop overt disease due to depletion of CD4+ helper T cells cripples the immune response.

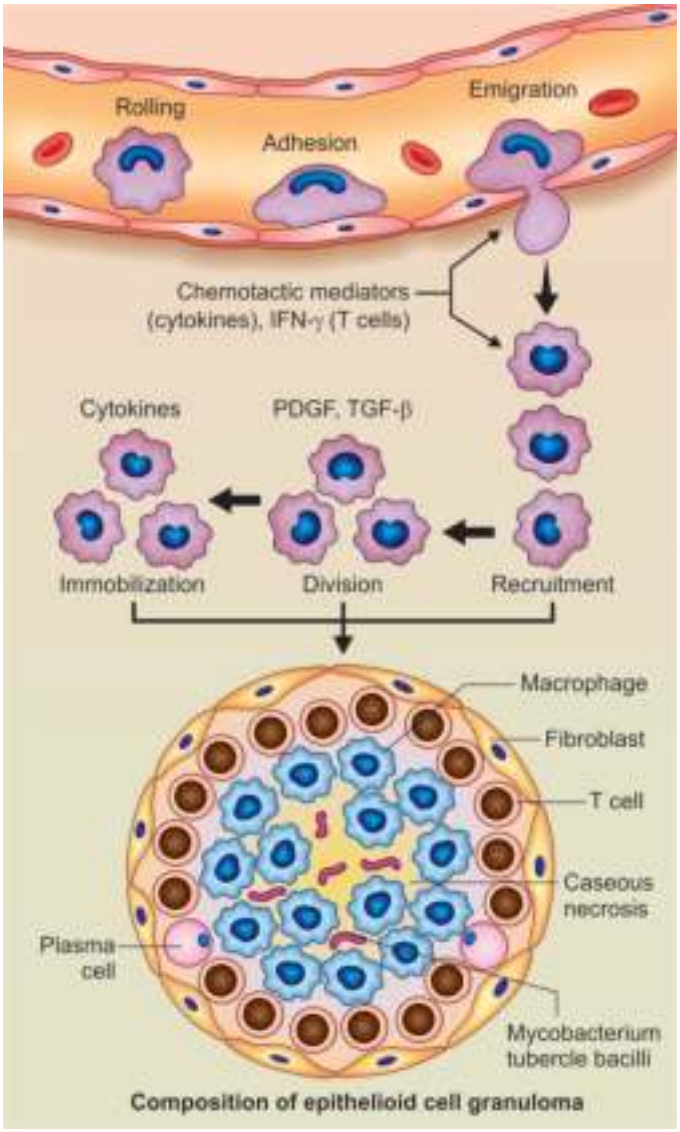


Fig. 7.16: Schematic representation of mechanism of epithelioid cell granuloma formation in tuberculosis.

CD4+ Helper T Cells Count <200/cu mm

Immunosuppression is more severe in HIV patients. Consolidation occurs in lower and middle lobe of lungs. Lymphadenopathy occurs in 50% of cases.
- Histologic examination of tubercular pneumonia lesion shows macrophages laden with numerous Mycobacterium tubercle bacilli, caseous necrosis but lack of epithelioid cell granulomas, which is called nonreactive tuberculosis due to poor immune response.
- Clinical presentation associated with *Mycobacterium avium-intracellulare* resembles that of tuberculosis.

CD4+ Helper T Cells Count >300/cu mm

Immunosuppression is less severe in HIV patients. Apical regions of lungs are affected. Extrapulmonary organs involvement occurs in 15% of cases.

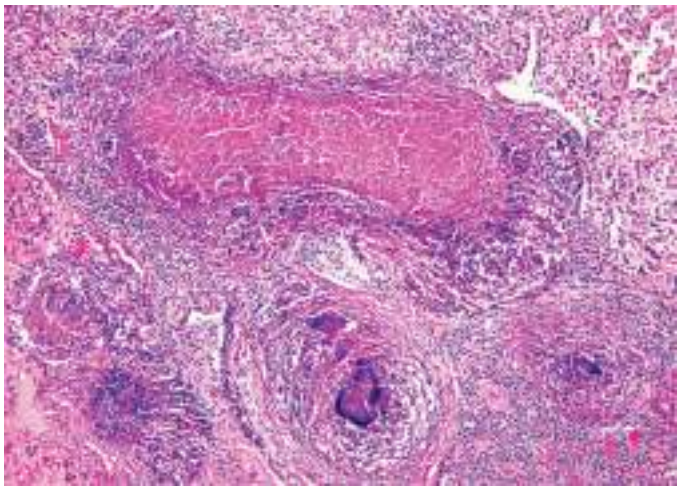


Fig. 7.17: Histologic examination of tubercular lesion shows epithelioid granulomas. Langhans' giant cells and caseous necrosis (400X).

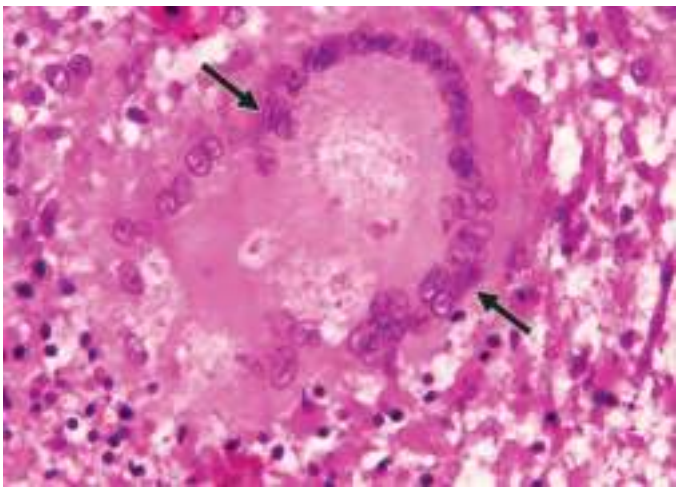


Fig. 7.18: Langhans' giant cells are demonstrated in a case of tuberculosis (arrows) (400X).

Table 7.11 Summary of patterns of tuberculous infection of the lungs

Primary Tuberculosis (Ghon's Focus + Regional Lymph Nodes = Primary Complex)
▪ Reparative mid-zone subpleural tubercular focus that usually heals by fibrosis and calcification
▪ Quiescent healed tubercular lesion (Mycobacterium tubercle bacilli present dormant in organs and lymph nodes)
▪ Progressive tubercular lesion • Pleural involvement (pleurisy/pleural effusion) • Airway dissemination of tubercular lesion (tuberculous laryngitis, tuberculous bronchopneumonia) • Tuberculosis involving lung segments (tubercular pneumonia) • Miliary tuberculosis (hematogenous dissemination to multiple organs)
Post-primary (Secondary) Tuberculosis
▪ Fibrocaseous apical cavitation due to high oxygen tension in apical region of lungs
▪ Reparative tubercular lesion that heals by fibrosis and calcification
▪ Quiescent lesion (Mycobacterium tubercle bacilli dormant)
▪ Progressive tubercular lesion • Local extension of tubercular lesion • Pleural involvement (pleurisy/pleural effusion) • Airway dissemination results in tubercular bronchopneumonia • Miliary tuberculosis (hematogenous dissemination to multiple organs)
Nonreactive Tuberculosis (Immunocompromised Persons)
▪ Tubercular lesion lacks epithelioid cell granulomas
▪ Ziehl-Neelsen stain demonstrates numerous Mycobacterium tubercle bacilli

EXTRAPULMONARY TUBERCULOSIS

Extrapulmonary tuberculosis occurs primarily in immunocompromised patients. Prominent examples of extrapulmonary tuberculosis include tuberculous meningitis, Pott's disease of the spine, paravertebral abscess, or psoas abscess. Other organs involved include kidney, adrenal glands, fallopian tubes, peritoneum, pericardium, breast, larynx and lymph node. Extrapulmonary tuberculosis in order of frequency is given in Table 7.15.

Tuberculous Meningitis

Tuberculous meningitis has insidious onset and may last for weeks or months, which is secondary to tuberculous infection occurring elsewhere in the body. It has a predilection for the base of the brain. Basal cisterns and the lateral sulci contain gelatinous whitish gray material. Inadequately treated tuberculous meningitis results in meningeal fibrosis, communicating hydrocephalus and vasculitis. Inflammation of striate arteries results in cerebral infarcts. Patient may develop focal mass in the brain is known as '**tuberculoma**'.

Pathogenesis

Tuberculous meningitis develops in two steps: (a) In the first step, Mycobacterium tubercle bacilli enter the host by droplet inhalation and escalate within the lungs, then disseminate to the regional lymph nodes, and via hematogenous route to meninges or brain parenchyma resulting in the formation of small subpial or subependymal foci disseminated caseous necrosis lesion, termed Rich foci, and (b) In the second step, development of tuberculous meningitis is an increase in the size of a Rich focus until it ruptures in the subarachnoid

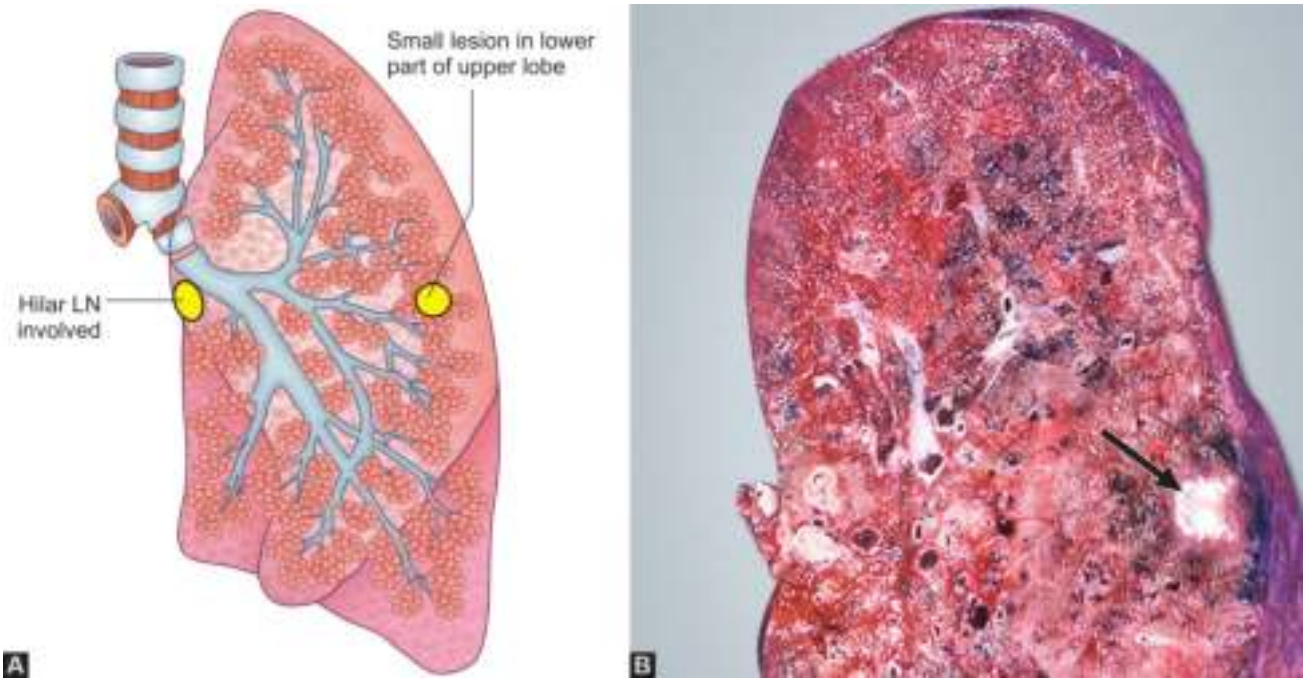


Fig. 7.19A and B: Primary pulmonary tuberculosis of Ghon's complex. It is showing subpleural mid-zonal Ghon's focus and hilar lymph node enlargement. Gross examination of lung showing subpleural mid-zonal Ghon's focus (arrow). (*Courtesy*: Department of Pathology, Dr. DY Patil Medical College, Pune, Maharashtra).

space of brain and spinal cord. Expansion of the tubercle '**Rich focus**' determines the type of central nervous system involvement.

Clinical Features

Tuberculous meningitis involves the central nervous system. Patient presents with headache, neck rigidity, drowsiness, vomiting, irritability, confusion and focal neurological symptoms possibly leading to coma.

Laboratory Diagnosis

Lumbar puncture reveals increased CSF pressure yielding straw-colored CSF. Proteins content is markedly increased. Light microscopy of CSF shows numerous lymphocytes. Acid-fast bacteria may be grown on culture media. Cerebrospinal fluid findings in tuberculous meningitis is given in Table 7.16.

Clinical Pearls: Brain Tuberculoma—Diagnosis

Brain tuberculoma is an important clinical entity. It is main challenge in the management to diagnose it.

- Computed tomography (CT) scan of brain demonstrates solitary or multiple ring-enhancing lesion with moderate perilesional edema, but these findings are not specific for brain tuberculoma.
  - Neurocysticercosis (NCC), coccidioidomycosis, toxoplasmosis, metastasis and few other diseases have identical appearance on CT scan of brain.
  - Cerebrospinal fluid examination is most often normal.
- Magnetic resonance imaging (MRI) of brain with magnetic resonance spectroscopy (MRS) have advantage over CT scan in diagnosis of brain tuberculoma.
- Magnetic resonance spectroscopy demonstrates a specific lipid peak in cases of brain tuberculoma, which is not seen in any other differential diagnosis of brain tuberculoma.
- Histologic examination of biopsy and tissue culture from tubercular lesion can establish the diagnosis. The tubercular lesion consists of epithelioid cell granulomas, caseous necrosis and Langhans' giant cells.

Intestinal Tuberculosis

Mycobacterium tubercle bacilli reach the intestine either ingestion of infected sputum or ingestion of unpasteurized milk. The tubercular lesion begins in the Peyer's patches (lymphoid follicles) and spread through lymphatic channels resulting in tubercular ulcer in terminal ileum especially in the **ileocecal region**.

Surgical Pathology: Intestinal Tuberculosis

Gross Morphology

- Intestinal tuberculosis produces circumferential ulcers perpendicular to long axis of small intestine.
  - Fibrosis in this region is responsible for 'stricture formation'.
  - Draining lymph nodes of small intestine are enlarged and matted with caseous necrosis known as 'tabes mesenterica', which most often heals by fibrosis and dystrophic calcification.

- Hyperplastic ileocecal tuberculosis is a variant of intestinal tuberculosis characterized by thickening of terminal ileum, caecum and ascending colon with mucosal ulceration. On clinical examination, hyperplastic ileocecal tuberculosis is palpable and mistaken for caecal carcinoma. Stricture formation in the intestine in tuberculosis is shown in Fig. 7.23.

Light Microscopy

Histologic examination of ileocecal tuberculosis lesion reveals caseating epithelioid granulomas with Langhans' giant cells.

Tuberculous Osteomyelitis

Tuberculous osteomyelitis is most commonly secondary to hematogenous extension from a primary tubercular focus in the lung, which principally targets the vertebral column, called Pott's disease and paraspinal cold abscess. Vertebral body destruction may result in impingement on the spinal cord. Tuberculosis also targets hip bone, long bones (femur, tibia) and small bones (hands, feet). Patient presents with fever, fatigue, bone pain, some tissue swelling and discharging sinuses.

Tuberculous Pyelonephritis

Kidneys are most common extrapulmonary site in tuberculosis, which can cause asymptomatic pyuria (white blood cells in the urine) and can spread to the reproductive organs and adversely affect reproduction. In men, tuberculous epididymitis (inflammation of the epididymis) may occur.

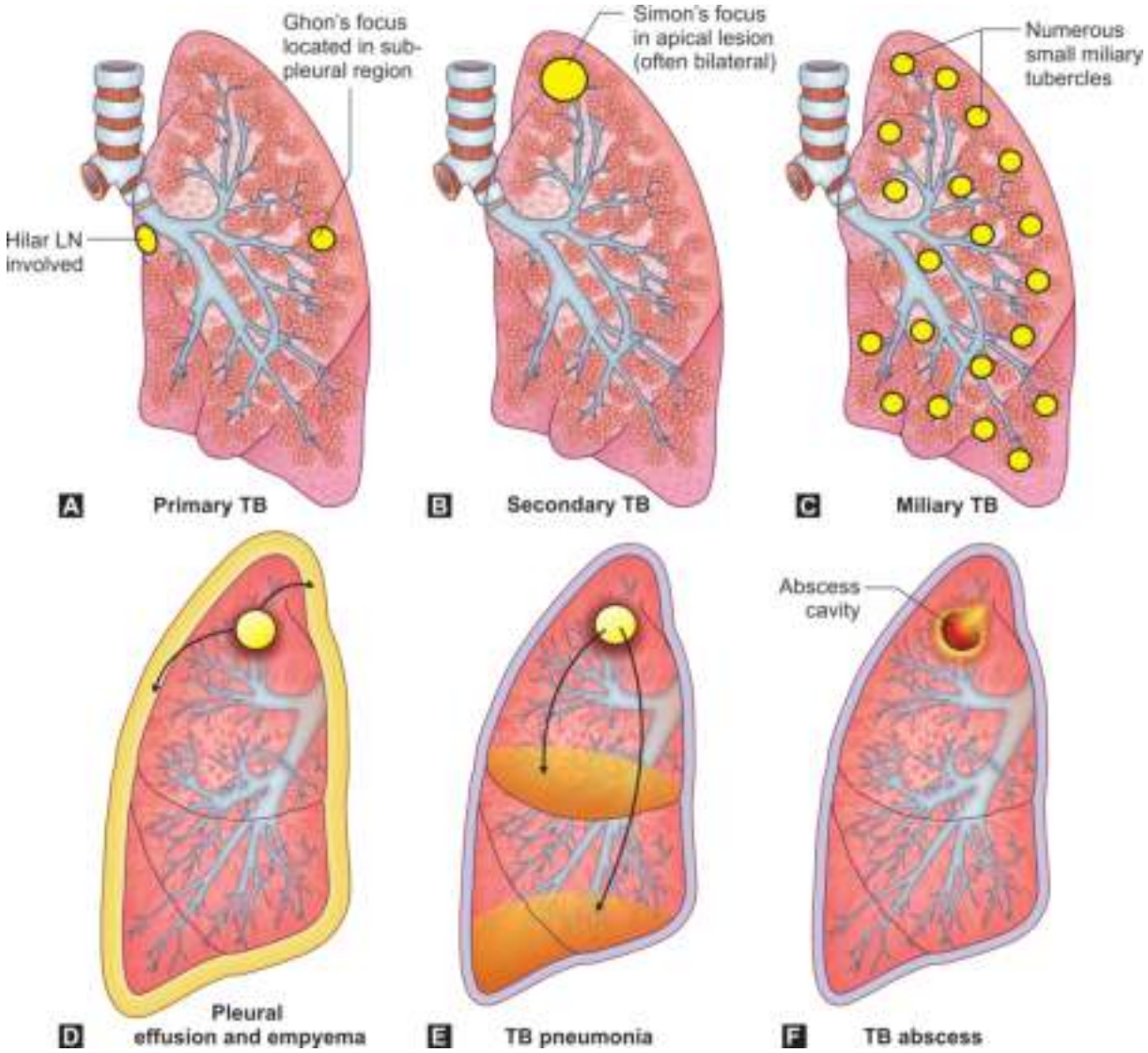


Fig. 7.20: Morphological patterns in a case of pulmonary tuberculosis. (A) Primary tuberculosis shows subpleural mid-zone and lesion hilar lymph node enlargement, (B) apical lesion is seen in secondary tuberculosis, (C) small miliary tubercles are demonstrated in lung, (D to F) pleural effusion, empyema, consolidation (pneumonia) and abscess are complications of tuberculosis.

Table 7.12 Causes of hemoptysis

Etiology	Disorders
Inflammatory lesions	▪ Tuberculosis* ▪ Bronchiectasis* ▪ Pneumonias*
Neoplastic lesions	▪ Primary lung cancers* ▪ Metastatic lung cancers* ▪ Bronchopulmonary adenoma
Other lesions	▪ Pulmonary embolism and infarction* ▪ Mitral stenosis ▪ Left ventricular failure ▪ Anticoagulant therapy ▪ Bronchial adenoma ▪ Idiopathic pulmonary hemosiderosis

**Most common causes of hemoptysis are tuberculosis, bronchiectasis, pneumonias, bronchogenic carcinoma, pulmonary embolism and infarction.*

Surgical Pathology: Tuberculous Pyelonephritis

Gross Morphology

Kidney is enlarged with hydronephrotic changes. Cut surface reveals fibrous-walled abscess cavities filled with caseous material filling the renal pelvis. Tuberculous pyelonephritis with hydronephrosis is shown in Fig. 7.24.

Light Microscopy

Histopathologic examination of tuberculous pyelonephritis shows caseous necrosis, epithelioid cell granulomas and Langhans' giant cells.

Adrenal Gland Tuberculosis

Tuberculosis of the adrenal glands can lead to adrenal insufficiency, which is the inability to increase steroid production in times of stress, causing weakness and collapse.

Table 7.13 Differences between primary and secondary (post-primary) pulmonary tuberculosis

Parameters	Primary Tuberculosis	Secondary Tuberculosis
Exposure to AFB	First time exposure	Reactivation of tubercular lesion
Age group	Children/younger age group	Any age group
Consolidation	Solitary lesion	Multifocal lesions (poorly defined)
Location	Mid-zone (Ghon's focus in subpleural lesion), other sites (tonsils, ileocecal region, skin)	▪ Upper lobes (apical region), Simon's focus in lungs: 2 cm gray white to yellowish well circumscribed consolidation in apical regions of one or both lungs ▪ Sputum for AFB is positive
Lymphadenopathy	Lymphadenopathy common	Lymphadenopathy rare
Severity of lesion	Less severe, healing by fibrosis and dystrophic calcification	More severe progressing to cavitation, lobar pneumonia, extension in lumen of bronchi, trachea, and larynx with vocal cord involvement
Pleural effusion	Pleural effusion common	Empyema common
Cavitation	Cavitation rare	Cavitation common
Miliary tuberculosis*	Miliary tuberculosis can involve organs	Miliary tuberculosis can involve organs

**Miliary tuberculosis can involve lung, liver, bone marrow, spleen, adrenal glands, meninges, kidneys, fallopian tubes, epididymis.*

Table 7.14 Differences between tuberculosis and sarcoidosis

Characteristics	Tuberculosis	Sarcoidosis
Etiology	Mycobacterium tubercle bacilli	Unknown etiology
Granuloma	Caseating granuloma	Noncaseating granulomas
Cytoplasmic inclusions in giant cells	Absence of Schaumann bodies, asteroid bodies and birefringent crystals	Presence of Schaumann bodies, asteroid bodies and birefringent crystals
Steroid therapy	Worsens the disease	Improves the disease
Diagnosis	Acid-fast bacilli present	Excluding causes of granulomatous lesions

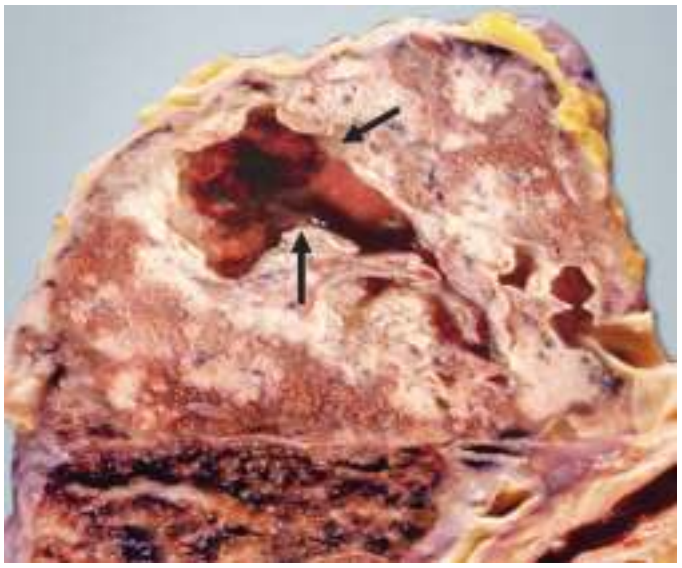


Fig. 7.21: Lung cavity in a case of tuberculosis. Cut surface of the lung shows a large cavity and pulmonary consolidation (arrows). (*Courtesy*: Department of Pathology, Dr. DY Patil Medical College, Pune.)

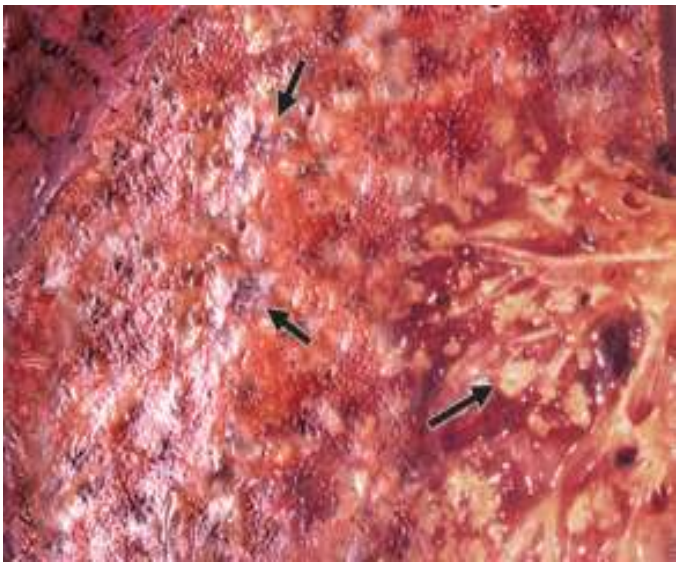


Fig. 7.22: Miliary tuberculosis in lung. Lung is showing millet-like seedlings in miliary tubercles (arrows). (*Courtesy*: Department of Pathology, Dr. DY Patil Medical College, Pune.)

Table 7.15 Extrapulmonary tuberculosis in order of frequency

Extrapulmonary Tuberculosis	Mechanism of Involvement	Comments
Tuberculous lymphadenitis	Hematogenous lymphatic spread	▪ Painless enlarged cervical lymph nodes in cervical and supraclavicular regions ▪ Cervical lymph node may form fistulous tract in skin
Pleural tuberculosis	Pleural tuberculosis can be part of pulmonary tuberculosis	▪ Pleural effusion ▪ Empyema ▪ Pleural fibrosis ▪ Restrictive lung disease
Tuberculosis of upper respiratory airways	Hematogenous spread	▪ Involvement of the larynx, pharynx and epiglottis in advanced secondary tuberculosis ▪ Symptoms, e.g. hoarseness, productive cough and dysphagia
Genitourinary tuberculosis	Hematogenous spread	▪ Tuberculous pyelonephritis ▪ Genital tuberculosis in females can affect the fallopian tube and endometrium causing infertility and menstrual abnormalities ▪ Genital tuberculosis in males affects epididymis producing a tender mass that may form a fistulous tract
Skeletal tuberculosis	Hematogenous or lymphatic spread	▪ Weight-bearing joints tuberculosis ▪ Spinal tuberculosis (Pott disease) may cause abscess formation that track along muscles and ligaments
Tuberculous meningitis and tuberculoma	Hematogenous spread or rupture of subependymal tubercle	▪ Tuberculous meningitis is more common in children than adults present with headache and altered sensorium and/or paresthesia ▪ Tuberculoma can cause seizures and focal symptoms
Intestinal tuberculosis	Swelling of infected sputum or ingestion of infected milk with *Mycobacterium bovis*	▪ *Mycobacterium bovis* trapped in the lymphoid follicles of intestinal mucosa causing bowel inflammation and ulceration ▪ Ileocecal tuberculosis
Tuberculous pericarditis	Progression of a pericardial focus or rupture of a nearby tubercular lymph node	▪ Tuberculous pericarditis may progress to constrictive pericarditis ▪ Tuberculous pericardial effusion
Miliary (disseminated) tuberculosis	Hematogenous spread to multiple organs	▪ Small yellow tubercles that may coalesce forming large areas of consolidation ▪ Nonspecific symptoms such as fever, night sweats and weight loss

Table 7.16 Cerebrospinal fluid findings in tuberculous meningitis

Features	Tuberculous Meningitis	Normal CSF
Causative organism	Mycobacterium tubercle bacillus	No organisms
CSF pressure	Increased	80–180 cm of H_2O (20 drops/minute)
Color	Straw colored	Clear, colorless
CSF proteins	Increased due to increased vascular permeability	Normal 15–40 mg%
CSF sugar	Normal range	50–80 mg%
White blood cells in CSF	Lymphocyte count is increased	0–4 Lymphocytes/mm^3
CSF culture	Bacterial growth on LJ medium	Sterile
Ziehl-Neelsen staining	Acid-fast bacteria demonstration	Organisms absent, hence insignificant

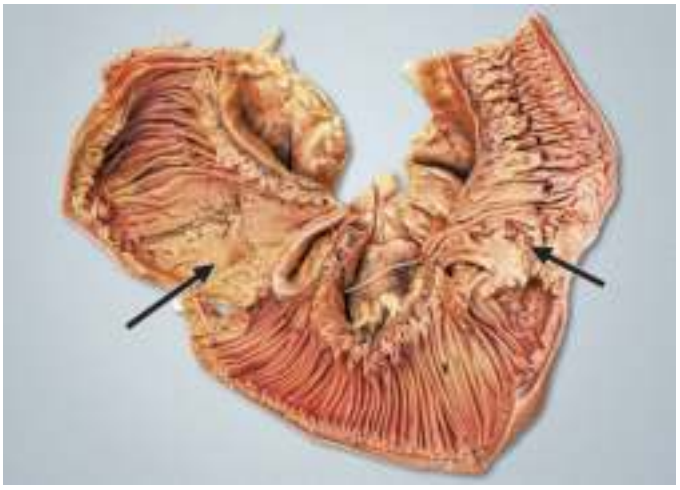


Fig. 7.23: Tubercular stricture of intestine in tuberculosis (arrows). (*Courtesy*: Department of Pathology, Krishna Institute of Medical Sciences, Krar, Maharashtra.)

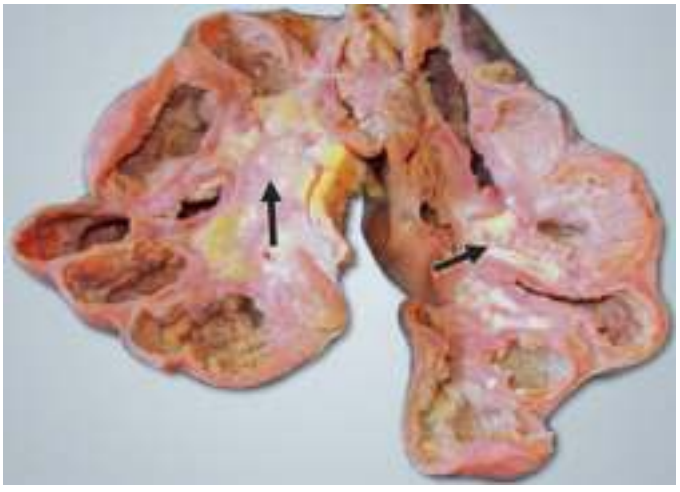


Fig. 7.24: Tuberculous pyelonephritis with hydronephrosis. Fibrous-walled abscess cavity filled with caseous material is shown on cut surface. This patient had a previous well-established history of pulmonary tuberculosis. Section from the patient's nephrectomy specimen shows a tuberculous granuloma, with central caseating necrosis and peripheral scarring. (*Courtesy*: Department of Pathology, Sapthagiri Institute of Medical Sciences, Bengaluru, Karnataka.)

Mammary Tuberculosis

Mammary tuberculosis usually occurs due to extension from a rib in females. Patient presents with breast abscess and fever. Breast abscess contains caseous material. Light microscopy reveals epithelioid cell granulomas, caseous necrosis and Langhans' giant cells.

Laryngeal Tuberculosis

Laryngeal tuberculosis is extremely infectious as it affects larynx or the vocal cords. Patient presents with hoarseness (80–90%), odynophagia (50–70%), dysphagia, stridor, cough and hemoptysis.

Tuberculous Salpingitis

Tuberculous salpingitis occurs due to extension of tuberculous infection of endometrium through intraluminal route causes adhesions of lining epithelium, which is important cause of infertility. On histopathologic examination, fallopian tube shows epithelioid cell granulomas, Langhans' giant cells and caseous necrosis.

Tuberculous Peritonitis

Tuberculous peritonitis affects peritoneum leading to increased production of fluid (exudate) within the peritoneal cavity. Increased fluid leads to abdominal distension and pain. Patients are moderately ill and having fever.

Tuberculous Pericarditis

The pericardium membrane surrounding the heart is involved in tuberculosis. Excessive production of fluid in the pericardial cavity impairs normal cardiac functions.

Tuberculous Lymphadenitis

Lymph nodes contain macrophages that capture the Mycobacterium tubercle bacilli. Any lymph node can

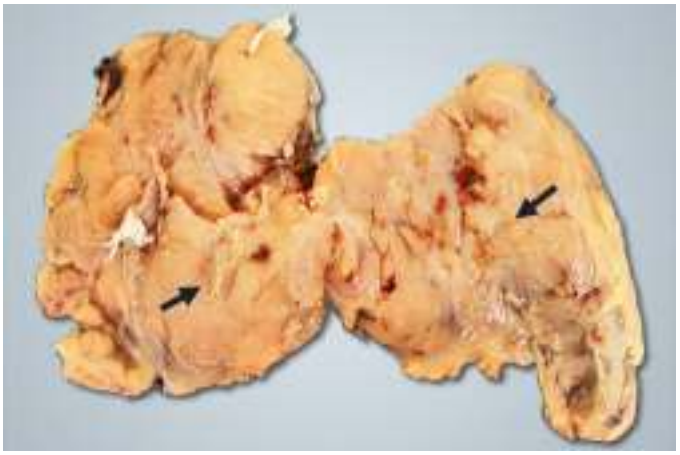


Fig. 7.25: Gross morphology of tuberculous lymphadenitis. Cut surface tuberculous lymphadenitis shows cheesy appearance on cut surface (arrows). (*Courtesy*: Department of Pathology, Dr. DY Patil Medical College, Pune, Maharashtra.)

harbor uncontrolled replication of bacteria, causing the lymph node to become enlarged. The infection can develop a fistula (passage way) from the lymph node to the skin. Gross morphology of tuberculous lymphadenitis is shown in Fig. 7.25.

LABORATORY DIAGNOSIS

Tuberculosis is caused by Mycobacterium tubercle bacilli. Tuberculosis should be suspected in persons who present with unexplained weight loss, anorexia, night sweats, fever and fatigue.

- In addition to these symptoms, patient with pulmonary tuberculosis presents with cough for longer than three weeks, hemoptysis and chest pain. Extrapulmonary tuberculosis presents with symptoms based on the area affected.
- Clinicians should ask about the patient's history of tuberculosis exposure, infection or disease.
- A physical examination can provide valuable information about the patient's overall condition and other factors such as HIV infection or other illnesses that may adversely affect tuberculosis outcome.

Imaging Techniques

- **Chest X-ray:** It reveals cavitation, calcification (healed disease), and lymph nodes in the upper lobes, but cannot confirm the diagnosis.
- **Computed tomography (CT):** It is more sensitive than chest radiography for detection of pulmonary cavities, lymphadenopathy, miliary disease, bronchiectasis, bronchial stenosis, bronchopleural fistula, and pleural effusion.
- **Magnetic resonance imaging (MRI):** It is preferred for diagnosis of extrapulmonary disease such as skeletal and intracranial tuberculosis.

Sputum Smear Prepared from Samples

Smears are prepared from specimens, i.e. sputum for three consecutive days, laryngeal, bronchoalveolar and gastric lavage. Smears prepared are stained with Ziehl-Neelsen staining and 'auramine–rhodamine O' for examination by immunofluorescence microscopy.

- **Sputum collection:** Sputum should be collected for three consecutive days to demonstrate acid-fast bacilli.
- **Laryngeal swab:** By laryngoscope, two swabs are collected and put in 10% H_2SO_4 in test tube for 5 minutes for killing contaminants. Then transfer these swabs to another test tube containing 2% NaOH for 5 minutes to kill other bacilli. Laryngeal swab material is stained with Ziehl-Neelsen stain.
- **Bronchoalveolar lavage:** Smears are prepared and stained with Ziehl-Neelsen stain.
- **Gastric lavage:** It is indicated in children and older patients, who are unable to cough out sputum. Specimen is collected by Ryle's tube and processed immediately to avoid the effect of gastric secretions. Neutralize the gastric contents with N/10 NaOH and put in refrigerator for 15 minutes. Remove the supernatant and centrifuge at 3000 rpm for 15 minutes. Smears are made from deposits.

Sputum Smear Staining for Tubercular Acid-fast Bacilli

Tubercular acid-fast bacilli can be demonstrated by Ziehl-Neelsen stain and fluorescent-like stains such as auramine and rhodamine 'O' stain in sputum samples.

- No acid-fast bacilli in 100 oil immersion fields: negative
- <9 acid-fast bacilli in 100 oil immersion fields: 1+
- 10–99 acid-fast bacilli in 100 oil immersion fields: 2++
- 1–9 acid-fast bacilli in oil immersion fields: 3+++
- >10 acid-fast bacilli in oil immersion fields: 4++++

Fine Needle Aspiration Cytology

Fine needle aspiration cytology smears are stained with May-Grunwald-Giemsa stain. Microscopic examination reveals epithelioid cell granulomas, caseous necrosis and Langhans' giant cells. Mycobacterium tubercle bacilli are demonstrated by Ziehl-Neelsen staining.

Histologic Examination

Biopsy is done to establish diagnosis of tuberculosis. Histologic examination of the tissue consists of epithelioid cell granulomas, caseous necrosis and Langhans' giant cells. Noncaseating tuberculosis lacks epithelioid cell granulomas but numerous Mycobacterium tubercle bacilli show strong positivity with Ziehl-Neelsen stain.

Fluid Examination

Fluid examination is done in extrapulmonary tuberculosis, i.e. cerebrospinal fluid, pleural fluid, peritoneal fluid and synovial fluid.

Culture Techniques

Liquid media-based culture takes 2 weeks. **Löwenstein-Jensen** culture medium takes 10 weeks. Drain off excess of fluid and inoculate in two test tubes of Löwenstein-Jensen medium.

- Aeration is done for every week for more than 8 weeks to remove water of condensation. Growth starts appearing in 10–14 days.
- Human strains of Mycobacterium tubercle bacilli (eugonic) grow more than bovine strains (dysgonic).
- Addition of 0.75% glycerol promotes the growth of human strains without any effect on bovine strain. After culture, smears are made from colonies and stained with Ziehl-Neelsen stain.

Gas Chromatographic Method

Gas chromatographic method is useful diagnostic tool to demonstrate tuberculostearic acid, when AFB is not demonstrated.

Nucleic Acid Amplification Test (NAAT)

Direct tests of nucleic acid amplification (NAA) are rapid, widely available, and can be performed in a day (within 2–7 hours).

- The amplified *Mycobacterium tuberculosis* direct test (gene-probe) targets mycobacterial ribosomal RNA by transcription-mediated amplification. DNA probes are used, which are highly specific for *M. tuberculosis* species.
- The amplified *Mycobacterium tuberculosis* direct test (gene-probe) is best used (and only approved for use) in patients in whom Mycobacterium tubercle bacilli smears are positive. Nucleic acid amplification test is highly specific for *Mycobacterium tuberculosis*. Specificity is greater than 95% in either acid-fast bacilli smear negative or smear positive samples.

Polymerase Chain Reaction Technique

Polymerase chain reaction (PCR) technique amplifies even very small portions of a predetermined target region of *M. tuberculosis*-complex DNA. The test uses an automated system that can rapidly detect as few as one organism from sputum, bronchoalveolar lavage, blood, cerebrospinal fluid, pleural fluid, or other fluid and tissue samples and has shown sensitivity and specificity of nearly 90% in pulmonary disease.

MYCOBACTERIUM LEPROSY (HANSEN'S DISEASE)

Leprosy is an infectious disease caused by a bacillus, *Mycobacterium leprae*, which multiplies slowly. Mycobacterium is slender rod-shaped weakly acid-fast bacillus and cannot be grown on culture medium or cell culture.

- *Mycobacterium leprae* are transmitted from human body by nasal discharge and digital impregnation of skin, as bacilli can be carried under nails and are inoculated under the skin by scratching. The incubation period is usually 3–5 years. Skin and peripheral nerves are commonly involved.
- After entering body, *Mycobacterium leprae* spread through circulation and reach cooler parts of body such as skin of extremities, peripheral nerves, mouth and eyes. These organisms may be demonstrated in bone marrow, liver, spleen and lymph nodes.
- *Mycobacterium leprae* target Schwann cells (SCs) leading to injury of the nerve, demyelination, loss of axonal conductance and consequent disability. The pathogen can invade the Schwann cells by a specific laminin-binding protein known as phenolic-glycolipid 1 (**PGL-1**).
- Leprosy is curable with multidrug therapy (MDT). If leprosy is untreated, it can cause progressive and permanent damage to the skin, nerves, limbs, and eyes.

CLASSIFICATION

Based on clinical, histologic and immunological status, leprosy is classified within two poles of the disease with transition into five categories: tuberculoid polar leprosy (TT), borderline tuberculoid (BT), mid-borderline (BB), borderline lepromatous (BL), and lepromatous polar leprosy (LL). Schematic representation of Ridley-Jopling and WHO classification of leprosy is shown in Fig. 7.26.

Indian Leprosy Classification

According to Indian classification, leprosy is classified into five forms: indeterminate, tuberculoid, borderline, lepromatous and pure neuritic leprosy (PNL). It is essential to pay more attention to **pure neuritic leprosy** and diagnose and treat patients earlier to prevent deformities and sequelae of nerve damage.

Myrid Leprosy Classification

According to Myrid classification, leprosy is classified into two categories: (a) immunological stable (lepromatous and tuberculoid leprosy), and (b) immunological unstable (indeterminate and borderline leprosy).

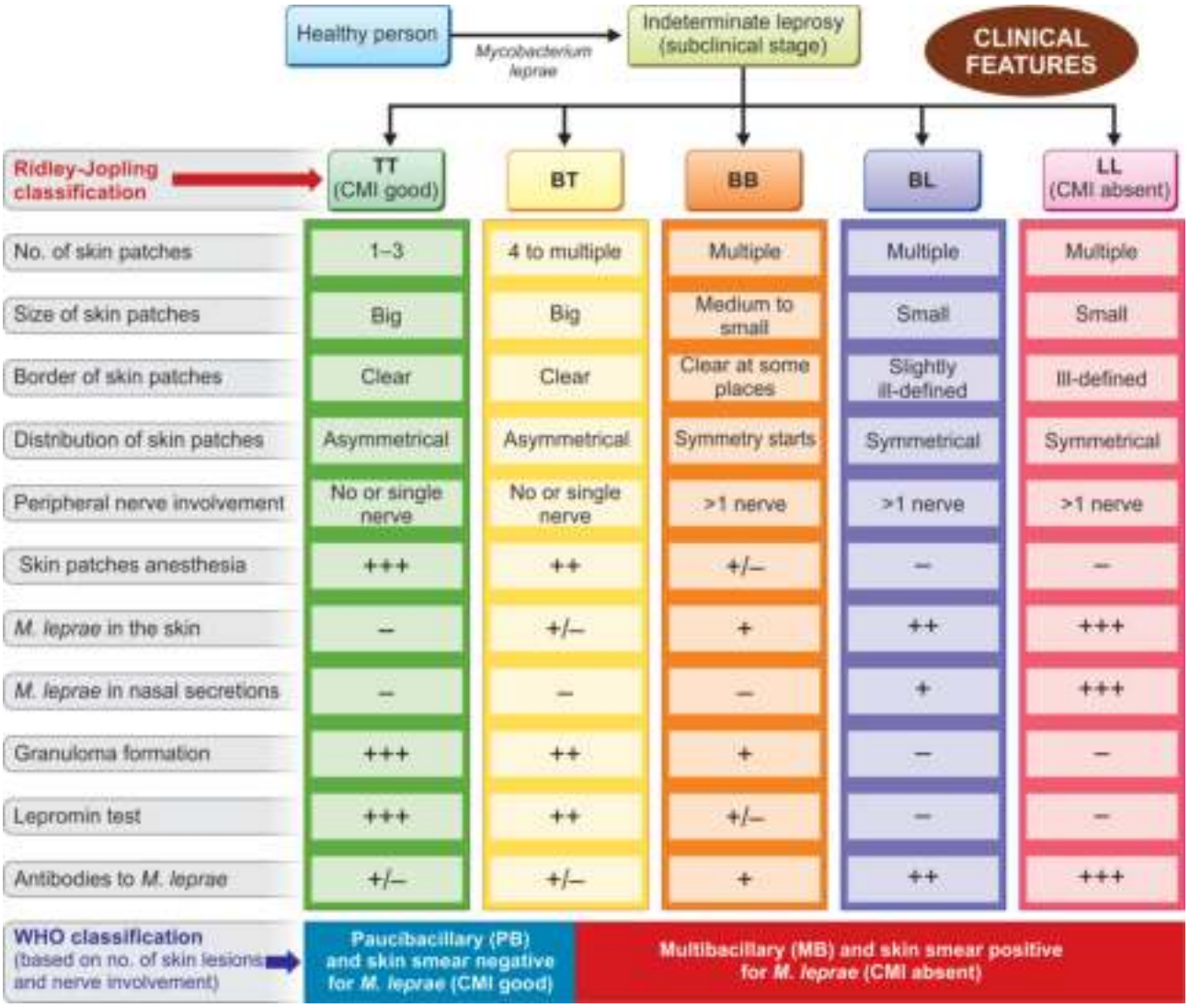


Fig. 7.26: Schematic representation of Ridley-Jopling and WHO classification of leprosy.

Clinical Pearls: Immune Status in Leprosy

- Cell-mediated and humoral immunity have been implicated in leprosy.
- CD4+ helper T cells participate in cell-mediated immunity. Increased titers of IgG, IgA and IgM have been demonstrated in these patients, which are not protective.
- Tuberculoid leprosy has good cell-mediated immunity with granulomatous reaction.
- Lepromatous leprosy has poor cell-mediated immune response without granulomatous reaction.
- Borderline leprosy is an intermediate form between tuberculoid and lepromatous leprosy.

High-immune Response (Indeterminate, TT and BT)

Patient presents with few well-defined skin lesions with regular borders with asymmetrically distribution pattern and nerve enlargement. Slit-skin smear may show scant *Mycobacterium leprae*. Lepromin test is positive.

Low-immune Response (BL and LL)

Patient presents with symmetrical distribution of numerous skin lesions ranging from macules–papules with ill-defined margins. These skin lesions may be hypoanesthetic.

Mid-immune Response (BB)

Patient shows features of both the spectrums of leprosy. This BB is immunologically unstable, and hence prone to reactions.

WHO Leprosy Classification

According to WHO system of working classification, leprosy is classified into paucibacillary (PB) or multibacillary (MB) because, they entail different treatment regimens.

- **Paucibacillary leprosy:** It is characterized by 1–5 skin lesions, one nerve involvement and slit-skin smear negative for AFB.

- **Multibacillary leprosy:** It is characterized by 6 or more skin lesions, involvement of more than two nerves and slit-skin smear positive for AFB.

Ridley-Jopling Leprosy Classification

The most widely accepted scheme of classification of leprosy is Ridley-Jopling classification based on a combination of clinical findings, bacteriological index (BI), immunological reactivity (lepromin test) and the histologic findings. Ridley-Jopling leprosy classification ranges from a spectrum of high- to low-resistance pattern.

Borderline Leprosy

Borderline leprosy includes borderline tuberculoid, mid-borderline and borderline lepromatous leprosy.

- **Borderline tuberculoid (BT) leprosy:** Patients present with multiple large anesthetics less-defined discrete; satellite papules similar to those seen in tuberculoid leprosy. Several peripheral nerve trunks are symmetrically enlarged and neuropathy often occurs. Bacterial index ranges from 0 to 2.
- **Mid-borderline (BB) leprosy:** Patient presents with numerous asymmetrical annular dimorphic plaques with poorly-defined outer borders and sharply defined central 'punched out' skin lesions, which exhibit both tuberculoid and lepromatous lesions. These patients may develop nerve hypertrophy and/or neuritis. Bacterial index ranges from 3 to 4.
- **Borderline lepromatous (BL) leprosy:** Majority of borderline lepromatous leprosy patients have variable immunological response. BL patients do not fall into polar group. BT (borderline tuberculoid) tends to be more towards TT (tuberculoid leprosy) and BL towards LL (lepromatous leprosy). Patient presents with dimorphic, symmetric bilaterally distributed lesions with widespread small macules, papules and nodules of variable sizes and shapes on skin including symmetric peripheral nerve involvement. Bacterial index is 4 or 5.

Tuberculoid Leprosy

Tuberculoid leprosy is characterized by presence of immunological response against *Mycobacterium leprae*. Patient develops large macular lesions on cooler parts especially nose, outer ears, testes and superficial nerve endings. There is presence of cell-mediated immune response. Infectivity is low in tuberculoid leprosy. Schematic representation of genesis of cell-mediated immunity in tuberculoid leprosy is shown in Fig. 7.27.

Lepromatous Leprosy

Lepromatous leprosy has least capacity to mount immunological response against *Mycobacterium leprae*. Patient develops extensive erythematous macules, papules or nodules with extensive destruction of skin. Immune response is severely depressed. There is high infectivity in lepromatous leprosy. Schematic representation of genesis of cell-mediated immunity in lepromatous leprosy is shown in Fig. 7.28.

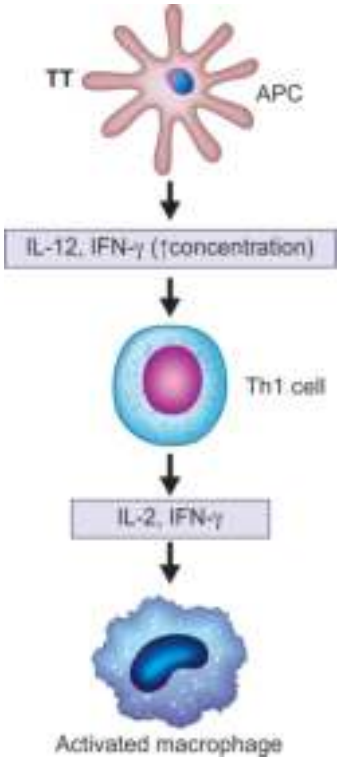


Fig. 7.27: Schematic representation of genesis of cell-mediated immunity in tuberculoid leprosy. It induces good immune response. Infectivity is low in tuberculoid leprosy.

Pure Neuritic Leprosy

Pure neuritic leprosy (**PNL**) constitutes a significant proportion of cases in India. It is essential to pay more attention to this form of leprosy and diagnose and treat patients earlier to prevent deformities and sequelae of nerve damage.

Pathology Pearls: Histology of Skin Biopsy in Leprosy

- Histologic examination reveals granulomatous inflammation and nerve inflammation and acid-fast bacilli on Ziehl-Neelsen staining.
- Langhans' giant cells are present in tuberculoid leprosy and borderline tuberculoid (BT) leprosy lesions.
- Noncaseating epithelioid cell granulomas are present in tuberculoid (TT) leprosy, mid-borderline (BB) leprosy lesions and to a lesser extent in borderline lepromatous (BL) leprosy.
- Histiocytes and foamy macrophages are present in borderline lepromatous leprosy and lepromatous leprosy (LL) lesions.
- Clear subepidermal zone is observed in mid-borderline, borderline lepromatous leprosy, and lepromatous leprosy lesions. Lamination of the perineum is more characteristic of borderline leprosy and lepromatous leprosy.

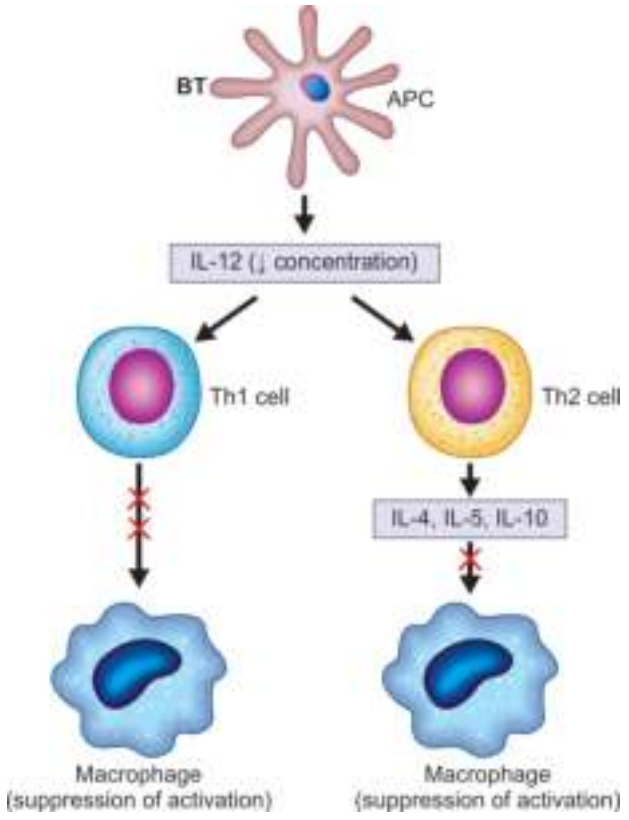


Fig. 7.28: Schematic representation of genesis of cell-mediated immunity in lepromatous leprosy (LL). It induces poor immune response. Infectivity is high in lepromatous leprosy.

CLINICAL FEATURES

Activity of leprosy disease is assessed by various signs: erythema/infiltration, extension or appearance of new lesions, extension/new appearance of anesthesia, paresis and paralysis, tenderness and pain in nerves, morphologic index and occurrence of reaction. Even though the nerves affected in leprosy are mixed nerves, the sensory loss is more marked compared to motor dysfunction.

Indeterminate Leprosy

The initial cutaneous manifestations of indeterminate leprosy are subtle and not specific. Initially erythematous patch, which in darker-skinned individuals appears pale; most cases resolve, some advance into more severe disease. It is most common type of leprosy in Indian children. Most cases resolve, but some advance into more severe disease.

- Patient presents with an ill-defined, bizarre hypopigmented macule(s) with a smooth or scaly surface. The sensations over the macule may or may not be impaired. The nerve proximal to the patch may or may not be thickened.
- Histologic examination of indeterminate leprosy shows scant lymphocytes and histiocytes in the superficial and deep in the dermis with some tendency to localize around skin appendages; and increased mast cells. Histology of indeterminate leprosy is shown in Fig. 7.29.

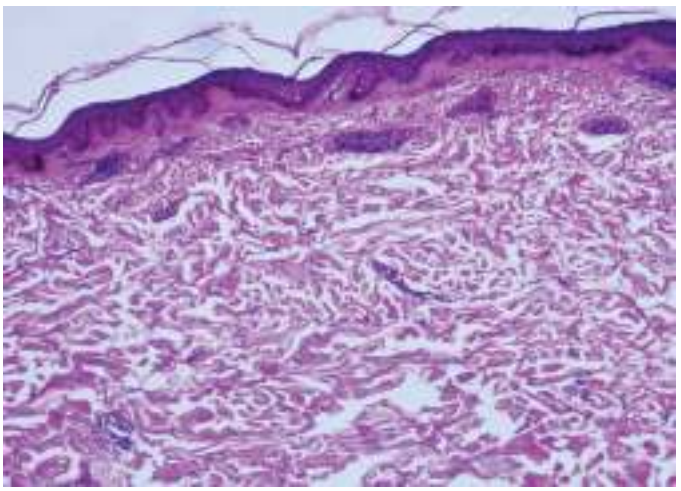


Fig. 7.29: Histology of indeterminate leprosy. Histologic examination of indeterminate leprosy shows scant lymphocytes and histiocytes around neurovascular bundles in the superficial and deep in the dermis with some tendency to localize around appendages; and increased mast cells (400X). (*Courtesy*: Dr. Geeta Pachori, Senior Professor and Head of Pathology, Jawahar Lal Nehru Medical College, Ajmer, Rajasthan.)

Borderline Leprosy

Borderline leprosy is an intermediate form between tuberculoid and lepromatous leprosy. Patient has hypopigmented macules and involvement of nerves exhibiting onion-skin appearance.

Borderline Tuberculoid Leprosy

Borderline tuberculoid leprosy (BT) is characterized by noncaseating epithelioid cell granulomas and numerous lymphocytes in dermis. Diagnosis of BT leprosy is achieved using real-time polymerase chain reaction (PCR) to amplify *Mycobacterium leprae* specific DNA sequence and to detect serum antibodies specific to *Mycobacterium leprae* antigens. Clinical photograph of borderline tuberculoid leprosy is shown in Fig. 7.30. Histology of borderline tuberculoid leprosy is shown in Fig. 7.31.

Borderline Lepromatous Leprosy

Majority of borderline lepromatous (BL) leprosy patients have variable immunological response. BL patients do not fall into polar group. Borderline tuberculoid (BT) leprosy tends to be more towards tuberculoid (TT) leprosy and borderline lepromatous leprosy (BL) towards lepromatous leprosy (LL). Patient presents with dimorphic, symmetric bilaterally widespread small macules, papules and nodules of variable sizes

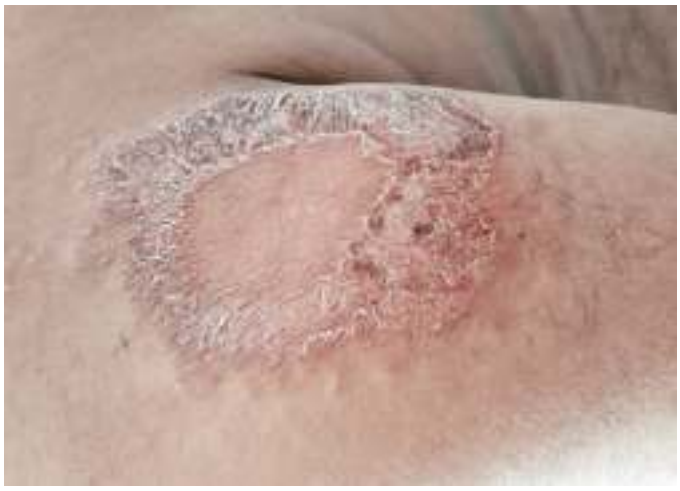


Fig. 7.30: Clinical photograph of borderline tuberculoid leprosy. Clinical examination shows more widespread and less sharply defined lesions than in tuberculoid form. These lesions are usually symmetrical on trunk but may be asymmetric on face. Nerve involvement is less prominent. (*Courtesy*: Dr. Krishna Deb Barman, Director Professor of Dermatology, Maulana Azad Medical College and Associated Lok Nayak Hospital, New Delhi.)

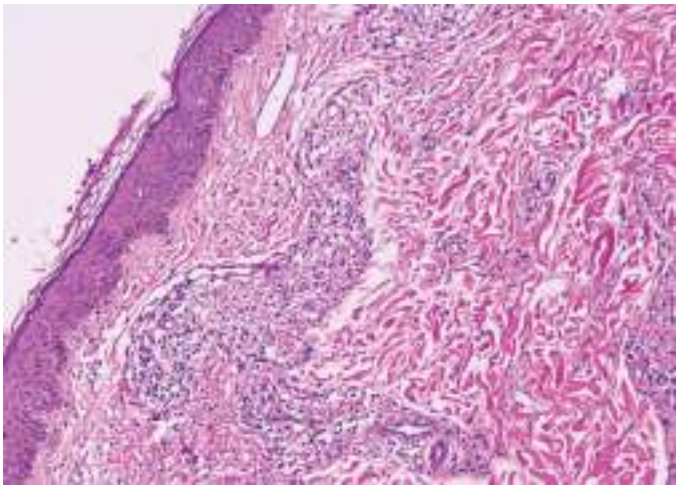


Fig. 7.31: Histology of borderline tuberculoid leprosy. Hematoxylin and eosin-stained section shows formation of noncaseating epithelioid cell granulomas (400X).

and shapes on skin including symmetric peripheral nerve involvement. Bacterial index is 4 or 5. Clinical photograph of borderline lepromatous leprosy is shown in Fig. 7.32. Histology of borderline lepromatous leprosy is shown in Fig. 7.33.

Tuberculoid Leprosy

Tuberculoid leprosy is a milder, less severe form of leprosy (called paucibacillary leprosy). It has robust cell-mediated immune response with prompts granulomatous inflammation and characterized by infiltration of dermis and subcutaneous fat by noncaseating granulomas predominantly centered

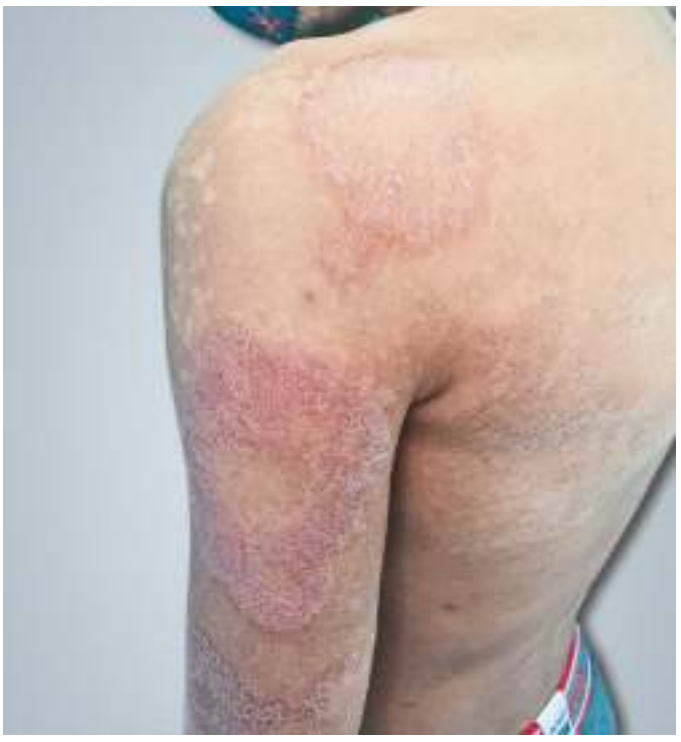


Fig.7.32: Clinical photograph of borderline lepromatous leprosy. Clinical examination shows symmetric bilateral widespread, well-defined, hypopigmented to erythematous hypoesthetic small macules, papules and nodules of variable sizes and shapes with downward slopping edges seen over the back. Small satellite lesion is also in the periphery of periphery. These skin lesions are mostly lepromatous in nature but also contain aspects of tuberculoid lesions. (*Courtesy*: Dr. Krishna Deb Barman, Director Professor of Dermatology, Maulana Azad Medical College and Associated Lok Nayak Hospital, New Delhi.)

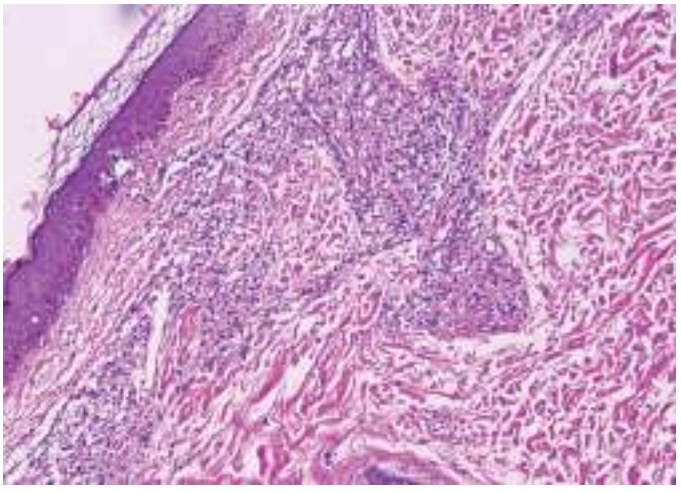


Fig. 7.33: Histology of borderline lepromatous leprosy. Hematoxylin and eosin-stained section shows formation of noncaseating epithelioid cell granulomas (400X).

around small cutaneous nerves. Nerves are completely destroyed in the process and replaced by noncaseating epithelioid cell granulomas. Cell-mediated hypersensitivity reaction eradicates bacteria from the lesion in

tissue. Therefore, *Mycobacterium leprae* bacilli are scarce and usually not identified with modified acid-fast stains (Wide-Fite stain).

- Patient presents with one or multiple erythematous or scaly, well-circumscribed macules or patches; usually hypoanesthetic or anesthetic on extremities, buttock and face, thickened nerves, loss of sensation and diminished sweating. All patients have nerve involvement. Inflammation of Schwann cells leads to thickening of peripheral nerves. Clinical course is generally benign.
- On histologic examination, tuberculoid leprosy demonstrates noncaseating epithelioid cell granuloma, Langhans' giant cells, CD4+ helper T cell infiltration, and involvement of peripheral nerve. The close association of granulomatous response with the cutaneous nerves is a helpful diagnostic feature in tuberculoid leprosy. Tuberculoid leprosy is less contagious than other forms. Histology of tuberculoid leprosy is shown in Fig. 7.34.

Lepromatous Leprosy

Patient presents with symmetrically distributed papular and nodular lesions on nose and ears; on cooler parts (face, ears, wrists, elbows, buttocks, and knees) sparing inguinal and axillary regions. These lesions often start on nose and ears, later involve hands, arms, buttocks. Facial lesions can be markedly swollen with loss of eyebrows (**Lucio sign**). Facial nerve involvement, hoarseness, loss of eyebrows and eyelashes, and nasal collapse (**saddle nose**) secondary to septa perforation may occur in advanced cases of disease. Nasal secretions are rich in organisms. Differences between tuberculoid and lepromatous leprosy are given in Table 7.17.

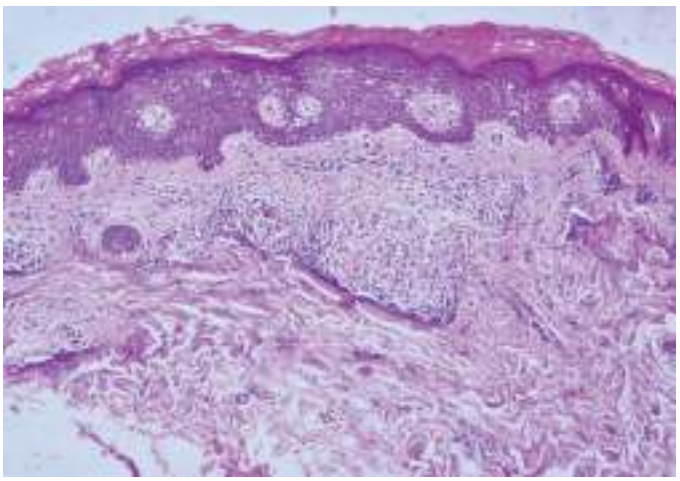


Fig. 7.34: Histology of tuberculoid leprosy. Hematoxylin and eosin-stained section shows noncaseating epithelioid granuloma, Langhans' giant cells, T-helper lymphocytic infiltration, and involvement of peripheral nerve (400X). (*Courtesy*: Dr. Geeta Pachori, Senior Professor and Head of Pathology, Jawahar Lal Nehru Medical College, Ajmer, Rajasthan.)

Surgical Pathology: Lepromatous Leprosy

Light Microscopy

- The predominant histologic feature in lepromatous leprosy is a diffuse histiocytic infiltrate in the dermis that is not necessarily centered around nerves.
- A grenz zone of sparing is usually present in the papillary dermis. There are no discrete granulomas in the dermis. The histiocytes are arranged in poorly circumscribed clusters.
- Lymphocytes are scarce and those are present usually CD8+ cytotoxic T cells. The histiocytes may demonstrate vacuolated or foamy cytoplasm with a grayish-blue tinge on hematoxylin and eosin-stained sections due to clusters of *Mycobacterium leprae* bacilli.
- With modified Ziehl-Neelsen (Wide-Fite) stain, *Mycobacterium leprae* bacilli can be visualized in large numbers in histiocytes surrounding cutaneous nerves, blood vessels and eccrine glands. In some cases of lepromatous leprosy, histiocytes are spindle-shaped and arranged in storiform pattern mimicking fibrous histiocytoma. This variant is referred to as histoid leprosy.
- Histology of lepromatous leprosy is shown in Fig. 7.35 and Ziehl-Neelsen (Wide-Fite) stained section demonstrates numerous *Mycobacterium leprae* bacilli as shown in Fig. 7.36.

Pathology Pearls: Histoid Leprosy—Rare Variant of Lepromatous Leprosy

- Histoid leprosy is a rare variant of lepromatous leprosy characterized by unique clinical, histopathologic, and morphological features.
- Patient presents with cutaneous and subcutaneous nodules. Histoid leprosy patients represent probable residual bacilli and a highly active lepromatous leprosy process. These cases may act as reservoirs of the disease and lead to further spread of leprosy.
- Continual occurrence of these cases does not bode well for eradication of leprosy. Histology of histoid leprosy is a type of lepromatous leprosy as shown in Fig. 7.37.

Erythema Nodosum Leprosum

Leprosy reactions are significant cause of morbidity in leprosy patients. Erythema nodosum leprosum (**ENL**) is an immunological complication affecting 50% of patients with lepromatous leprosy (LL) and affecting 10% of borderline lepromatous (BL) leprosy.

- Erythema nodosum leprosum is associated with clinical manifestations such as tender red plaques and nodules together with areas of erythema over skin, neuritis, orchitis, dactylitis, osteitis, lymphadenitis, facial mutilation, eye inflammation, neurotrophic ulcers, flexion contractures of hands, foot drop and nephritis.
- The eruption is generally widespread and accompanied by fever, malaise and arthralgia. Erythema

Table 7.17 Differences between tuberculoid and lepromatous leprosy

Characteristics	Tuberculoid Leprosy	Lepromatous Leprosy
Host resistance	Present	Absent
T cell immunity	Present	Absent
Lepromin test	Strongly positive	Negative
Fernandez reaction (24–48 hours)	Positive	Negative
Mitsuda reaction (3–4 weeks)	Positive	Negative
Skin lesions	▪ Macular lesions with central depressed areas and hypopigmented margins ▪ Asymmetrical lesions not involving both sides	▪ Macular/papular/nodular lesions on hand and face (leonine face) ▪ Symmetrical lesions involving both sides
Touch sensation	Hypoanesthetic/anesthetic lesions	Absent (anesthetic patches)
Clinical appearance	Disfigurement minimal	Disfigurement maximum (leonine facies, claw hands and pendulous ear lobes)
Nerves involved	Ulnar, facial and peroneal nerves	Ulnar and peroneal nerves
Other organs	Other organs not involved	Anterior chamber eye, upper respiratory tract, testes, lymph nodes, liver, spleen and gynecomastia in males
Infectivity	Low	Very high
Skin histology	▪ Epidermis nonatrophic ▪ Rete ridges present ▪ Dermal papillae normal ▪ Clear zone between epidermis and dermis is absent ▪ Epithelioid cell granulomas present ▪ CD4+ helper T cells: Present at the periphery of granulomas ▪ CD8+ cytotoxic T cells: Very few at the center of lesion ▪ Fite stain: AFB (*M. lepare* bacilli) few (4–5) in macrophages	▪ Epidermis atrophic ▪ Rete ridges absent ▪ Dermal papillae flattened ▪ Clear zone between epidermis and dermis is present ▪ Epithelioid cell granulomas absent ▪ CD4+ helper T cells are absent ▪ CD8+ cytotoxic T cells are present in large number in diffuse manner ▪ Fite stain: AFB (*M. lepare* bacilli) abundant in macrophages
Complications	These are related to nerve damage like paralysis, distinct sensory losses	Antigen–antibody complex mediated erythema nodosum leprosum, vasculitis, glomerulonephritis besides nerve related
Prognosis	Milder disease, hence good prognosis	Extensive progressive disease, hence poor prognosis

nodosum leprosum is treated mainly with corticosteroids. Prolonged administration of corticosteroids can cause morbidity and mortality. Clinical photograph of erythema nodosum leprosum is shown in Fig. 7.38.

- Histologic examination of hematoxylin and eosin-stained sections demonstrates an inflammatory neutrophilic infiltrate, vasculitis and/or panniculitis, immune-complex deposit and complement deposit associated with antigens of *Mycobacterium leprae*. Histology of erythema nodosum leprosum is shown in Fig. 7.39.
- The clinical course is prolonged with recurrent episodes of *Mycobacterium leprae* over a period of 12–24 months or even longer than seven years in some cases.

LEPROMIN TEST

Lepromin test is used to demonstrate immune status in leprosy patients. Antigenic extract obtained from *Mycobacterium leprae* is administered by intradermal route. The injection site is evaluated at 1–2 days and 3 weeks. Delayed hypersensitivity is demonstrated in tuberculoid leprosy (TT). Lepromin test is negative

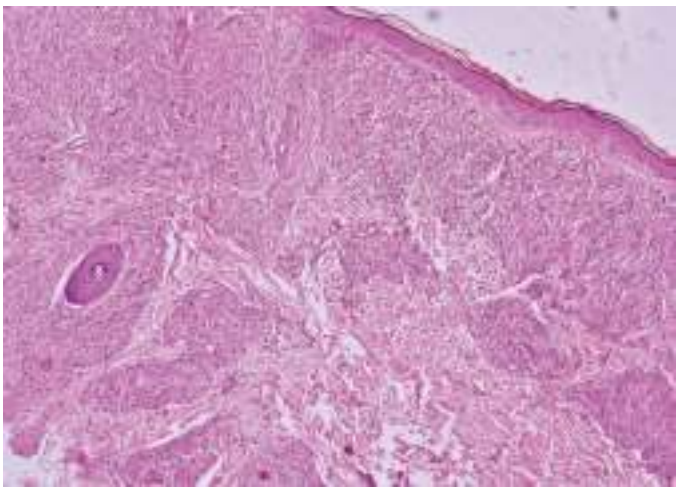


Fig. 7.35: Histology of lepromatous leprosy. Epidermis shows atrophy. There is loss of rete ridges of epidermis. Dermal papillae are flattened. There is loss of dermal appendages. The predominant histologic feature in lepromatous leprosy is a diffuse histiocytic infiltrate arranged in nodular or diffuse patterns. In the dermis that is not necessarily centered around nerves. Grenz zone (clear zone) is demonstrated at the junction of epidermis and dermis in lepromatous leprosy. There are no discrete granulomas in the dermis. The histiocytes are arranged in poorly circumscribed clusters (400X).

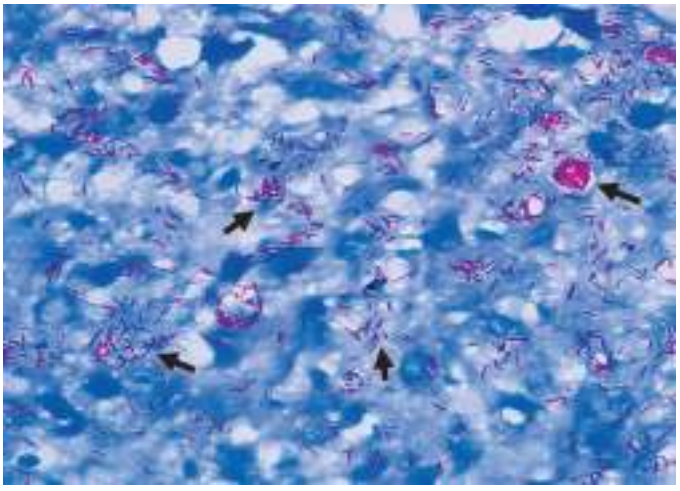


Fig. 7.36: Lepromatous leprosy. Modified Ziehl-Neelsen (Wide-Fite) stain is showing macrophages laden with *Mycobacterium leprae* bacilli (arrows) (400X). (*Courtesy*: Dr. Geeta Pachori, Senior Professor and Head of Pathology, Jawahar Lal Nehru Medical College, Ajmer, Rajasthan.)

in lepromatous leprosy (LL). Lepromin positive reaction is of two types: (a) Fernandez reaction, and (b) Mitsuda reaction.

Fernandez Reaction

Fernandez reaction occurs within first 2 days and represents a delayed-type of hypersensitivity reaction to intradermally injected heat-killed leprosy bacilli. Patient develops skin induration within 24–48 hours at the site of injection.

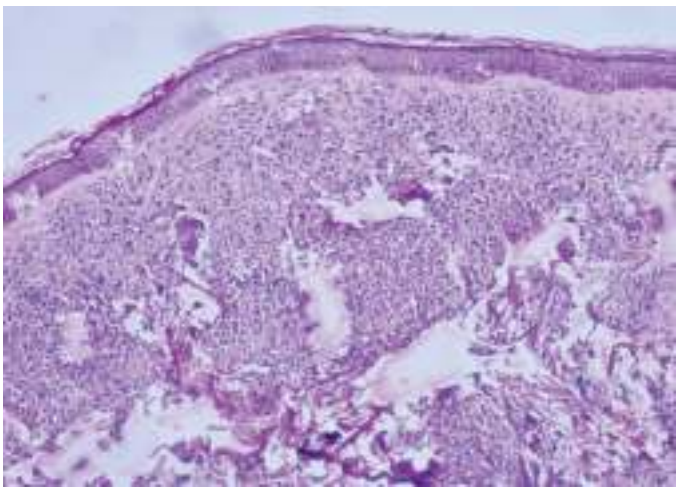


Fig. 7.37: Histology of histoid leprosy. It is a variant of lepromatous leprosy. In some cases of lepromatous leprosy, histiocytes are spindle-shaped and arranged in storiform pattern mimicking fibrous histiocytoma around blood vessels and adnexal structures (400X). (*Courtesy*: Dr. Geeta Pachori, Senior Professor and Head of Pathology, Jawahar Lal Nehru Medical College, Ajmer, Rajasthan.)

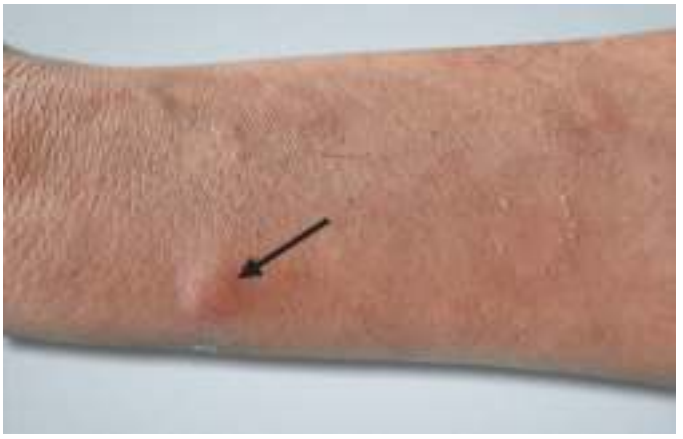


Fig. 7.38: Clinical photograph of erythema nodosum leprosum. The skin lesions are characterized clinically by tender red plaques and nodules together with areas of erythema. The eruption is generally widespread and accompanied by fever, malaise and arthralgia (arrow). (*Courtesy*: Dr. Krishna Deb Barman, Director Professor of Dermatology, Maulana Azad Medical College and Associated Lok Nayak Hospital, New Delhi.)

Mitsuda Reaction

Mitsuda reaction measures the granulomatous immune response to intradermally injected heat killed leprosy bacilli. A positive Mitsuda reaction is described as an indurated skin lesion of more than 4 mm that histologically shows noncaseating epithelioid cell granuloma formation after 3–4 weeks at the site of injection. A positive Mitsuda reaction corresponds to the acquisition of cell-mediated immunity against the *Mycobacterium leprae* and occurs in tuberculoid leprosy patients. Borderline leprosy patients show an indurated skin lesion of less than 3 mm.

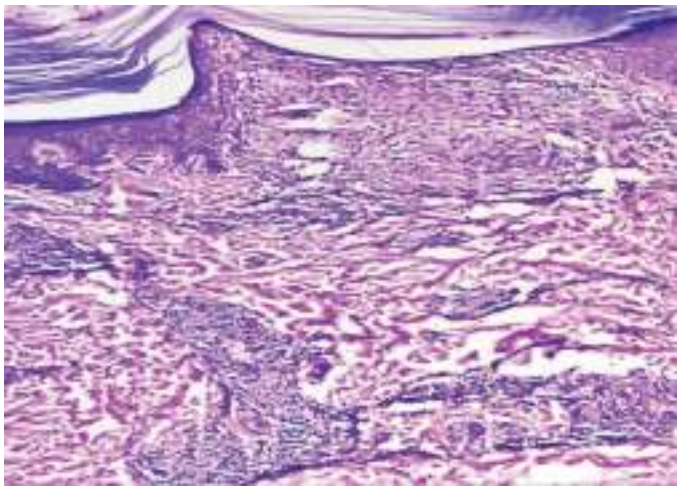


Fig. 7.39: Histology of erythema nodosum leprosum (ENL). Histologic examination of H&E-stained sections demonstrates an inflammatory neutrophilic infiltrate, vasculitis and/or panniculitis, immune-complex deposit and complement deposit associated with antigens of *Mycobacterium leprae* (400X). (*Courtesy*: Dr. Geeta Pachori, Senior Professor and Head of Pathology, Jawahar Lal Nehru Medical College, Ajmer, Rajasthan.)

DIAGNOSTIC APPROACH

Diagnostic approach includes clinical examination of skin patches with definite sensory impairment, peripheral nerves enlargement, skin biopsy and positive skin smears for AFB. Involvement of nerves is seen in 100% of patients. The type C fibers which mediate sensations towards heat and cold or different temperatures are lost first before loss of pain or light touch fibers.

Histologic Examination of Skin Biopsy: Tuberculoid (TT) Leprosy and Lepromatous Leprosy (LL)

Histopathologic examination of skin biopsy is performed. Fite stain is done to demonstrate leprae bacilli.

Tuberculoid (TT) Leprosy

- **Well-formed noncaseating epithelioid cell granulomas:** Skin biopsy from patient with tuberculoid leprosy shows discrete noncaseating epithelioid cell granulomas surrounded by zone of lymphocytes. Langhans' giant cells are demonstrated.
- **Absence of grenz zone (clear zone):** There is absence of clear zone at the junction of epidermis and dermis in tuberculoid leprosy. Clear zone is demonstrated in lepromatous leprosy.
- **Perineural inflammation:** Histopathologic examination of dermal nerve twigs shows infiltration by lymphocytes and macrophages, which indicates destruction of nerves responsible for sensory loss.
- **Fite stain:** *Mycobacterium leprae* is usually negative on Wade-Fite stain.

Lepromatous Leprosy (LL)

- **Epidermis and dermis:** Epidermis shows atrophy. There is loss of rete ridges of epidermis. Dermal papillae are flattened. There is loss of dermal appendages.
- **Grenz zone (clear zone):** Clear zone is demonstrated at the junction of epidermis and dermis in lepromatous leprosy.
- **Foamy macrophages:** Dermis shows collection of macrophages arranged in nodular or diffuse patterns.
- **Fite stain:** Macrophages in dermis are laden with *Mycobacterium leprae* on Wade-Fite staining.

TREATMENT

Many different agents available for treating leprosy. According to currently WHO recommendations, leprosy is treated based on tuberculoid or lepromatous leprosy:

- **Tuberculoid leprosy:** Dapsone 100 mg daily and rifampicin 600 mg monthly for 6–9 months are administered in these patients.
- **Lepromatous leprosy:** Dapsone 100 mg daily; clofazimine 150 mg, and rifampicin 600 mg monthly for 12–18 months are administered in these patients.

SPIROCHETES

Spirochetes are gram-negative, motile, spiral bacteria, which have axial filaments depending on the species. These cause syphilis. Lyme disease, relapsing fever and leptospirosis. Most of the spirochetes are characterized by their distinct shapes and unique motility.

SYPHILIS

Syphilis is a systemic bacterial disease caused by *Treponema pallidum* affecting persons across world. *Treponema pallidum* is the only organism that causes venereal disease transmitted through sexual contact, anogenital and orogenital contact.

- Primary and secondary syphilis during pregnancy lead to neonatal infection and adverse pregnancy outcomes if not timely treated. Vertical transmission occurs via transplacental route resulting in congenital syphilis. Sir Jonathan Hutchinson from England described a triad in late congenital syphilis consisting of notched incisors, interstitial keratitis and eighth cranial nerve deafness.
- The infection progresses through four stages that can affect many organs. There are many histopathologic features of syphilis such as interstitial chronic inflammatory lymphoplasmacytic infiltrate, endothelial swelling, irregular acanthosis, elongated rete ridges. Methenamine silver stain can detect spirochetes at any site in 30–70% of cases. Immunohistochemistry

has a sensitivity of 70% of accuracy in identification of the *Treponema pallidum*.

- Fortunately, spirochete *Treponema pallidum* is still sensitive to penicillin. The organism has a rich outer phospholipid membrane and slow metabolic rate as it takes an average of 30 hours to multiply.
- Syphilis is an important synergistic infection for human immunodeficiency virus (HIV) acquisition and has been closely linked with HIV. Untreated cases affect the course of HIV infection with higher virus replication and lowers CD4+ helper T cells counts and a rapid progression to late syphilis.

Pathophysiology

Treponema pallidum is a very tiny organism that is not visible on light microscopy. Thus, it can be identified by its distinct spiral movements on darkfield microscopy. The organism cannot survive outside the body.

Primary Syphilis

Patient presents with a solitary nontender genital chancre in response to invasion by the *Treponema pallidum*. However, patient can have multiple nongenital chancres on digits, nipples, oral mucosa and tonsils accompanied by nontender lymphadenopathy. The lesion primary syphilis may heal by scarring without scarring.

Secondary Syphilis

After 3–12 weeks from the resolution of a chancre, untreated primary syphilis can progress to secondary syphilis via hematogenous dissemination of the spirochetes, which has many clinical and histopathologic findings.

- Patient presents with condylomata lata (papulosquamous eruptions with central surface and a white ring of a scaling edge known as Biett's collarette) on hands and feet, macular rash and lymphadenopathy, headache, myalgia, malaise, arthralgia, pharyngitis, alopecia, and hepatosplenomegaly.
- The secondary lesion in secondary syphilis may heal without scarring. The infection can be detected at this stage with serological testing.

Tertiary Syphilis

Untreated patients in secondary syphilis stage progress to the tertiary syphilis stage, characterized by irreversible cardiovascular syphilis, neurosyphilis and latent benign syphilis. The incubation period is about 20–90 days.

- **Cardiovascular syphilis:** Cardiovascular syphilis can manifest as syphilitic aortitis, aortic valve regurgitation, cardiomegaly **'cor bovinum',** carotid **ostial stenosis** and granulomatous lesions (gummas) in various organs.
- **Meningovascular syphilis (neurosyphilis):** *Treponema pallidum* does not invade the central nervous system early, but symptoms appear late in tertiary syphilis, neurosyphilis can manifest as meningitis, cerebral stroke, cranial nerve palsies during early neurosyphilis or tabes dorsalis, dementia, general paresis during late neurosyphilis.
- **Latent benign syphilis:** It is the stage at which there are no clinical signs of syphilis and the cerebrospinal fluid is normal. Latent benign syphilis begins when the first attack of secondary syphilis passed and that persists for lifelong, which is usually detected by reactive serologic tests for syphilis.

Congenital Syphilis

Congenital syphilis occurs if *Treponema pallidum* spirochete is transmitted from the mother to the fetus. If vertical transmission of infection occurs within the first trimester of pregnancy, the consequences may be premature delivery, spontaneous abortion, stillbirth, nonimmune hydrops or perinatal mortality. If vertical transmission of infection during second to third trimesters of pregnancy, newborns appear healthy and develop clinical manifestations months to years later. Thus, congenital syphilis may be differentiated in early congenital syphilis (clinical manifestations ≤2 years of age) or late congenital syphilis (clinical manifestations ≥2 years of age).

- **Early congenital syphilis:** Clinical manifestations of early congenital syphilis include skin exfoliative rash (pemphigus syphiliticus), splenomegaly, lymphadenopathy, condylomata lata, chorioretinitis, cataracts, periostitis, osteochondritis, nephrotic syndrome, myocarditis, pancreatitis, malabsorption syndrome and hypopituitarism (diabetes insipidus).
- **Late congenital syphilis:** Clinical manifestations of late congenital syphilis include Hutchinson's triad (i.e. notched teeth, Mulberry molar teeth, interstitial keratitis), healed chorioretinitis, intellectual disability, hydrocephalus, seizures, optic nerve atrophy, cranial nerve palsies, juvenile general paresis, frontal bossing, saddle nose deformity, protuberant mandible, short maxilla, high palatal arch, saber shin, sternoclavicular joint thickening (Higouménakis' sign) and Clutton joints (swelling of synovial membrane).

Prognosis

Prognosis of syphilis depends on the stage and extent of organ involvement. Untreated cases have significant

morbidity and mortality. These patients usually develop cardiovascular syphilis and neurosyphilis, which can be fatal. Congenital syphilis is associated with miscarriage, stillbirth and fulminant pulmonary hemorrhage in neonates. Without treatment during pregnancy, syphilis is almost always transmitted to the fetus.

LYME DISEASE

Lyme disease is caused by four main species of bacteria *Borrelia burgdorferi* and *Borrelia mayoni* cause Lyme disease in the United States while *Borrelia afzelii* and *Borrelia garinii* cause Lyme disease in Europe and Asia. Lyme disease is transmitted by the bite of an infected black-legged tick, commonly known as a deer tick. Disease is more common in persons, who spend time in grassy and heavily wooded regions where blacklegged ticks bite the persons to cause Lyme disease.

Clinical Features

Initially patient presents with skin rash (erythema migrans) within 3–30 days after tick bite, fever, chills, fatigue, body aches, headache, neck stiffness, and lymphadenopathy. Skin rash may expand and attain 30 cm in size. Untreated cases develop symptoms within weeks to months such as skin rash (erythema migrans) on the other regions of body, migratory severe joint pain and swelling and neurological symptoms (e.g. meningitis and Bell's palsy).

Complications

Untreated Lyme disease can cause arthritis of the knee, neurological symptoms such as facial palsy and neuropathy, cardiac arrhythmia and cognitive defects such as impaired memory.

ANAEROBIC BACTERIA

Anaerobic bacteria do not grow in the presence of oxygen, which are most commonly found in gastrointestinal tract. They play a key role in pathogenesis of appendicitis, diverticulitis and perforation of the bowel. Anaerobes are classified as facultative, microaerophilic and obligate types.

- Facultative anaerobes preferentially utilize oxygen as terminal electron acceptor and metabolize in the absence of oxygen by reducing other compounds.
- Microaerophilic anaerobes can only proliferate in low concentration of oxygen (2–10%).
- Obligatory anaerobes are completely incapable of aerobic metabolism but they are variably tolerant to oxygen.

ANAEROBIC BACTERIAL ABSCESSES

Anaerobes can cause a perirectal abscess or facial abscess in children, or dental abscess.

- **Brain abscess:** Anaerobes are commonly isolated from brain abscesses, which result from a complication of sinusitis, otitis media and dental infections. Anaerobes commonly isolated are Fusobacterium, Bacteroides and Prevotella.
- **Intra-abdominal abscess:** It is caused by mixed anaerobic and aerobic organisms. Perforated appendix gives enteric anaerobic abscess leading to peritonitis. The common anaerobic bacteria implicated in abdominal infections are Bacteroides, Lactobacillus and *Clostridium* species.
- **Liver abscess:** The common anaerobic bacteria implicated in liver abscess include Bacteroides and *Fusobacterium* species.
- **Pelvic inflammatory disease:** Anaerobic bacteria are implicated in pelvic inflammatory disease (PID). Common anaerobe bacteria implicated in pelvic inflammatory disease are Prevotella, *Clostridium* species and Porphyromonas.
- **Pulmonary infections:** Pulmonary infections are common in children, who lack normal cough reflex, or they suffer from cerebral palsy and tracheoesophageal malformations. Aspiration results in pneumonia, which can develop into pulmonary abscess if untreated. The predominant pathogens involved in aspiration pneumonia are part of the oropharyngeal flora such as Fusobacterium, *Bacteroides fragilis* and Prevotella and Peptostreptococcus.
- **Skin and soft tissue infections:** Anaerobic bacteria can cause perirectal abscess or facial abscess in children. Perirectal abscess is caused by *Bacteroides fragilis* and *Clostridium* species. Oral infections are caused by Prevotella, Porphyromonas and Fusobacterium.

CLOSTRIDIAL INFECTIONS

Life-threatening soft tissue infections are caused by anerobic bacteria *Clostridium* species found in soil, which enter the human body via punctured wounds. Clostridium bacteria produce spores that can survive in the environment for a very long time and can cause diseases in human beings such as tetanus (*Clostridium tetani*), botulism (*Clostridium botulinum*) and **gas gangrene** (*Clostridium perfringens*).

Tetanus (Clostridium Tetani)

Tetanus is an infection caused by bacterium called *Clostridium tetani*. The incubation period is usually

between 3 and 21 days (average 10 days). Most cases acquire tetanus within 14 days of wound contaminated by *Clostridium tetani*. In general, tetanus has shorter incubation period due to heavily contaminated wounds, which is linked to serious disease with fatal outcome.

Mode of Transmission

Spores of *Clostridium tetani* are present in the environment such as soil, dust and manure. The spores can enter the body from contaminated objects through broken skin such as punctured wounds, burns, crush injuries, scraping of superficial wounds, surgical procedures, compound fractures and intravenous drug addicts. The spores of *Clostridium tetani* develop into bacteria.

Clinical Features

Clostridium tetani produce toxin that causes painful skeletal muscle contractions. The most common initial sign is spasms of the muscles of neck and jaw resulting in 'lockjaw' of patients.

- Patient presents with jaw cramping, sudden involuntary muscle spasms, painful skeletal muscle stiffness all over the body, difficulty in swallowing, jerking or seizures, headache, fever, sweating, changes blood pressure and tachycardia.
- Vaccines are recommended to infants, children, adolescents and adults to prevent tetanus.

Botulism (Clostridium Botulinum)

Clostridium botulinum is an anaerobic, rod-shaped spore forming bacterium, which synthesizes and releases potent neurotoxin (eight distinct toxins designated types A through H) that can cause food poisoning 'botulism' arising from improperly sterilized food products. Botulism is classified into various types: (a) food-borne botulism is associated with home-canned or fermented foods, (b) infant botulism is associated with honey ingestion, (c) wound botulism is associated with injection drug use of black-tar' heroin, (d) iatrogenic botulism occurs due to use of botulism toxins A and B for therapeutic and cosmetic purpose, and (e) botulism is a potent bioterrorism agent deployed by aerosol or ingestion.

Clinical Features

More than 90% of patients with botulism have 3–5 of the following signs and symptoms such as nausea, vomiting, dysphagia, skeletal muscle weakness, difficulty in breathing, diplopia (double vision), blurred vision, drooping of eyelids, dilated/fixed pupils, difficulty in moving eyes, and an extremely dry mouth.

Laboratory Diagnosis

Presumptive diagnosis of botulism is based on clinical presentation such as acute onset of bilateral cranial neuropathies with symmetrical descending weakness.

- Mouse bioassay is gold standard for demonstration of botulinum toxin, in which patient samples are injected intraperitoneally. The presence of botulinum toxin results in rapid development of disease symptom such as skeletal muscle weakness and respiratory failure within 1–5 days.
- Culture of serum, stool and environmental samples requires strict anaerobic conditions. Characteristic electrophysiological study findings are suggestive of botulism.

Gas Gangrene (Clostridium Perfringens)

Clostridium perfringens synthesizes and releases exotoxin that can cause gas gangrene (also called clostridial myonecrosis), enteritis necroticans and food poisoning in human beings.

Pathogenesis

As *Clostridium perfringens* grows inside the body, it produces gas and toxin that can damage body tissue cells and blood vessels. Gas gangrene develops suddenly at the site of trauma following surgery, most often in the gastrointestinal tract or biliary tract and following aseptic abortions. *Clostridium perfringens*, *Clostridium septicum* and *Clostridium histolyticum* are the principal causes of trauma-associated gas gangrene especially during wars, hurricanes, earthquakes and mass-casualty conditions.

Clinical Features

Gas gangrene can occur anywhere on the body, which most commonly affects arms or legs. Patient presents with fever, tachycardia, and air under the skin. The skin in the affected becomes pale and later changes to dark red or purple. These symptoms usually develop 6–48 hours after initial infection with *Clostridium perfringens* and progress rapidly.

Treatment

Patients are treated by administration of antibiotics and surgical removal of dead tissue. Surgery consists of debridement and sometimes amputation.

OBLIGATE INTRACELLULAR BACTERIA

Obligate intracellular bacteria cannot live outside the host cell. Examples of obligate intracellular bacteria are *Chlamydia trachomatis* and *Rickettsia*. Chlamydial cells are unable to carry out energy metabolism and lack biosynthetic pathways and therefore are dependent on

the host cell to supply them with adenosine triphosphate (ATP) and other intermediate products. The rickettsia is found in ticks, fleas, mites and mammals.

CHLAMYDIAL INFECTIONS

Chlamydia disease is sexually transmitted infection caused by bacterium *Chlamydia trachomatis* in men and women. Many persons, who have Chlamydia, do not develop symptoms, but they can still infect others through sexual contact. Untreated cases can develop serious complications later.

- *Chlamydia trachomatis* infection in women may induce genital pain, discharge from vagina, trachoma, lymphogranuloma venereum, cervicitis, salpingitis and pelvic inflammatory disease. On the contrary, men present with burning sensation during urination, yellow or green discharge from the penis, pain in lower abdomen and pain in the testes.
- *Chlamydia trachomatis* is treated by azithromycin in single dose. Doxycycline antibiotic is administered twice daily for about one week.

RICKETTSIAL INFECTIONS

Rickettsial infections are caused by unusual bacteria that can live and multiply only inside the cells of host and cannot survive on their own in the environment. Many species of these bacteria live in human beings, rats, cattle, sheep and goats. Most of Rickettsial infections are transmitted through bite of ticks, mites, fleas, or lice. Each species of rickettsiae and Rickettsia-like bacteria has its own host and usually vectors. Some of these bacteria occur across world.

- Human beings are the usual host for *Rickettsia prowazekii*, which causes epidemic typhus. Hosts may or may not be ill from the infection.
- Some of these bacteria infect vascular endothelial cells of small blood vessels causing occlusion or bleeding into the surrounding tissue.
- *Coxiella burnetii* reside in cattle, sheep or goats, which cause Q fever, which can be transmitted through the air or in contaminated food and water. The bacteria do not require a vector. Patient presents with severe headache and skin rash. The patients are treated by antibiotics.

Rocky Mountain Spotted Fever

Rocky Mountain spotted fever is a bacterial infection caused by *Rickettsia rickettsii* and transmitted by a tick. The disease is most prevalent in Southeastern part of the United States and other parts of Canada, Mexico, Central America and South America.

- Initially patient presents with severe headache, high-grade fever, chills, muscle aches, nausea, vomiting, confusion and neurological symptoms. A few days later, a red, nonpruritic skin rash appears on the wrists and ankles.
- Rocky Mountain spotted fever responds to prompt treatment with antibiotics. Untreated cases can cause serious damage smallest blood vessels to internal organs such as heart, lungs, kidneys, brain.
- In addition to severe headache, it can cause encephalitis, which can cause confusion, seizures and delirium.
- It can cause inflammation of heart and lungs leading to cardiopulmonary failure in severe cases.
- It can damage small blood vessels supplying kidneys leading to renal failure.
- It can damage small blood vessels supplying fingers and toes. Amputation of fingers and toes would then be necessary. About 80% of untreated cases, who have fatal outcome.

Epidemic Typhus

Epidemic typhus is potentially lethal, louse-borne disease caused by *Rickettsia prowazekii*. Disease has also been called camp fever, jail fever, and war fever that occurs due to overcrowding and improper sanitation. Patient presents with prolonged high-grade fever, chills, intractable headache, rapid breathing, body and skeletal muscle aches, maculopapular rash, cough, nausea, and vomiting.

Ehrlichiosis

Ehrlichiosis is caused by several forms of the bacterium, which affects human beings through the bite of tick on skin surface. Rarely, blood transfusions or organ transplants infected with the bacterium have caused ehrlichiosis. Without treatment, this infection may cause serious complications.

- Patient develops symptoms a week or more after an infected tick bites a person. Severity of disease varies from mild to severe.
- Patient presents with flu-like symptoms such as fever, chills, muscle aches, skin rash, fatigue, diarrhea, nausea, vomiting, headache, confusion and red eyes (more in children).
- Severe ehrlichiosis may cause seizures, breathlessness, organ failure and superinfections from viruses and fungi. Patients are diagnosed by taking careful history, physical examination, biochemical tests and hemogram.

Anaplasmosis

Anaplasmosis is a disease caused by the bacterium *Anaplasma phagocytophilum* transmitted by tick bites

primarily from the blacklegged tick (*Ixodes scapularis*) and the Western blacklegged tick (*Ixodes pacificus*). Patient presents with fever, headache, chills and muscle aches within 1–2 weeks after the bite of an infected tick.

- Tick bites are usually painless. Persons with anaplasmosis will initially present with fever, chills, headache, skeletal muscle aches, nausea, vomiting, loss of appetite and diarrhea.
- Later, these patients can develop severe illness such as respiratory failure, organ failure, bleeding tendencies and fatal outcome.

Q Fever (Coxiella Burnetii) Infection

Q fever is a disease caused by the bacterium *Coxiella burnetii*, which is usually as mild disease with flu-like symptoms. Many persons remain asymptomatic. However, in a small percentage of persons, the infection can resurface years later.

- Patient presents with fever, chills, sweats, fatigue, headache, skeletal muscle aches, nausea, vomiting, diarrhea, chest pain and pain in gastric region.
- Q fever is diagnosed by indirect immunofluorescence antibody using *Coxiella burnetii* antigen to demonstrate a significant rise of antibody titer.

VIRAL INFECTIONS

VIRAL INFECTIONS:OVERVIEW

Viruses are infectious particles lacking organelles. A virus particle comprises nucleic acid core either DNA or RNA and not both and is surrounded by a protein shell, or capsid. Combination of nucleic acid and capsid is called **nucleocapsid**. Depending on the viral morphology, viruses can be categorized into complex, enveloped or naked. Basic types of viral morphology may be complex, enveloped or naked given in Table 7.18. Important human DNA and RNA virus-induced diseases are given in Table 7.19.

Table 7.18 Basic types of viral morphology

Basic Type of Virus	Examples
Complex viruses	▪ Poxvirus (large DNA virus) ▪ Flexible-tailed bacteriophage
Enveloped viruses	▪ With a helical nucleocapsid (e.g. mumps virus and rhabdovirus) ▪ With an icosahedral nucleocapsid (e.g. herpesvirus and HIV)
Naked viruses	▪ Helical capsid (e.g. plum poxvirus) ▪ Icosahedral capsid (e.g. poliovirus and papillomavirus)

Table 7.19 Important human DNA and RNA virus-induced diseases

Common Name of Genus Members	Name of Disease
DNA viruses	
Variola	Smallpox
MCV1 and MCV2	Molluscum contagiosum
Herpes simplex 1 (HSV-1)	Fever, blister, cold sores
Herpes simplex 2 (HSV-2)	Genital herpes
Varicella-zoster virus	Chickenpox
Human cytomegalovirus	CMV infections
Human adenovirus	Adenovirus infections
Human papillomaviruses	Several types of warts
JC virus (human polyomavirus 2)	Progressive multifocal leukoencephalopathy
Hepatitis B virus (HBV or Dane particle)	Serum hepatitis
Parvovirus B19	Erythema infectiosum (slapped cheek syndrome)
RNA viruses	
Poliovirus	Poliomyelitis
Coxsackievirus	Hand-foot-mouth disease
Hepatitis A virus	Short-term hepatitis
Human adenovirus	Common cold, bronchitis

Contd...

Table 7.19 Important human DNA and RNA virus-induced diseases (*Contd...*)

Common Name of Genus Members	Name of Disease
Norwalk virus	Viral diarrhea, Norwalk vial syndrome
Yellow fever virus	Yellow virus
Rubella virus	Rubella (German measles)
Dengue fever virus	Dengue
Ebola Marburg virus	Hemorrhagic fever
Human rotavirus	Rotavirus gastroenteritis
Influenza type A virus	Influenza or flu
Mumps virus	Mumps
Measles virus	Measles
Rabies virus	Rabies (hydrophobia)
Human T cell leukemia virus (HTLV)	T cell leukemia
HIV (human immunodeficiency virus 1 and virus 2)	Human immunodeficiency syndrome (AIDS)
SARS virus	Severe acute respiratory syndrome

Clinical Pearls: Viral Infections—Clinical Course

Depending on clinical course of viral diseases, viral infection can be acute, persistent and transforming viral infections.

Acute (Transient) Viral Infection

An acute viral infection is characterized by rapid onset of disease (common flu, respiratory tract infection), which persists for a brief period and resolves within days.

Persistent Viral Infection

Persistent viral infections occur when the virus is not cleared but remains in specific cells of infected persons. Persistent viral infections may involve stages of both silent and productive infection without rapidly fatal disease or even producing excessive damage of the host cell. There are three types of overlapping persistent virus–host interaction that may be defined as latent, chronic and slow viral infection.

- **Latent viral infection:** A latent viral infection occurs when the virus persists in the body in dormant or inactive state and does not replicate within the host.
- **Chronic viral infection:** Chronic viral infection underlies important diseases that either follow directly from primary infection or may require months, years, or even decades to develop. Pathogens associated with significant disease include hepatitis C virus, human immunodeficiency virus (HIV) and a number of herpesviruses.
- **Slow viral infection:** Slow viral infection is characterized by a prolonged incubation period followed by progressive disease.

Transforming Viral Infection

Transforming viral infections are also referred to as malignant tumor-forming viruses, which can be either cytotoxic (usually in the case of RNA viruses) or persistent (usually in the case of DNA viruses). Integration of HBV (hepatitis B virus), HPV (high-risk human papillomavirus) and MCV (Merkel cell polyomavirus) into the human genome induces cellular and viral responses and further contributes to carcinogenesis.

- **Hepatitis B virus (HBV):** HBV integrates into the host genome that results in elevated expression of cellular cancer-related genes such as TERT (telomerase reverse transcriptase), MLL4 (mixed-lineage) and CCNE1. Integrated HBV sequences encode HBx and/or truncated envelope pre-S2/S proteins. These cellular alterations cause genomic instability and cell cycle deregulation contributing to carcinogenesis.
- **Human papillomavirus (HPV):** HPV integrates into the host genome that results in disruption of E2 resulting in significantly increased expression of the E6 and E7 oncoproteins. These cellular alterations cause deregulated cellular proliferation, inhibition of apoptosis, and increased genomic instability.
- **Merkel cell polyomavirus (MCV):** MCV integrates into the host genome that results in clonal expansion of tumor cells contributing to Merkel cell carcinoma.

PATHOGENESIS

Virus enters human cell leading to formation of complete viral particles. Enveloped virus attaches to its host cell by specific binding of its spikes to cell receptors.

- The virus is engulfed into a vesicle leading to uncoating of its envelope and freeing the virus RNA into the cell's cytoplasm.
- Under the control of viral genes, the cell synthesizes the basic components of new viruses such as RNA molecules, capsomers and spike proteins. Nucleocapsid is formed from RNA and capsomers.
- Enveloped virus is released by budding of the cell membrane carrying away an envelope with spikes. This released complete virus is ready to infect another human cell.
- Released virus infects another human cell and induces cytopathic changes. Viral cytotoxicity is either direct or immunologically mediated.

- Major events in the multiplication cycle of an enveloped RNA virus in human are shown in Fig. 7.40. Cytopathic changes in selected virus infected cells are given in Table 7.20.

PROTECTION OF CELLS AGAINST VIRUSES

Both humoral and cellular arms of the immune system protect against the harmful effects of viral infections. Presentation of viral proteins to immune

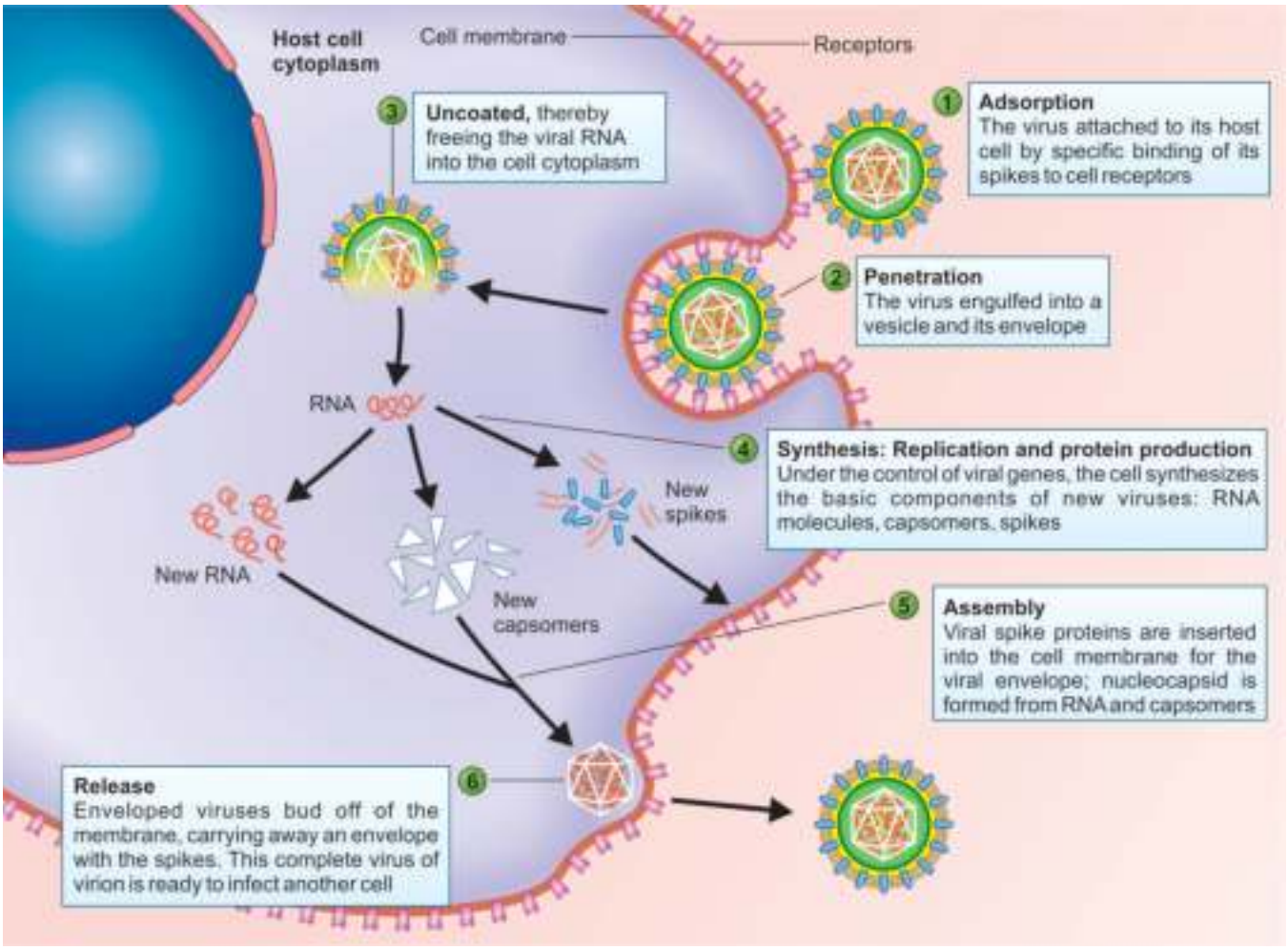


Fig. 7.40: Major events in the multiplication cycle of an enveloped RNA virus in human.

Table 7.20 Cytopathic changes in selected virus infected cells

Virus	Shape of Cell	Inclusions in Cytoplasm or Nucleus
Smallpox virus	Cell round	Cytoplasm
Herpes simplex virus	Cells fuse to form multinucleated syncytium	Nucleus
Adenovirus	Clumping of cells	Nucleus
Poliovirus	Cell enlarged	Absent
Influenza virus	Cell round	Absent
Rabies virus	No change in cell shape	Cytoplasm (Negri bodies)
Measles virus	Multinucleated cell forming syncytium	Absent
Cytomegalovirus	Cell enlarged	Nucleus (owl eye inclusion)

system immunizes the body against the invader and elicits natural killer cells and production of antiviral antibodies. Humoral and cellular arms of the immune system eliminate virus-infected cells by either inducing apoptosis or directing complement-mediated cytolysis.

RNA VIRUSES

RNA virus contains single-stranded RNA and double-stranded RNA as its genetic material. RNA viruses have high mutation rates when compared to DNA viruses, because viral RNA polymerases lack the proofreading ability of DNA polymerases.

- The genetic diversity of RNA viruses is the main reason why it is difficult to prepare effective vaccine against them.
- Human diseases caused by RNA viruses include hepatitis C virus, Ebola virus, Marburg virus. Severe acute respiratory syndrome (SARS) caused by coronavirus, influenza, polio, measles, orthomyxovirus, retrovirus including adult human T cell lymphotrophic type 1 (HTLV-1) and human immunodeficiency virus (HIV).

SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 (SARS-COV-2) FOR COVID-19 PANDEMIC

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the pathogen responsible for the coronavirus disease 2019 (COVID-19) disease across world, which has resulted in global healthcare crises and strained health resources. COVID-19 disease is now recognized as a multiorgan disease with a broad-spectrum of clinical manifestations.

- The spike protein (S) plays a key role in the receptor recognition and cell membrane fusion. The spike protein (S) of SARS-CoV-2 is composed of two subunits, S1 (receptor-binding domain) and S2 (viral cell membrane fusion domain). Most persons experience mild-to-moderate respiratory illness and recover without requiring special medical treatment.
- Elderly persons and those with underlying medical illness like diabetes mellitus, cardiovascular disease, chronic respiratory diseases, and cancer are more prone to develop serious illness.
- COVID-19 disease spreads primarily through droplets of saliva or discharge from the upper respiratory tract when an infected person coughs or sneezes on the surfaces.
- Best way to prevent and slow down transmission of COVID-19 by avoiding contact with these infected patients and washing hands or using an alcohol-based rub and not touching face.

SARS-CoV-2 Virus Genomic Arrangement

Single-stranded RNA genome of SARS-CoV-2 virus has ~3000 nucleotides and consists of two large genes: ORF1a and ORF1b. SARS-CoV-2 virus encodes 16 nonstructural proteins (NSP1–NSP16) and four structural proteins: (a) nucleocapsid (N) protein, (b) membrane (M) protein, (c) envelope (E) protein, and (d) spike (S) protein. Schematic genomic representation of the SARS-CoV-2 virus is shown in Fig. 7.41.

Structural Proteins

Structural genes of the SARS-CoV-2 virus encode structural proteins: (a) nucleocapsid (N) protein, (b) membrane (M) protein, (c) envelope (E) protein and (d) spike (S) protein.

- **Nucleocapsid (N) protein:** SARS-CoV-2 virus nucleocapsid (N) is a structural protein that forms complexes with genomic RNA, which interacts with the viral membrane (M) protein during virion assembly and plays a pivotal role in enhancing the efficiency of SARS-CoV-2 virus transcription and assembly in host cells. Recent studies have confirmed that nucleocapsid (N) protein is a multifunctional protein.
- **Membrane (M) protein:** The membrane protein plays a key role in virus assembly, turning cellular membranes into workshops where SARS-CoV-2 virus and host factors come together to produce new virus particles.
- **Envelope (E) protein:** The envelope protein is a small, integral membrane protein involved in several aspects of SARS-CoV-2 virus life cycle such as assembly, budding, envelope formation, pathogenesis, and virus release.
- **Spike (S) protein:** Spike protein consists of the S1 subunit (14–685 residues) and S2 subunit (686–1273 residues), which are responsible for receptor binding and membrane fusion, respectively. Spike protein is located on the surface of envelope by which spike protein binds to the receptor. S1 domain contains the RBD, which is mainly responsible for binding of the receptor on host cell, while S2 domain mainly contains the HR domain, including HR1 and HR2, which is closely related to virus.
  - **S1 subunit of spike protein:** The binding of SARS-CoV-2 virus particle with the help of spike protein S1 subunit to the cell ACE2 receptors on the surface of the host cell distributed on lung (alveolar type 2), heart and intestine, that results in the formation of endosomes, which triggers viral fusion activity under low pH. Therefore, receptor is an important determinant of viral entry and a drug design target for therapy.

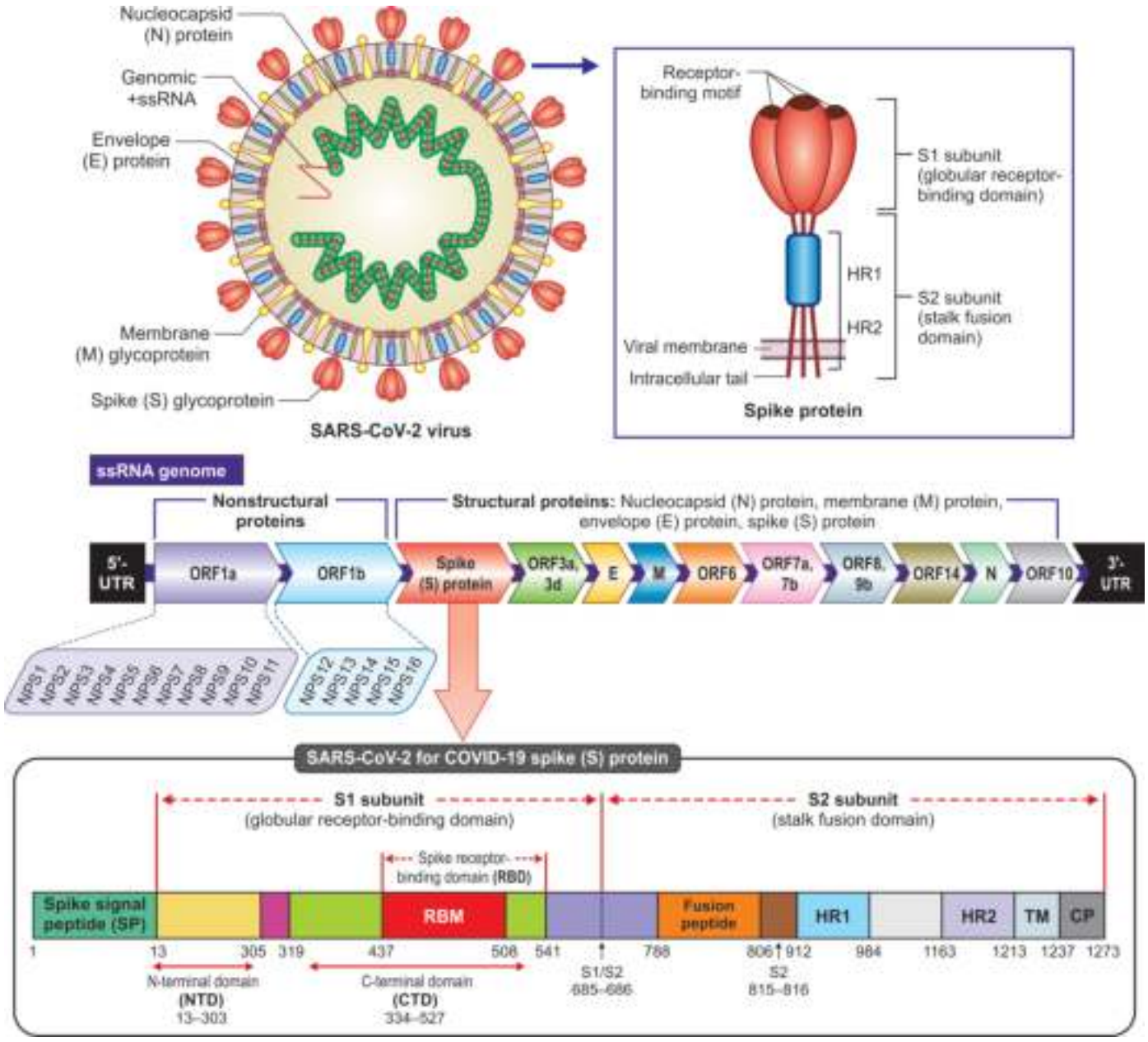


Fig. 7.41: Schematic genomic representation of the SARS-CoV-2 virus. The genomic organization of the SARS-CoV-2 demonstrates sequential arrangement of various structural, nonstructural and accessory genes as follows: 5'-cap-leader UTR-replicase spike (S), envelope (E), membrane (M), nucleocapsid (N)-3'UTR-poly(A) tail with accessory genes such as ORF3a, ORF3d, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF14, and ORF10 interspersed among the structural genes preceding 3'end of the viral RNA genome. The spike (S) protein binds to the receptor ACE2 expressed on lung, heart and intestine, which mediates virus cell binding and its fusion with host cell fusion.

- **S2 subunit of spike protein:** Spike membrane S2 unit of virus is composed of a fusion peptide (FP), heptapeptide repeat sequence 1 (HR1), heptapeptide repeat sequence 2 (HR2), TM domain, and cytoplasmic domain fusion (CT) is responsible for viral fusion and entry.

Nonstructural Proteins (NSPs)

Apart from aforementioned structural proteins there are several nonstructural proteins in SARS-CoV-2 virus, namely NSP-1 to NSP-10 and NSP-12 to NSP-16 encoded by genes located within the 5'-region of viral RNA genome. Nonstructural proteins (NSPs) of severe acute respiratory distress syndrome (SARS)-CoV-2 virus for COVID-19 disease are given in Table 7.21.

Accessory Proteins

There are nine accessory proteins in SARS-CoV-2: ORF3a, ORF3b, ORF3d, ORF6, ORF7a, ORF7b and ORF8, ORF9b, ORF14 and ORF10 encoded by ORF

Table 7.21 Nonstructural proteins (NSPs) of severe acute respiratory distress syndrome (SARS)-CoV-2 virus for COVID-19 Disease

Nonstructural Proteins (NSPs)	Proteins	Comments
NSP1	N-terminal product of the viral replicase	SARS-CoV-2 NSP1, is also known as leader protein that binds the ribosomal mRNA channel to inhibit translation and also degrade host mRNA
NSP2	N-terminal product of the viral replicase	▪ NSP2 binds to two host proteins such as prohibitin 1 (PHB1) and prohibitin 2 (PHB2) and disrupts the host cell environment ▪ Normally, PHB1 and PHB2 play roles in cell cycle progression, cell migration, cellular differentiation, apoptosis and mitochondrial biogenesis
NSP3	Papain-like protease	NSP3 is responsible for release of NSP1, NSP2 and NSP3 from the N-terminal region of pp1a and pp1ab
NSP4	Membrane spanning protein containing trans-membrane domain 2	NSP4 plays key role in viral replication–transcription complex and helps modify endoplasmic reticulum membrane
NSP5	Proteinase	NSP5 cleaves at multiple distinct sites to yield mature and intermediate nonstructural proteins
NSP6	Putative transmembrane domain	▪ NSP6 generates autophagosomes from endoplasmic reticulum (ER) as well as double membrane vesicles ▪ Autophagosomes facilitate assemble of replicase proteins ▪ NSP6 limit autophagosome/lysosome expansion, which in turn prevents autophagosomes from delivering viral components for degradation in lysosomes
NSP7	RNA-dependent RNA polymerase	NSP7 is required to form a complex with NSP8 and NSP12 to yield the RNA polymerase activity of NSP8
NSP8	Multimeric RNA polymerase; replicase, single-stranded	NSP8 makes heterodimer with NSP8 and NSP12, which ultimately forms the RNA polymerase complex
NSP9	RNA-binding viral protein	NSP9 interacts with the DEAD-box RNA helicase 5 (DDX5) cellular protein and plays role in viral replication
NSP10	Growth factor protein consists of 13 amino acids and possesses two zinc-binding motifs	NSP10 interacts with NSP14 in SARS-CoV-2, which stimulates activity of NSP14
NSP11	Consists of 13 amino acids	Function unknown
NSP12	RNA-dependent RNA polymerase	NSP12 is the RNA-dependent RNA polymerase that copies viral RNA, which forms complex with NSP7-NSP8 and plays role in replication and methylation
NSP13	RNA-dependent RNA polymerase (RdRp)	▪ NSP13 has high concentration of ATP and has zinc binding domain involved in replication and transcription ▪ NSP13 possesses 5'-triphosphatase activity which is responsible for introducing the 5'-terminal caps of the viral mRNA
NSP14	3' to 5'-endonuclease, N7-methyltransferase	NSP14 has exoribonuclease activity acting in a 3'–5' direction and N7-guanine methyltransferase activity
NSP15	EndoRNAs; NSP15-A1 and NSP15-B-NeuroUEndoRNAase	NSP15 is Mn (2+)-dependent endoribonuclease activity
NSP16	2'-O-ribose-methyltransferase	NSP16 is methyltransferase that mediated mRNA cap 2'-O-ribose methylation to the 5'-cap structure of viral mRNAs

encoding accessory genes. These proteins along with above mentioned nonstructural proteins (NSPs) play important role in SARS-CoV-2 viral replication.

- **ORF3a and ORF3b proteins:** The accessory protein ORF3a of SARS-CoV-2 consists of 274 amino acid residues and is encoded by ORF3a located in between the spike (S) and envelope (E) genes.
  - The ORF3a accessory protein is an O-linked glycosylated possessing three transmembrane domains. ORF3a forms dimer and its six transmembrane

helices together create ion channel in the host cell membrane, which is highly conductive for Ca^{++}/K^{+} cations compared with Na^{+} ion. ORF3a also participates in the SARS-CoV-2 virus release, apoptosis and pathogenesis.
  - Similarly, ORF4d encodes ORF4d protein of SARS-CoV-2 which consists of 154 amino acids long polypeptide chain. ORF4d is located in the nucleolus and mitochondria.
- **ORF6 protein:** The accessory ORF6 protein is a long membrane associated protein comprising 61 amino acids located in endoplasmic reticulum (ER) and Golgi apparatus in virus infected host cells in lung and intestine of patients.
- **ORF7a and ORF7b proteins:** ORF7a and ORF7b accessory proteins are synthesized by SARS-CoV-2 bicistronic RNA-7. ORF7a protein is a type 1 of transmembrane protein, which consists of 15 amino acids signal peptide sequence, an 81-amino acid luminal domain, 21 amino acids transmembrane domain and a short C-terminal tail. On the other hand, ORF7b of SARS-CoV-2 consists of 44 amino acids, which is integral membrane protein expressed in virus infected host cells in the Golgi apparatus. Furthermore, patients develop anti-ORF7b antibody in their serum indicative of its expression in infected SARS-CoV-2 patients. In addition, ORF7b protein has been demonstrated to be closely associated with intracellular virus particles.
- **ORF8 protein:** ORF8 accessory protein of SARS-CoV-2 is encoded by ORF8 gene.
  - ORF8 protein consists of 121 amino acid residues. The 1–17 amino acid residues comprise N-terminal signal sequence, prerequisite for transport to endoplasmic reticulum (ER).
  - ORF8 has been demonstrated to interact with major histocompatibility complex I (MHC-I), thereby mediating their gradation in cell culture and therefore may help in immune evasion.
- **ORF9b protein:** ORF9b accessory protein of SARS-CoV-2 consists of 97 amino acid residues encoded by sgRNA of N gene. ORF9b protein tends to be associated with TOMP70 adaptor protein, which suppresses interferon 1 (INF-1)-mediated antiviral response. Analysis of ORF9b-IFN-1 interaction helps in designing therapeutic response.
- **ORF10 protein:** ORF10 accessory protein of SARS-CoV-2 is encoded by leaky scanning of sgRNA of N gene. ORF10 protein is detected in infected host cells.
- **ORF14 protein:** ORF10 accessory protein of SARS-CoV-2 is encoded by leaky scanning of sgRNA of N gene. ORF14 protein plays a key role in virus replication and evasion of virus from immune system. Researcher has demonstrated the association of ORF9b protein of SARS-CoV-2 to host mitochondrial import receptor subunit (TOMP70) and thereby suppresses type 1 interferon signaling.

Life Cycle of SARS-CoV-2

Life cycle of SARS-CoV-2 consists of invasion of virus into host cell, expression of viral genes, formation of progeny and eventual exit via exocytosis. Life cycle of virus can be divided into six steps: (a) attachment of SARS-CoV-2 to host cell surface ACE2 receptors, (b) SARS-CoV-2 fusion to host cell membrane, (c) viral penetration into host cell and uncoating, (d) replication–transcription complex (RTC) formation, (e) synthesis of viral RNA, and (f) molecular assembly and exit of SARS-CoV-2 through exocytosis. Schematic representation of life cycle of the SARS-CoV-2 in host cells is shown in Fig. 7.42.

Attachment of SARS-CoV-2 to Host Cell Surface

Life cycle of the SARS-CoV-2 begins by binding with the spike protein (RBD region of the S1 subunit mediating viral attachment to the host cells in the form of trimer) on the host cell ACE2 receptors expressed on nasal epithelium, lung alveolar type 2, kidney, heart and intestine. Nasal epithelium is one of the first sites of infection with SARS-CoV-2.
- The researchers probed for the expression of the cell surface enzyme angiotensin-converting enzyme 2 (ACE2), which have been proven to bind to SARS-CoV-2 spike (S) protein and promote internalization of the virus into the human cells.
- Numerous studies have highlighted the low-rates of SARS-CoV-2 infection and less severe symptoms in children compared with adults, it is because of to low expression of ACE2 receptors on the nasal epithelium in children.

Fusion of SARS-CoV-2 to Host Cell Membrane

Receptor binding of S1 subunit to ACE2 (angiotensin-converting enzyme 2) drives conformational change in the S2 subunit and thereby facilitating its fusion with the plasma membrane of the host cell resulting in release of the viral genome into the cell. Cleavage of the SARS-CoV-2 spike proteins S1 and S2 subunits is the basis of fusion. The spike protein is cleaved into two parts, the S1 subunit and S2 subunit, by host proteases, and the subunits exist if noncovalent form until viral fusion occurs.

Penetration of SARS-CoV-2 into Host Cell and Uncoating

After fusion of SARS-CoV-2 spike (S) protein with ACE2 receptors on host cells, there is subtle conformational alterations, releasing viral nucleocapsid (N) into the

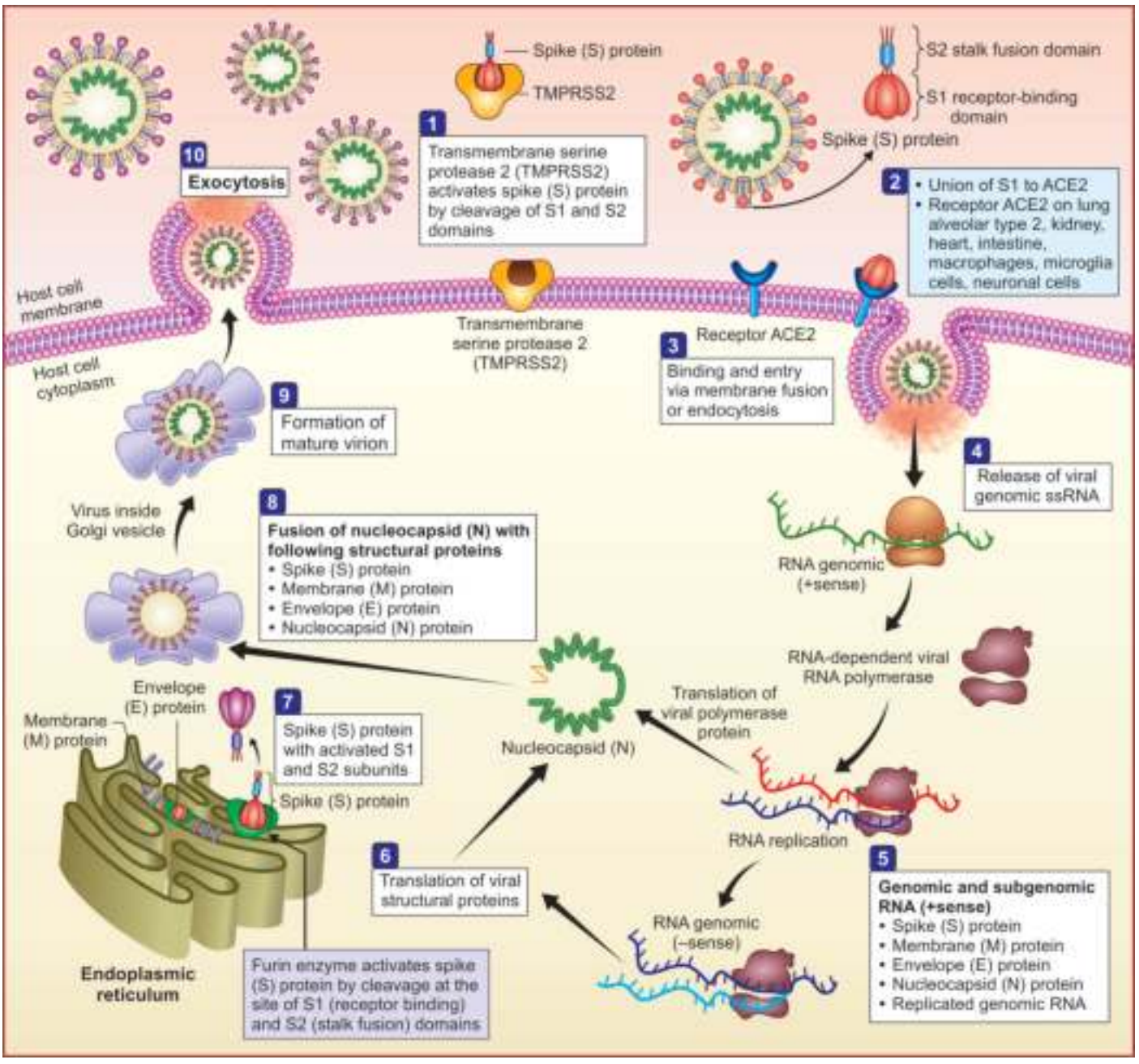


Fig. 7.42: Schematic representation of life cycle of the SARS-CoV-2 in host cells. Life cycle of virus can be divided into six steps: (a) attachment of SARS-CoV-2 to host cell surface ACE2 receptors, (b) SARS-CoV-2 fusion to host cell membrane, (c) viral penetration into host cell and uncoating, (d) replication–transcription complex (RTC) formation, (e) synthesis of viral RNA, and (f) molecular assembly and exit of SARS-CoV-2 through exocytosis.

host cell cytosol. This process is assisted by several host factors, including transmembrane, serine protease 2 (TMPRSS2) and cathepsin L.

Replication–Transcription Complex (RTC) Formation

Immediately, after release of viral nucleocapsid (N), single-stranded RNA (+ssRNA) serves as a functional messenger RNA (mRNA) with respect to ORF1a and ORF1b encoding **polyprotein pp1a** (440–500 kDA) and **pp1ab** (740–810 kDA) respectively.

- However, polyprotein pp1a is expressed more than pp1ab owing to differential efficiency of frameshift between ORF1a and ORF1b genes. These pp1a and pp1ab polyproteins undergo autoproteolytic cleavage yielding 16 nonstructural proteins (16 NSPs) which together form the 'replication–transcription complex' for viral RNA synthesis.
- This functional replication–transcription complex (**RTC**) leads to formation of a nested set of single guide RNA (**sgRNA**) via discontinuous transcription.

SARS-CoV-2 RNA Synthesis

The 'replication–transcription complex' sets molecular process in motion resulting in synthesis of multiple copies of SARS-CoV-2 virus into the host cell.

- The negative single-stranded RNA (**ssRNA**) serves as intermediate template, which is eventually replicated to form multiple copies of guide RNA (**gRNA**) as well as nested single guide RNA (**sgRNA**) by discontinuous transcription.
- Thereafter, they associate with ribosome that leads to synthesis of various structural and accessory proteins building multiple virus structure in the endoplasmic reticulum–Golgi intermediate compartment (**ERGIC**) and eventually exit cell via exocytosis.

Molecular Assembly and Virus Exit

Most of the structural and accessory proteins associated with membrane such as spike (S) protein, membrane (M) protein and envelope (E) protein are synthesized by endoplasmic reticulum (ER) bound ribosomes, whereas viral proteins, including nucleocapsid (N) protein, are translated by free cytosolic ribosomes of host cells.

- The structural (S) proteins also undergo post-translational modification that modulate their functions. Virions are assembled at endoplasmic reticulum–Golgi intermediate compartment.
- Membrane (M) protein interacts with other structural proteins, such as envelope protein (E) and facilitates virion morphogenesis.
- Further, membrane (M) protein–nucleocapsid (N) interactions mediate condensation of nucleocapsid (N) with the envelope (E) protein.
- Post-molecular assembly, progeny virions are transported in smooth-wall vesicle and using secretory pathway that are trafficked to plasma membrane of host cells eventually exit through exocytosis and spread to other regions of body.

Complications and Sequelae

Acute complications of SARS-CoV-2 usually persists until four weeks from the onset of symptoms affecting lungs, kidneys, heart and black fungal infection, known as mucormycosis.

- On the other hand, post-acute sequelae of SARS-CoV-2 is defined as persistent symptoms and/or long-term complications beyond four weeks from the onset of symptoms such as fatigue, dyspnea, chest pain, palpitations, muscular weakness, persistent oxygen requirement, anxiety/depression, sleep disturbances, cognitive disturbances, headache, arthralgia, thromboembolism, chronic renal disease, and alopecia; which adversely affect the quality of life.
- SARS-CoV-2 infection induces synthesis of inflammatory cytokines and procoagulant factors leading to cellular damage and sequelae. Therefore, holistic approach is essential for follow-up care and well-being of post-SARS-CoV-2 recovering patients. SARS-CoV-2 acute complications and sequelae in COVID-19 survivors are shown in Fig. 7.43.

Pathology Pearls: Severity of SARS-CoV-2 (COVID-19) Disease

Nonsevere Patients

- Absence of signs of severe or critical disease
- Patients are managed by supportive measures

Severe Patients

- SpO_2 <90% on room air
- Respiratory rate >30 in adults
- Increased respiratory rate in children
- Signs of respiratory distress

Critically Ill Patients

- Patients require life-sustaining treatment
- Acute respiratory distress syndrome
- Sepsis
- Septic shock

Pulmonary Manifestations

Acute pulmonary complications of COVID-19 disease include pneumonia, acute respiratory distress syndrome (ARDS), dyspnea, decreased exercise capacity and hypoxia, pneumocyte damage and dyspnea, increased vascular permeability, thrombotic microangiopathy and oxygenation dysfunction.

- Follow-up COVID-19 disease survivors demonstrate persistent dyspnea and chest pain, reduced diffusion capacity, restrictive pulmonary physiology and ground-glass opacities and fibrotic changes on radiograph or high-resolution computed tomography imaging of the chest.
- Assessment of progression or recovery of pulmonary disease and functions is analyzed by home pulse oximetry, six-minute walk tests (6MWTs), pulmonary function tests (PFTs), high-resolution computed tomography imaging of the chest and computed tomography pulmonary angiogram as clinically appropriate.
- Pathophysiology of accumulation of fluid in the lungs in a patient of SARS-CoV-2 (COVID-19) disease is shown in Fig. 7.44. Computed tomography scan demonstrates lungs involvement in a patient of SARS-CoV-2 (COVID-19) disease is shown in Fig. 7. 45.

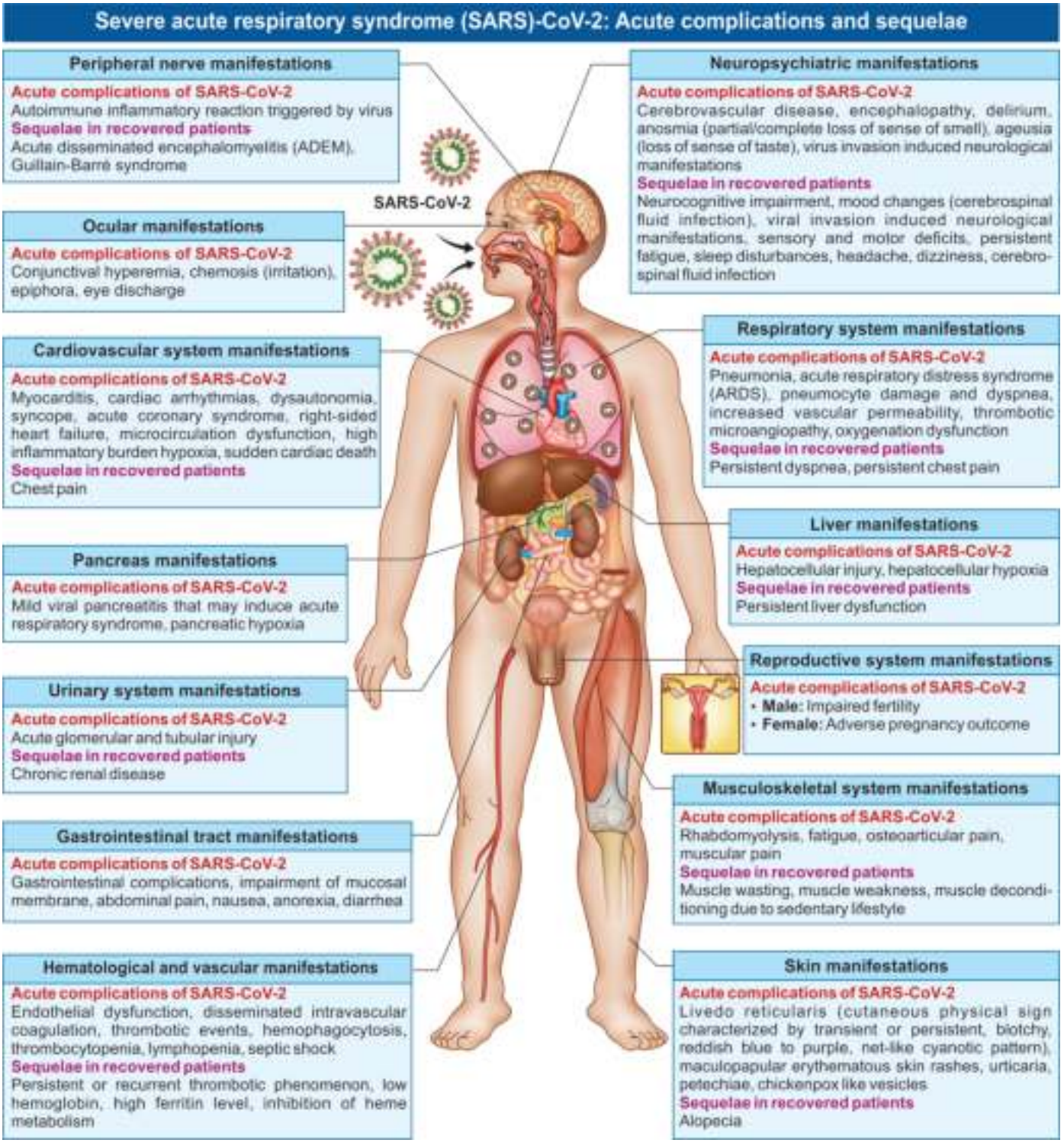


Fig. 7.43: SARS-CoV-2 acute complications and sequelae in COVID-19 survivors.

Cardiovascular System Manifestations

Acute cardiovascular system complications in COVID-19 disease patients include myocarditis, chest pain, cardiac arrhythmias, dysautonomia, syncope, acute coronary syndrome, right-sided heart failure, microcirculation dysfunction, high inflammatory burden hypoxia and sudden cardiac death.

- Long-term sequelae may include increased metabolic demand of heart, cardiac arrhythmias, persistent chest pain, autonomic dysfunction and myocardial fibrosis (detectable via cardiac MRI).
- Patients develop cardiovascular complications during acute COVID-19 infection. Those patients experiencing persistent cardiovascular system

SARS-CoV-2 (COVID-19): Autoimmune hemolytic anemia and pleural effusion

- Normal life span of red blood cells is around 120 days. SARS-CoV-2 (COVID-19) infection causes autoimmune leading to pleural effusion. At the time of autoimmune hemolytic anemia onset, all patients demonstrate elevated markers of inflammation (i.e. fibrinogen, D-dimers and C-reactive protein).
- Heme oxygenase is an enzyme that catalyzes the degradation of heme to produce biliverdin, ferrous ion and carbon monoxide.
- Biliverdin reductase (BVR) is an enzyme present in all tissues especially in reticuloendothelial cells in liver and spleen. Biliverdin reductase facilitates the conversion of biliverdin to bilirubin via the reduction of a double-bond between the second and third pyrrole ring into a single-bond.

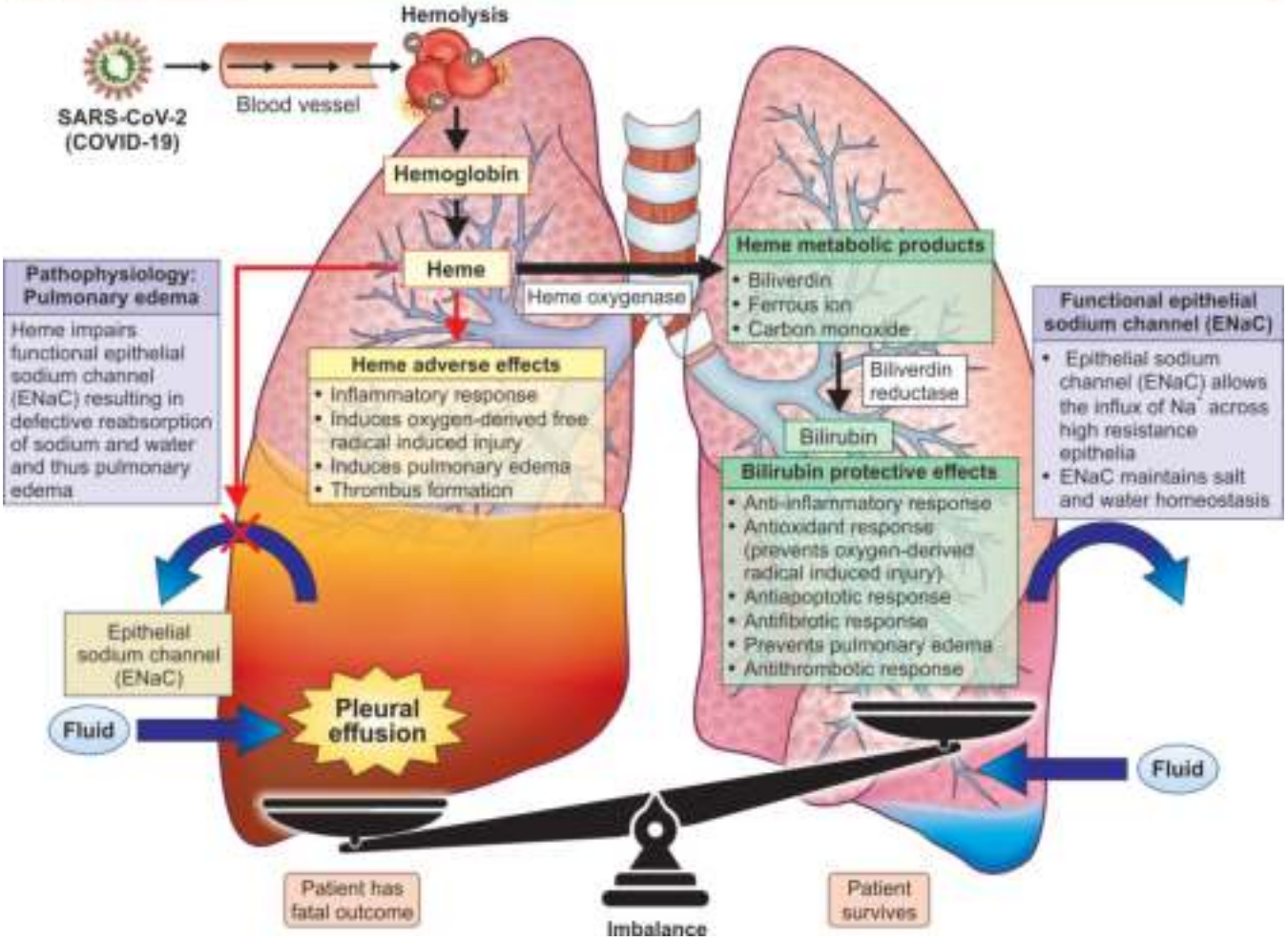


Fig. 7.44: Pathophysiology of accumulation of fluid in the lungs in a patient of SARS-CoV-2 (COVID-19 disease).

manifestations must be monitored with serial clinical echocardiogram and electrocardiogram follow-up.

Neuropsychiatric Manifestations

Acute neuropsychiatric complications of COVID-19 disease include cerebrovascular disease, encephalopathy, delirium, anosmia (partial/complete loss of sense of smell), ageusia (loss of sense of taste) and virus invasion induced neurological manifestations.

- Patients develop persistent neuropsychiatric manifestations such as neurocognitive impairment, mood changes, fatigue, myalgia, headache, dysautonomia, cerebrospinal fluid infection, viral invasion induced neurological manifestations, sensory and motor deficits, persistent fatigue, sleep disturbances, headache, dizziness and cerebrospinal fluid infection.
- About 30–40% of COVID-19 disease survivors develop anxiety, depression, sleep disturbances and post-traumatic stress disorder (PTSD).
- Neuropsychiatry manifestations occur due to immune dysregulation, inflammation, microvascular thrombosis, iatrogenic adverse effects of medications and psychological impacts of COVID-19 disease.

Peripheral Nervous System Manifestations

Acute peripheral nervous system complication of COVID-19 disease includes autoimmune inflammatory reaction triggered by virus. COVID-19 disease survivors

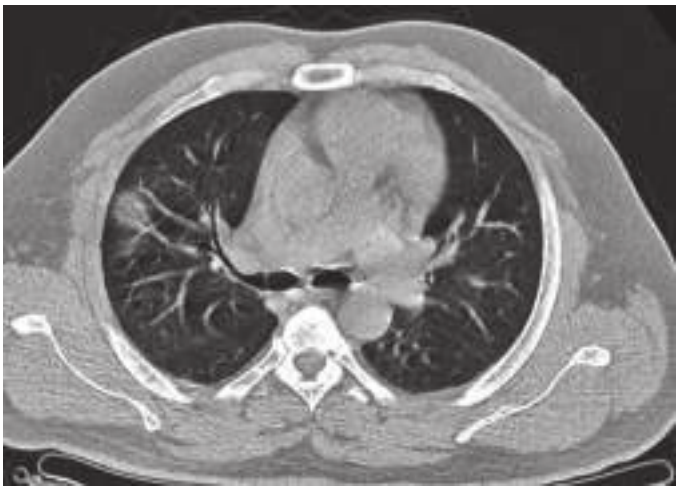


Fig. 7.45: Computed tomography scan demonstrates lungs involvement in a patient of SARS-CoV-2 (COVID-19 disease). (*Courtesy:* Dr. Vigyat, Dr. DY Patil Medical College, Pune, Maharashtra).

may develop acute disseminated encephalomyelitis (ADEM) and Guillain-Barré syndrome. The clinical presentation of COVID-19 associated Guillain-Barré syndrome is similar to non-COVID-19 cases. Patient presents with weakness and tingling sensations in extremities, which quickly progress to paralysis. Most patients with this disorder must be hospitalized to receive treatment.

Ocular Manifestations

Acute ocular complications of COVID-19 disease include conjunctival hyperemia, chemosis (irritation), epiphora and eye discharge.

Gastrointestinal Tract Manifestations

Prolonged viral fecal shedding can occur in COVID-19 disease even after negative nasopharyngeal swab testing. COVID-19 disease has potential to change the gut microbiome, including enrichment of opportunistic organisms and depletion of beneficial commensals. Acute gastrointestinal system complications of COVID-19 disease include impairment of mucosal membrane, abdominal pain, nausea, anorexia and diarrhea.

Hepatobiliary System Manifestations

Acute hepatobiliary system complications of COVID-19 disease include hepatocellular injury and hypoxia. COVID-19 disease survivors develop persistent liver dysfunction.

Pancreas Manifestations

Acute pancreas complications of COVID-19 disease include mild viral pancreatitis that may induce acute respiratory syndrome and pancreatic hypoxia.

Hematologic Manifestations

Acute hematologic complications of COVID-19 disease include endothelial dysfunction, disseminated intravascular coagulation, thromboembolic events, hemophagocytosis, thrombocytopenia, lymphopenia and septic shock.

- About 5% of post-COVID-19 disease cases may develop thromboembolic phenomenon in retrospective studies. Exact mechanism of persistent or recurrent thromboembolic event is not known. Therefore, the patients should be administered oral anticoagulants and low-molecular heparin to prevent thromboembolic phenomenon.
- D-dimer levels should be analyzed to predict severity of the disease. Elevated levels of D-dimer are demonstrated in severely infected COVID-19 disease patients.

Renal Manifestations

Acute renal complication of COVID-19 disease includes acute glomerular and tubular injury in majority of patients. These renal changes may resolve, however reduced glomerular filtration rate has been reported at months follow-up. COVID-19 disease survivors develop persistent impaired renal function. Collapsing glomerulopathy may be predominant pattern of renal injury in African descent.

Musculoskeletal System Manifestations

Acute musculoskeletal system complications in COVID-19 disease patients include rhabdomyolysis, fatigue, osteoarticular pain and muscular pain. Long-term sequelae in COVID-19 disease survivors include muscle wasting, muscle weakness and muscle deconditioning due to less physical activity.

Dermatologic Manifestations

Acute complications in COVID-19 disease patients include livedo reticularis (cutaneous physical sign characterized by transient or persistent, blotchy, reddish blue to purple, net-like cyanotic pattern, maculopapular erythematous skin rashes, urticaria, petechiae and chickenpox-like vesicles. Hair loss is the predominant symptom and has been reported in about 20% of COVID-19 disease survivors.

Endocrine System Manifestations

Endocrine system post-COVID-19 disease sequelae may include new or worsening control of diabetes mellitus, subacute thyroiditis and bone demineralization. Patients with newly diagnosed diabetes mellitus in the absence of risk factors for type 2 diabetes mellitus, suspected hypothalamic-pituitary-adrenal axis suppression or

hypothyroidism should undergo appropriate laboratory testing and should be referred to endocrinologists.

Reproductive System Manifestations

COVID-19 disease, men and women survivors may develop infertility. Women may have adverse pregnancy outcome.

Multisystem Inflammatory Syndrome in Children

Multisystem inflammatory syndrome in children (MIS-C) is a serious disorder linked to COVID-19 disease. Diagnostic criteria include: less than 21 years old presents with fever, elevated inflammatory markers, multiorgan dysfunction, recent SARS-CoV-2 infection and exclusion of other plausible diagnoses. It most often occurs in children less than seven years of age and disproportionately of African, Africa-Caribbean or Hispanic origin. These children may develop complications such as coronary artery aneurysm, headache, encephalopathy, cerebral stroke and seizures.

SARS-CoV-2 Genetic Mutations

SARS-CoV-2 virus has mutated overtime, leading to genetic variation in the population of circulating viral strains over the course of the COVID-19 pandemic.

- Molecular antigen, and serology tests are affected by viral mutations differently due to the inherent design differences of each test.
- Genetic mutation of the severe respiratory syndrome coronavirus 2 (SARS-CoV-2) is a change in the genetic sequence of the SARS-CoV-2 when compared with genetic sequence such as Wuhan-Hu1 (the first genetic sequence identified) or USA-WA1/2020 (the first identified genetic sequence in the United States).
- A new genetic variant of SARS-CoV-2 may have one or more mutations that differentiate it from the reference genetic sequence of predominant virus variants.
- New genetic variants of SARS-CoV-2 can have different characteristics. Some variants of SARS-CoV-2 may spread more easily or show signs of resistance to existing treatment options.

SARS-CoV-2 Delta Variant

SARS-CoV-2 Delta variant has K417 mutation in the spike (S) protein of the SARS-CoV-2 virus, and thus named delta virus by World Health Organization.

- SARS-CoV-2 Delta variant is highly contagious, which spreads faster than early forms of SARS-CoV-2, which virus strain drove the **second wave of infections** in India. The spike protein helps the virus enter and infect human cells.
- The K417 mutation in SARS-CoV-2 Delta variant has been associated with immune escape that makes the virus less susceptible to the vaccine or any form of drug therapy.
- Some data suggest the SARS-CoV-2 Delta variant causes more severe illness than previous strains in unvaccinated persons. Unvaccinated persons remain the greatest concern. Fully vaccinated persons with SARS-CoV-2 Delta variant breakthrough infections can spread the virus to others. However, vaccinated persons appear to be more infectious for a shorter period. It is essential to wear multilayered masks to prevent transmission of SARS-CoV-2.

SARS-CoV-2 Omicron (B.1.1.529) Variant

SARS-CoV-2 Omicron (B.1.1.529) variant was first reported to the World Health Organization from South Africa on 24 November 2021, which has a large number of genetic mutations, some of which are concerning. Current SARS-CoV-2 polymerase chain reaction (PCR) diagnostics continue to detect SARS-CoV-2 Omicron (B.1.1.529) variant except one of three genes, i.e. spike gene (32 mutations affect spike proteins). World Health Organization has designated SARS-CoV-2 (B.1.1.529) Omicron variant.

SARS-CoV-2 Vaccines

Vaccines are highly effective to prevent SARS-CoV-2 infections, including Delta variant in majority of persons. But these vaccines are not 100% effective and some fully vaccinated persons can become infected (called a breakthrough infection) and experience illness. For such persons, the vaccine still provides them strong protection against serious illness and death. There are three main approaches to design vaccine: (a) whole virus approach using whole pathogen, (b) subunit approach using very specific subunit of pathogen, and (c) genetic approach using nucleic acid.

- Whole virus vaccine: Whole SARS-CoV-2 virus is used to prepare vaccine by three methods: inactivation of virus, weakened version of virus and viral proteins.
- Subunit vaccine: A subunit vaccine is prepared by using the very specific subunit of a SARS-CoV-2 virus that the immune system needs to recognize.
- Genetic material vaccine: Genetic material vaccine provides the instructions for making specific proteins. The nucleic acid approach is a new way of developing vaccines against SARS-CoV-2 virus.

INFLUENZA VIRUS INFECTION

Influenza A viruses are single-stranded RNA viruses, which are three types of influenza viruses: A, B and C. Out of these influenza viruses, influenza A virus is most important to cause illness, whereas influenza types B and C produce minor illness. Researchers suggest that most adults have considerable immunity against influenza A virus.

- Influenza A virus is inhaled into respiratory tract, which evades the mucoprotein 'trap' and eviction by ciliated epithelium. Virus invades columnar ciliated cells and induces cell death due to virus replication and apoptosis. Spread of influenza virus infection to adjacent epithelial cells results in cell death producing areas of necrosis. Nonproductive influenza A virus infection of leukocytes, lymphocytes and monocytes compromises their function. There is increased risk of secondary bacterial infection, e.g. *Staphylococcus aureus*.
- Patient presents with flu-like symptoms within one to four days after exposure to the influenza A virus that resolve itself within five to seven days as the immune system fights it off.

POLIOVIRUS INFECTION

Human beings are the only known reservoir of polio RNA virus. The single-stranded RNA core is surrounded by a protein capsid without a lipid envelope, which makes poliovirus resistant to lipid solvents and stable at low pH. There are distinct serotypes of poliovirus, types 1, 2 and 3. The poliovirus is transmitted via droplets or aerosols from the throat and via fecal contamination of hands, utensils, water and food. Immunization against poliovirus infection represents one of the world's great medical achievements.

Clinical Features

Brain stem nuclei are affected by poliovirus resulting in poliomyelitis, which is characterized by degeneration and necrosis of anterior horn cells of the spinal cord. Poliomyelitis can lead to a spectrum of clinical presentations ranging from subclinical infection to paralysis and mortality. About 90–95% of all poliovirus infections remain asymptomatic. Paralytic poliomyelitis has been classified into spinal, bulbar and bulbospinal types, depending on the site of the affected motor neurons.

- **Spinal poliomyelitis:** Patient presents with meningitis followed by severe myalgia and localize sensory and motor symptoms leading to weakness and asymmetric and flaccid paralysis involving proximal skeletal muscles predominantly and distal muscles within 1–2 days.
- **Bulbar poliomyelitis:** Patient suffers from serious form of disease resulting from paralysis of the skeletal muscles innervated by cranial nerves. Patient presents with dysphagia, nasal speech, pooling of secretions and breathlessness. Rarely, patient can develop encephalitis. Bulbar poliomyelitis is linked to mortality in 5–15% of cases.
- **Post-polio syndrome:** It is poorly understood condition. Post-polio syndrome is characterized by the onset of fatigue, skeletal muscle weakness and wasting in patients, who have recovered from paralytic polio, starting several years after acute polio disease.

COXSACKIEVIRUS INFECTION

Coxsackievirus can spread from person-to-person contact, usually on unwashed hands and surfaces contaminated by feces, where Coxsackievirus can survive for several days to eight weeks. The incubation period lasts about 1–2 days. Patient presents with fever, anorexia, breathlessness, sore throat, cough, malaise and small blisters on the palms and feet. These skin blisters may undergo ulceration. The patients may shed virus for many days.

HEPATITIS A VIRUS INFECTION

Hepatitis A is an enteric picornavirus. Its HAV genome contains single-stranded RNA molecule that codes for a polyprotein which is processed to give rise to viral proteins **VP-1, VP-2, VP-3** and other proteins.

- Hepatitis A virus causes a highly contagious short-time mild to severe hepatitis A infection. Hepatitis A virus is primarily transmitted by fecal–oral route; and also, via close, physical contact (oral–anal sex) with an infected person.
- Incubation period of hepatitis A virus is usually 14–28 days. Patient presents with fever, malaise, loss of appetite, nausea, diarrhea, abdominal discomfort, dark-colored urine and jaundice. Hepatitis A virus infection is normally followed by recovery.

NOROVIRUS (NORWALK VIRUS) INFECTION

Norovirus survives acid barrier of the stomach, which invades terminal epithelial cells of the villi in the upper intestine and induces cell death due to replication and apoptosis.

- Norovirus replication spreads distally along the intestine. Villi remain intact, but are broadened and flattened. Villus function is compromised.
- Malabsorption of carbohydrates and fat leads to diarrhea. Autonomic signal to the stomach delays gastric emptying with associated projectile vomiting.

YELLOW FEVER VIRUS INFECTION

Yellow fever virus belongs to the genus Flavivirus, which is related to West Nile, Japanese encephalitis virus and St. Louis encephalitis virus. Yellow fever virus is transmitted to the persons through infected Aedes mosquitoes. The 'yellow' name refers to the jaundice that affects some patients. Yellow fever virus is endemic in tropical regions of Africa and Central America and South America due to heavily populated regions with high Aedes mosquito density.

Clinical Features

Patient presents with fever, headache, jaundice, skeletal muscle pain, abdominal pain, nausea, vomiting, fatigue, passage of dark colored urine. Bleeding can occur from the mouth, nose, eyes or gastric region. In most patients, symptoms disappear within 3–4 days. About half of the patients develop severe toxic symptoms and have fatal outcome with 7–10 days.

Laboratory Diagnosis

Polymerase chain reaction (**PCR**) analysis of blood and urine samples can sometimes detect the yellow fever virus in early stages of the disease. In the later stages, antibodies are demonstrated by enzyme-linked immunosorbent assay (**ELISA**) and plaque reduction neutralization test (**PRNT**) techniques.

Prevention

Yellow fever can be prevented by yellow fever vaccine, which is safe and affordable. Single dose of yellow fever vaccine gives lifelong immunity.

RUBELLA VIRUS INFECTION

Rubella is commonly called as German measles caused by rubella virus, which is more prevalent in China than across world. It is a contagious disease, that commonly affects skin and lymph nodes.

- Rubella virus is transmitted from person to person via coughing and sneezing of respiratory secretions such as mucus. Rubella is very dangerous for a pregnant woman and her developing baby transmitted via hematogenous route. It is essential to protect women from rubella virus before they become pregnant. Rubella virus infection causes the most severe damage when the mother is infected early in pregnancy, especially in the first 12 weeks (first trimester).
- Rubella virus is inhaled into respiratory tract, which invades the mucosa and lymphoid tissue of the nasopharynx, where replication occurs. Virus spreads directly to the regional lymph nodes or via a transient viraemia.
- With appearance of IgM and IgG antibodies, patents develop maculopapular rash. In the nonimmune mother in the first stage of pregnancy, rubella virus crosses the placenta, and replicates in a wide range of tissues of the fetus. There is a direct cytotoxic effect on cells of the myocardium, retinal epithelium and neural tissues.

Clinical Features

Most persons who get rubella virus infection usually present with low-grade fever, sore throat, skin rash progressing to generalized skin rashes, lymphadenopathy and joint pains. Children who have rubella recover within a week, but adults take longer time to recover.

Clinical Pearls: Congenital Rubella Syndrome (CRS)

- Congenital rubella syndrome occurs in a developing baby in the womb whose mother is infected with rubella virus.
- Pregnant women who contract rubella infection are at high risk for miscarriage or stillbirth, and their developing babies are prone to develop severe birth defects with devastating, lifelong consequences.
- The most common birth defects include cardiac defects, cataracts, deafness, intellectual disabilities, liver damage, spleen damage, low birth rate and skin rashes.
- Less common lifelong consequences include glaucoma, brain damage, endocrinopathies and inflammation of the lungs.
- It is essential to vaccinate women for rubella vaccine before they become pregnant. There is no definite cure of congenital rubella syndrome.
- The rubella test is done to detect antibodies in the blood that develop in response to a rubella or immunization. Rubella testing may be performed to confirm the presence of adequate protection against the rubella virus.

DENGUE VIRUS INFECTION

Dengue is a mosquito-borne human viral infection caused by dengue virus (DENV) between human beings and mosquito vector.

- Dengue virus (DENV) is transmitted by female mosquitoes mainly of the species *Aedes aegypti* and to a lesser extent, *Aedes albopictus*.
- Dengue virus infection occurs in tropical and subtropical regions across world. There are four serotypes of dengue virus: DENV-1, DENV-2, DENV-3 and DENV-4.

Clinical Features

Dengue virus infection produces acute flu-like illness that affects infants, young children and adults. Symptoms usually persists for 2–7 days and incubation period of 4–10 days after bite from an infected mosquito. Occasionally, patient develops potentially lethal complication, severe dengue infection associated with bleeding, organ impairment and/or plasma leakage.

- **Mild to moderate dengue fever:** Dengue fever should be suspected when patient presents with high-grade fever (40°C/104°F) accompanied by two of the symptoms during the febrile phase such as severe headache, myalgia, arthralgia, pain behind eyes, nausea, vomiting, skin rashes and lymphadenopathy.
- **Severe dengue fever**: Patient with severe dengue fever can progress to potentially severe disease due to plasma leaking, fluid accumulation, respiratory

distress, severe bleeding tendencies or organ impairment. Warning signs that medical professional should look for severe abdominal pain, persistent vomiting, rapid respiration, fatigue and hematemesis. These patients should be kept under close observation to prevent lethal complications.

Treatment

There is no specific treatment for dengue fever. Early detection of disease progression helps in prompt medical treatment. Dengue prevention and control depends on effective vector control measures.

EBOLA VIRUS AND MARBURG VIRUS INFECTION

Ebola and Marburg viruses are related filamentous filoviruses but distinct from each other that cause clinically similar diseases characterized by hemorrhagic fever and capillary leakage. Ebola virus infection is slightly more virulent than Marburg infection.

Mode of Transmission

Ebola is named for the river in **Africa** where the hemorrhagic fever disease was first recognized in 1976. Ebola virus has five species: (a) infectious viruses include Zaire Ebola virus, Sudan Ebola virus, Tai Forest Ebola virus, Bundibugyo Ebola virus, and (b) noninfectious Reston Ebola virus. Ebola virus outbreaks have been linked to consumption of meat and soup from wild animals like bats. Marburg virus has been identified in bats. Persons are exposed to bats in mines or caves.

Clinical Features

Patient develops abrupt hemorrhagic fever, chills, malaise, myalgia with 5–10 days of Ebola and Marburg virus infection. Over the time, symptoms increase in severity such as nausea, vomiting, bloody diarrhea, abdominal pain, weight loss, skin rashes, red eyes, cough, sore throat, and chest pain.

MUMPS VIRUS INFECTION

Mumps is caused by a Paramyxovirus, a member of the Rubulavirus family, that involves salivary glands. The incubation period ranges 12–25 days with average 16–18 days.

Clinical Features

Patient presents with pain, tenderness and swelling of unilateral or bilateral parotid salivary glands. Swelling usually peaks in 1–3 days and then subsides during the next week. Submandibular and sublingual glands may also be involved in 10% of cases. Nonspecific symptoms may precede parotitis such as low-grade fever, myalgia, anorexia, malaise and headache, that last within 5–10 days.

Complications

Patient with mumps can develop various complications that include orchitis, oophoritis, mastitis, meningitis, encephalitis, pancreatitis and hearing loss among adults than children.

MEASLES VIRUS INFECTION

Measles is a highly contagious serious disease caused by a virus in the Paramyxovirus family. Unvaccinated young children are at high risk of developing measles and its complications including mortality.

Mode of Transmission

Measles virus is transmitted by coughing and sneezing through inhalation of droplets, close personal contact or direct contact with infected nasal or throat infections. Virus remains active and contagious in the air and infected surface up to two hours.

Clinical Features

Patient presents with high-grade fever, which begins about an incubation period of 10–12 days and lasts for 4–7 days. Initially, patient presents with cough, runny nose, red and watery eyes, and small white spots inside the cheeks. After several days, skin rashes erupt usually on the face and upper region of neck. After three days, skin rashes appear on hands and feet, which last for 5–6 days and then fade away.

Complications

Most measles-related mortality occurs caused due to serious complications such as blindness, encephalitis, severe diarrhea related dehydration, otitis media and pneumonia in immunocompromised persons.

Prevention

Measles virus infects the respiratory tract, then spreads throughout the body. Accelerated immunization activities contribute to reduce morbidity and mortality in measles.

RABIES VIRUS INFECTION

Rabies virus is transmitted by the bite of animals as dogs, foxes and bats, whose saliva contains rabies virus, which enters a peripheral nerve and is transported by retrograde axoplasmic flow to the spinal cord and brainstem and spillage into the cerebellum and hypothalamus.

Clinical Features

Patient presents with hydrophobia (fear from water) characterized by violent muscle contractions and convulsions.

Histologic Examination

Histologic examination of brain shows eosinophilic intracytoplasmic inclusions (**Negri bodies**) in the hippocampus and Purkinje cells of the cerebellum. Brainstem and spinal cord shows neuronal degeneration, perivascular accumulations of mononuclear cells.

HEPATITIS C VIRUS INFECTION

Hepatitis C virus causes chronic liver inflammation, and is transmitted through blood-to-blood contact and sharing infected needles. If left untreated over many years, some patients can cause serious and potentially life-threatening cirrhosis and hepatocellular failure. Direct-acting antiviral medicines can cure >95% of persons with hepatitis C.

HUMAN IMMUNODEFICIENCY VIRUS INFECTION

Human immunodeficiency virus (HIV) may affect the brain, spinal cord, or peripheral nervous system before the onset of immunodeficiency syndrome. Reticuloendothelial cells are vehicles for viral entry into the nervous system. HIV infection results in AIDS dementia complex and is characterized by impairment of memory, coordination of balance and motor functions with progressive dementia. Patient is prone to opportunistic bacterial and fungal infections. Refer to Chapter 4, immunopathology for human immunodeficiency virus (HIV) infection in details.

HUMAN T CELL LEUKEMIA VIRUS TYPE 1 INFECTION

Human T cell leukemia virus type 1 (HTLV-1) is a retroviral infection that affects T cells. Majority of persons have no signs and symptoms. However, some persons may develop T cell leukemia, HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) or other medical disorders.

Mode of Transmission

Human T cell leukemia virus type 1 is transmitted by blood transfusions, sharing infected needles and sexual contact. HTLV-1 can also be transmitted from mother to the newborn during birth or breastfeeding. Majority of persons remain asymptomatic throughout life.

Clinical Features

Majority of the persons with HTLV-1 infection remain asymptomatic. About 2–5% of infected persons develop adult T cell leukemia. Depending on the subtype of acute T cell leukemia, patients have a life span of 6 months to two years of diagnosis. Although chemotherapy can produce a complete remission, yet it cannot alter the life expectancy. About 2% of persons can develop HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) or other medical disorders. These persons are unable to walk and need wheelchair.

Laboratory Diagnosis

Human T cell leukemia virus type 1 is diagnosed by detection of antibodies to the HTLV-1 during screening of blood donation and testing in patients having family history of the infection or a work up for HTLV-1 related disorders.

HUMAN ROTAVIRUS INFECTION

Human rotaviruses are responsible for severe acute watery diarrhea with dehydration, vomiting, fever and/or abdominal pain among infants and young children.

- Persons who are infected with rotavirus shed virus in their stool. Viruses in the environment can infect other persons. Infants and children in contact with infected persons get rotavirus infection during winter and spring seasons (January to June).
- Rotavirus vaccination is the best way to protect infants and children from rotavirus. Handwashing and cleanliness are important to prevent transmission of the virus.

DNA VIRUSES

DNA viruses have DNA genomes that are replicated by either host or virally encoded DNA polymerases. Upon uncoating, the genomes of DNA viruses are transcribed to produce an "early" set of mRNAs required for viral genome replication. After genome replication another set of mRNAs, the "late" mRNAs are expressed. Late genes encode structural proteins and other proteins that are packaged within virions.

VARIOLA (SMALLPOX)

Before smallpox was eradicated, it had been an acute contagious, disfiguring and deadly disease caused by the variola virus, a member of the Orthopoxvirus family. Edward Jenner developed smallpox vaccine in 1796. World Health Organization launched an intensified plan to eradicate smallpox in 1967 and achieved the target in 1980. Patients developed pus-filled blisters all over the body. Because smallpox no longer occurs naturally, scientists are only concerned that it could emerge through bioterrorism.

VARICELLA-ZOSTER VIRUS (CHICKENPOX) INFECTION

Varicella (chickenpox) is an acute viral infection of childhood caused by the varicella-zoster virus. The varicella-zoster is a DNA virus that is a member of herpesvirus group. The average incubation period for varicella is 14–16 days after exposure to varicella-zoster virus.

- Infants, adolescents, adults, pregnant women and immunocompromised persons are at risk for more serious disease and acquiring complications.
- Patient presents with fever, malaise and generalized pruritic skin rashes such as macules, papules and vesicles.
- The skin rashes usually appear first on the chest, back, and face, then spread over the entire body. Recovery from primary varicella zoster virus infection usually provides lifelong immunity.
- Following overt varicella, the virus can remain latent for years in dorsal root ganglia. Reactivation of latent infection causes herpes zoster (Shingles).

MOLLUSCUM CONTAGIOSUM VIRUS 1 AND 2 (MCV1/MCV2) INFECTION

Molluscum contagiosum is viral infection of the keratinocytes caused by MCV1 (DNA poxvirus) in 98% of affecting children (2–5 years) and immunocompromised persons. **MCV1** is transmitted by direct skin-skin contact or sharing infected clothes. **MCV2** is transmitted via **sexual contact** in adults in human immunodeficiency virus (HIV). In contrast to most double-stranded DNA viruses, poxvirus replicates in the cytoplasm. Incubation period is 2 weeks to six months. Average incubation period is 6 weeks. Henderson-Paterson bodies also known as 'molluscum bodies' are large intracytoplasmic eosinophilic virion inclusions in keratinocytes.

Clinical Features

Patient presents with umbilicated, dome-shaped papules over body, arms, legs, thighs, neck, face, axilla, trunk, genitalia and perianal region. Umbilication of skin lesion occurs as a result of intracytoplasmic viral inclusions extruding infected cell on the surface with central pore.

Surgical Pathology: Molluscum Contagiosum

Gross Morphology

Molluscum contagiosum is dome-shaped flesh colored skin papule. Central umbilication may be present or absent.

Light Microscopy

- Epidermis grows deeper down into the dermis and form closely packed lobules.
  - Epidermal cells contain large intracytoplasmic eosinophilic stained inclusion bodies known as molluscum bodies.
  - Molluscum bodies displace the nuclei at the periphery of epidermal cells.
  - In the center of the lesion, stratum corneum disintegrates and release the molluscum bodies together with keratinous debris resulting to formation of central crater.
- Dermis shows prominent acute and chronic inflammatory infiltrate and foreign body giant cells. Histopathologic features of molluscum contagiosum are shown in Fig. 7.46.

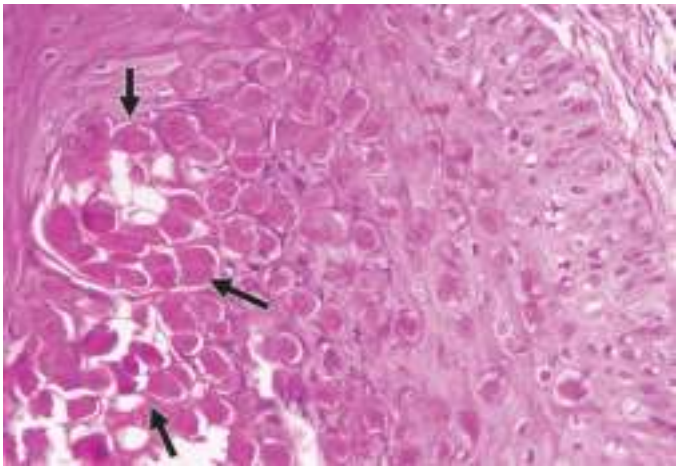


Fig. 7.46: Histopathological features of molluscum contagiosum. Epidermal cells contain large intracytoplasmic eosinophilic stained inclusion bodies known as molluscum bodies (arrows) (400X).

HERPES SIMPLEX VIRUS (HSV-1, HSV-2) INFECTION

The herpes simplex virus is categorized into two types: herpes simplex type 1 (HSV-1) and herpes simplex type 2 (HSV-2).

- **HSV-1** is mainly transmitted by oral-to-oral contact to induce oral herpes that presents as cold sores. HSV-1 can also induce genital herpes. **HSV-2** is sexually transmitted infection that induces genital herpes.
- Both HSV-1 and HSV-2 infections can occur throughout life. Most oral and genital herpes infections are asymptomatic and leading to painful blisters at the site of infection.
- Herpes infections are most contagious when in contact with symptomatic patients. However, herpes infections can be transmitted to others in the absence of symptoms. Infection with HSV-2 increases the risk for acquiring and transmitting human immunodeficiency virus (HIV) infection.
- Neonatal herpes simplex infection can occur when an infant is exposed to HSV-1 or HSV-2 in the genital tract during delivery.

Herpes Simplex Virus Type 1

Herpes simplex virus type 1 is inoculated onto mucosa of the mouth. Virus replication occurs in epidermis and dermis and the patient is usually asymptomatic.

- Virus enters sensory nerve endings and migrates via axon to cell bodies in the trigeminal ganglion. With high level replication in the ganglion, virus returns to the skin, and replicates there with herpetic vesicles developing. Immune response resolves skin lesions, but latent virus exists in the ganglion.
- Reactivation of latent HSV-1 and its traveling back to the skin manifests as new skin lesions. Oral herpes infection is most often asymptomatic.

- Patient with oral herpes simplex infection presents with painful blisters around the mouth with itching and burning sensation. Sores on the lips are commonly referred to cold sores.
- Neonatal herpes infection can occur when an infant is exposed to HSV-1 or HSV-2 in the genital tract during delivery.
- In immunocompromised persons, HSV-1 can induce more severe infection involving brain (encephalitis) and eye (keratitis). Herpes simplex virus targets the temporal lobes by binding on the plasma membranes of central nervous system cells. Herpes simplex virus 1 (HSV-1) has ability to remain latent or selective replication in distinct intracellular microenvironments. Patient presents with headache, hyperactivity, and/or general weakness.

Herpes Simplex Virus Type 2

Herpes simplex virus type 2 (HSV-2) is exclusively sexually transmitted infection inducing genital herpes across world. Genital herpes infection remains asymptomatic in 66% of cases lifelong and incurable.

- About one-third of genital herpes may have symptoms such as one or more blisters on genitalia and anal region. Patient presents with fever, body aches, sensations of mild tingling or shooting pain in the legs, hips and buttocks before appearance of genital ulcers and lymphadenopathy.
- HSV-2 infection increases the risk of acquiring a new human deficiency virus (HIV) infection in immunocompromised persons by approximately three-fold.
- Neonatal herpes infection can occur when an infant is exposed to HSV-2 or HSV-1 in the genital tract during delivery.
- HSV-2 infection can cause complications such as meningoencephalitis, esophagitis, hepatitis, pneumonitis, retinal necrosis or disseminated infection.

CYTOMEGALOVIRUS INFECTION

Cytomegalovirus (CMV) affects lungs, kidneys (tubules and glomerular endothelial cells), gastrointestinal tract (epithelial lining), brain (neurons) and eye, which is transmitted via droplets, saliva, sexual contact (vaginal discharge) and transplacental route (mother to fetus).

- **Congenital cytomegalovirus infection:** Vertical transmission of cytomegalovirus (mother to fetus) causes intrauterine growth retardation. Lesion in proximity to third ventricle and aqueduct cause hydrocephalus. Neonate presents with jaundice, hepatosplenomegaly, encephalitis, chorioretinitis, anemia, and thrombocytopenia.
- **Postnatal cytomegalovirus infection:** Postnatal CMV infection most often occurs in immunocompromised persons, which causes encephalomyelitis and affects lungs, kidneys, liver and salivary glands.
- **Cytomegalovirus infection in immunocompetent persons:** Cytomegalovirus infection in immunocompetent person is generally asymptomatic or may present as cytomegalovirus mononucleosis syndrome, which is characterized by long duration of fever but has less cervical lymphadenopathy.
- **Cytomegalovirus infection in immunocompromised persons:** Cytomegalovirus infection in immunocompromised persons cause substantial morbidity and mortality especially in persons with human immunodeficiency virus (HIV) infection and among transplant recipients.

Surgical Pathology: Cytomegalovirus Involving Organs

Light Microscopy

- The organ affected shows mononuclear cells infiltration with mild edema.
- The infected cells are large with prominent deep blue inclusion in nuclei surrounded by a clear space giving an 'owl eye appearance'.
- Necrosis and calcification are seen.

Histochemistry

Cytomegalovirus is highlighted by PAS and silver methenamine stain.

Immunohistochemistry

Monoclonal antibodies to cytomegalovirus are used for diagnosis.

HUMAN PAPILLOMAVIRUS INFECTION

Human papillomavirus (6, 11) causes vulvar wart known as 'condyloma acuminatum' on vagina and cervix. Koilocytes (intracytoplasmic vacuolation) are indicative of HPV-infected epithelial cells, which are apparent in cytopathologic (Papanicolaou smear) and histopathologic preparations. High-risk human papillomavirus (16, 18, 31, 33, 35, 39, 45) may contribute to the pathogenesis of squamous cell carcinoma of vulva and vagina. Oncogenic risk for anogenital human papillomavirus is given in Table 7.22. Papanicolaou smear prepared from cervical squamous cell infected by human papillomavirus (HPV) is shown in Fig. 7.47.

JOHN CUNNINGHAM VIRUS (JC VIRUS) INFECTION

John Cunningham virus (JC virus) or also called human polyomavirus 2 infects the white matter of the brain and attacks the cells responsible for producing

Table 7.22 Oncogenic risk for anogenital human papillomavirus

Oncogenic Risk for Cervical Cancer	Human Papillomavirus	Histologic Features	
High-risk anogenital human papillomavirus	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 68, 59, 66	▪ HSIL (high-grade squamous intra-epithelial lesion) ▪ Koilocytosis occasional present ▪ Aneuploidy usually	▪ Abnormal mitoses frequent ▪ Undifferentiated cells and mitoses in upper two-thirds of cervical epithelium
Low-risk anogenital human papillomavirus	6, 11, 42, 43, 44, 53	▪ LSIL (low-grade squamous intra-epithelial lesion) ▪ Koilocytosis frequently present ▪ Diploid or polyploid (most often)	▪ Abnormal mitoses absent ▪ Undifferentiated cells and mitoses in lower third of cervical epithelium

Medium risk human papillomavirus includes 33, 35, 39, 51, 52, 56, 57, 58, 59, 68.

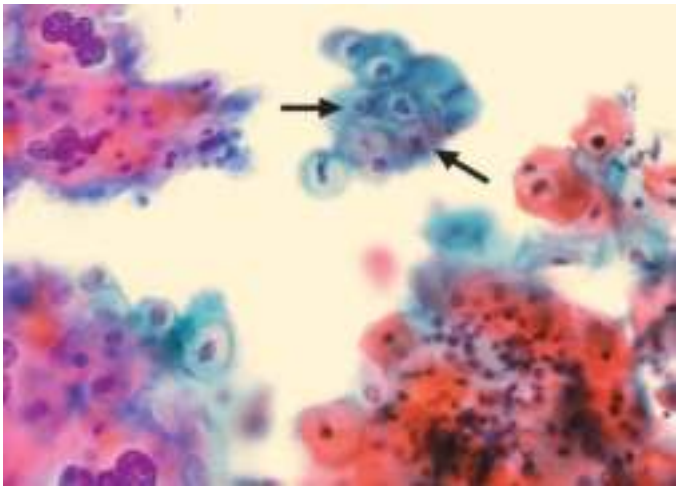


Fig. 7.47: Papanicolaou smear prepared from cervical squamous cell infected by human papillomavirus (HPV). It shows koilocytosis, the typical appearance. There is a 'halo' of cleared cytoplasm surrounding an enlarged nucleus (arrows) (400X).

myelin, the protective coating that covers and protects nerve cells. Imaging of the head and a spinal tap are performed.

Mode of Transmission

John Cunningham virus (JC virus) is transmitted via urine-oral route. The virus remains latent in the renal tubular epithelial cells, bone marrow hematopoietic stem cells (HSCs), tonsillar lymphoid cells and brain. Antibodies formed against JC virus cannot control JC virus reactivation and replication.

Clinical Features

Patient presents with clumsiness, weakness, progressing to dementia, partial vision loss and progressive multifocal leukoencephalopathy (**PML**) in immuno-compromised persons associated with fatal outcome within nine months.

HEPATITIS B VIRUS (HBV OR DANE PARTICLE) INFECTION

Hepatotropic viruses infecting liver include HAV, HBV, HCV, HDV and HEV. Acute viral hepatitis shows similar morphology irrespective of their precise etiology. HAV infection has a direct cytopathic effect. Hepatitis B is a potentially life-threatening liver infection caused by hepatitis B virus (HBV). Acute hepatitis has three phases: preicteric phase (prodromal phase), icteric phase (jaundice) and recovery phase (convalescence phase).

Pathogenesis

Hepatocellular injury occurs due to attack by CD8+ cytotoxic T cells and natural killer cells followed by antibodies formed against viral neoantigens expressed on virally infected hepatocytes. The mechanisms of hepatocellular injury have been most closely studied in HBV. It is thought that the extent of inflammation and necrosis depends on the person's immune response.

- **Prompt immune response:** A prompt immune response during the acute phase of HBV infection may cause hepatocellular cell injury but at the same time eliminate the virus.
- **Marginal immune response:** People with marginal immune response and fewer symptoms are less likely to eliminate the HBV, and hepatocytes expressing the viral antigens persist, leading to the chronic or carrier state.
- **Accelerated immune response:** It can be explained in terms of an accelerated immune response of HBV inducing severe hepatocellular necrosis.

Clinical Course

Patient may remain asymptomatic and can develop acute hepatitis without jaundice (anicteric hepatitis), acute hepatitis with jaundice (anicteric hepatitis), chronic hepatitis, chronic carrier stage and massive liver necrosis with acute hepatocellular failure.

PARVOVIRUS B19 INFECTION

Parvovirus B19 infects only human beings, that spreads from person to person. It most commonly causes classic 'slapped cheek' skin rash of erythema infectiosum (fifth disease), that mainly affects children. However, it may be observed in adults. Parvovirus B19 can also cause severe anemia.

Clinical Features

Patient presents with mild skin rash (erythema infectiosum), fatigue, low-grade fever, headache, and swollen and painful joints (more common in adults). Clinical course can vary in intensity and usually vanishes in 7–10 days, but it can come and go for several weeks. There is no vaccine available for Parvovirus B19.

HUMAN ADENOVIRUS INFECTION

Human adenoviruses are small nonenveloped DNA viruses with linear, double-stranded genome. In human, there are 88 human adenoviruses in seven species (human adenovirus A, B, C, D, E, F, G).

Mode of Transmission

Human adenoviruses are transmitted by two routes: (a) respiratory tract infections via contact with infectious material from another person, and (b) gastrointestinal tract infection occurs via fecal–oral route due to intake of contaminated food or water. Symptoms appear 1–2 days after exposure of children to adenovirus infection may persist for 1–2 weeks. Patient presents with abrupt onset of watery diarrhea, fever, abdominal tenderness and vomiting.

Clinical Features

Human adenoviruses are associated with common cold, croup, bronchitis or pneumonia, conjunctivitis and gastrointestinal tract infections. Adenovirus infection may occur at any age. Symptoms appear 2–14 days after infection. Patient presents with runny nose, sore throat, fever, severe cough, lymphadenopathy, headache, pink eyes.

Laboratory Diagnosis

Human adenovirus infection is diagnosed by complete medical history, physical examination, complete blood counts, nasal swab culture, stool culture and chest radiograph.

Complications

Complications of human adenoviruses infection include pneumonia, chronic obstructive pulmonary disease and intussusception.

FUNGAL DISEASES

Important fungal diseases include Candida, mucormycosis, Aspergillus, Cryptococcus, histoplasmosis, chromomycosis and rhinosporidiosis. Rhinosporidiosis mainly involves the mucosa of nose, nasopharynx and conjunctiva. Classification of pathogenic fungi and diseases is given in Table 7.23. Classification of pathogenic fungi is given in Table 7.24.

ASCOMYCOTA

Ascomycota are fleshy unicellular or multicellular fungi. Most of ascomycetes have reproductive structure called ascus. The ascus contains microscopic sexual reproductive cells are called **ascospores**. The mycelium is made up of chitin. Ascomycota has members such as **dermatophytes, molds, yeasts** and **dimorphic fungi**.

DERMATOPHYTES (SUPERFICIAL FUNGAL INFECTIONS)

Dermatophytes grow in the soil and on animals, which are often confined to the stratum corneum, hair and nails. The genera most often causing dermatophytosis include *Epidermophyton flocossum, Microsporum canis, Trichophyton interdigitale, Trichophyton rubrum*, which can produce a number of diverse and characteristic clinical lesions according to the area involved.

Cutaneous Dermatophytosis

Dermatophytes cause cutaneous dermatophytosis involving scalp, beard, skin and nails. Cutaneous dermatophytes and lesions are given in Table 7.25.

- **Tinea corporis:** It is commonly referred to as '**ringworm**', a term used by laypersons, to describe practically any annular or ring-like eruption on non-hairy regions of the trunk, but any region may be affected. Active site of growth of fungi occurs in the periphery of lesion, which may spread from other infected humans, or it may be autoinoculated from other regions of the body that are infected by tinea such as tinea pedis or tinea capitis.
- **Tinea capitis (scalp ringworm):** Dermatophyte fungal infection causes scalp and hair-shaft lesions. Patient presents with itchy, scaly bald (loss of hair) patches on scalp. Tinea capitis is highly contagious infection most often observed in toddlers and school-age children.

Table 7.23 Classification of pathogenic fungi and diseases

Fungus	Diseases
Ascomycota	
Dermatophytes	Dermatophytes (*Epidermophyton flocossum, Microsporum canis, Trichophyton interdigitale, Trichophyton rubrum*) cause cutaneous dermatophytosis: scalp, beard, skin and nails
Molds	*Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger* cause allergic bronchopulmonary aspergillosis, fungal balls in damaged lungs, invasive disease of lung, sinuses, brain in neutropenic patients
Yeast	Yeast (*Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*) causes oropharyngeal, mucocutaneous candidiasis
Dimorphic fungi	Dimorphic fungi cause pneumonia and disseminated infection involving organs ▪ *Blastomyces dermatitidis:* Skin, bone and genitourinary system ▪ *Coccidiodes immitis:* Central nervous system and bone ▪ *Histoplasma capsulatum:* Liver, spleen, spleen and bone marrow demonstrated in macrophages in immunocompromised states like HIV infection, corticosteroid therapy and immunosuppression therapy ▪ *Paracoccidioides brasiliensis:* Mucosa of mouth and nose
Basidiomycota	
Cryptococcus neoformans	*Cryptococcus neoformans* causes lung infections involving meninges in immunocompromised persons
Malassezia furfur	*Malassezia furfur* causes pityriasis of skin
Zygomycota*	
Absidia corymbifera	*Absidia corymbifera* causes rhinocerebral, thoracic and systemic infections in poorly controlled diabetes mellitus, burns and immunocompromised persons
Rhizomucor pusillus	*Rhizomucor pusillus* causes disseminated infections in immunocompromised persons
Rhizopus oryzae	*Rhizopus oryzae* invades and occlude blood vessels resulting in organ infarcts and hematogenous dissemination in immunocompromised persons
Dematiaceous fungi (pigmented fungi)	
Mycetoma	Mycetoma is chronic granulomatous disease of the skin and subcutaneous tissue, which sometimes involves skeletal muscle, bone and neighboring organs leading to tumefaction, abscess formation and fistulae in the lower extremities of farm workers
Phaeohyphomycosis	Phaeohyphomycosis is a clinical syndrome caused by melanized or dematiaceous fungi characterized by the presence of brown mycelial structures
Chromoblastomycosis	Chromoblastomycosis is a chronic fungal infection involving skin and subcutaneous tissue following traumatic injury and inoculation of the organism
Other fungi causing skin and subcutaneous infections	
Sporothrix schenckii	Sporotrichosis is also known as rose gardener disease caused by *Sporothrix schenckii* fungus
Cladosporium	*Cladosporium* species have been reported to cause infections of the skin and toenails as well as sinuses and lungs

* ***Zygomycota phylum members:*** *Absidia corymbifera, Rhizomucor pusillus and Rhizopus oryzae cause rhinocerebral, thoracic and systemic infections in poorly controlled diabetes mellitus, burns and immunocompromised persons.*

- **Tinea unguium:** It is common type of dermatophyte fungal infection called onychomycosis. The fungus causes infections in nails more commonly toe-nails, which become thickened and brittle. It is more common in older men, who have diabetes mellitus, peripheral vascular disease or immunocompromised persons.
- **Tinea cruris (jock itch):** Dermatophyte fungal infection causes skin lesions in moist areas such as upper thighs and genitalia. The disease is diagnosed by clinical manifestations and demonstration of fungus in potassium hydroxide wet mounted slide preparations.

Table 7.24 Classification of pathogenic fungi

Categories	Species
True pathogens	
Cutaneous infective fungi also called dermatophytosis	▪ *Epidermophyton flocossum* ▪ *Microsporum canis* ▪ *Trichophyton interdigitale* ▪ *Trichophyton rubrum*
Subcutaneous infective fungi (acquired through traumatic injury)	▪ *Actinomedura medurae* ▪ Cladosporium ▪ *Madurella grisea* ▪ *Sporothrix schenckii*
Fungi causing systemic infections	▪ *Blastomyces dermatitidis* ▪ *Coccidioides immitis* ▪ *Histoplasma capsulatum* ▪ *Paracoccidioides brasiliensis*
Opportunistic pathogens in immunocompromised persons	
Invading multiple organs	▪ *Aspergillus fumigatus* ▪ *Cryptococcus neoformans* ▪ *Candida albicans* ▪ *Absidia corymbifera* ▪ *Rhizomucor pusillus* ▪ *Rhizopus oryzae*

- **Tinea pedis (athlete foot):** It is a dermatophyte fungal infection that begins between the toes, which spreads to nails, which commonly occurs in persons whose feet become very sweaty while confined within tight-fitting shoes. Patient presents with scaly rash that usually causes itchy, stinging and burning sensations.

Laboratory Diagnosis

A scraping with KOH mount can be utilized to identify these fungi. Fungal cell walls are rich in mucopolysaccharides and stained with periodic acid-Schiff (PAS) stain resulting in bright pink to red appearance. Involved regions of skin and nails may fluoresce under ultraviolet light.

MOLDS

Molds (*Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger*) cause allergic bronchopulmonary aspergillosis, fungal balls in damaged lungs, invasive disease of lung, sinuses, brain in neutropenic patients.

Aspergillus Fumigatus

Aspergillus fumigatus grows and invades blood vessels and produces organ infarcts, which may also grow in preexisting cavities caused by tuberculosis or bronchiectasis. Invasive form of aspergillosis has predilection for growth into blood vessels, with consequent widespread hematogenous dissemination.

- The fungus proliferates to form fungus balls, which are also referred to as aspergillomas or mycetomas in patients with a previous history of **cavitating** pulmonary disease such as pulmonary tuberculosis.
- The fungus generally does not invade the lung parenchyma. Lung shows focal yellow areas of consolidation. There are three different types of pulmonary aspergillosis, namely allergic bronchopulmonary aspergillosis, aspergillomas, and invasive aspergillosis.

Aspergillus Flavus

Aspergillus flavus is an opportunistic pathogen of crops, which is important because *Aspergillus flavus* produces a variety of secondary metabolites such as aflatoxins, cyclopiazonic acid, aflavinin, aflatrem, kojic acid, aspergillic acid, neoaspergillic acid, β-nitropropionic acid and paspalinine in the seeds of crops before and

Table 7.25 Cutaneous dermatophytes and lesions

Disease	Fungi Responsible	Cutaneous Lesions
Tinea pedis (athlete foot)	▪ *Trichophyton rubrum* ▪ *Trichophyton mentagrophytes* ▪ *Epidermophyton flocossum*	Area between toes spreading to nails
Tinea corporis (ringworm)	▪ *Epidermophyton flocossum* ▪ *Trichophyton* species ▪ Microsporum	▪ Most often on nonhairy areas of the trunk, but any area may be affected ▪ Active site of growth of fungi in the periphery of lesion
Tinea capitis (scalp ringworm)	▪ *Trichophyton* species ▪ Microsporum	Scalp lesions with hair loss
Tinea cruris (jock itch)	▪ *Epidermophyton flocossum* ▪ *Trichophyton rubrum*	Skin lesions in moist areas such as upper thighs and genitalia
Tinea unguium	*Trichophyton rubrum*	Nails thickened and brittle

after harvests. Aflatoxin B1 is one of the highly toxic metabolites of *Aspergillus flavus*, which can cause hepatocellular carcinoma.

Aspergillus Niger

Aspergillus niger causes a disease called '**black mold**' on certain fruits and vegetables such as grapes, onions, apricots and peanuts, which is a common contaminant of food. Some strains of *Aspergillus niger* secrete ochratoxins-mycotoxins, which can cause nephrotoxicity and renal tumors on consumption of contaminated fruits and vegetables.

YEAST

Yeast (*Candida albicans, Candida glabrata, Candida krusei, Candida parapsilosis* is unicellular fungus, which is reproduced by budding and causes oropharyngeal, mucocutaneous candidiasis. Yeast can be demonstrated in tissues or exudates if cultured in an incubator at 37°C.

Candida Albicans

Candida albicans causes vulvovaginitis affecting 10% of women, which is a normal vaginal flora. *Candida albicans* infection is commonly associated with pregnancy, diabetes mellitus, broad-spectrum antibiotic therapy, oral contraceptive use, and immunosuppression resulting in fungal growth.

- In immunocompromised patients, invasive form of *Candida albicans* produces blood-borne dissemination leading to abscess formation in lungs, kidneys, renal liver and endocardium.
- Patient presents with white curd-like vaginal discharge that may cause intense itching. Per vaginal examination shows white patches on the mucosal surface of vagina covered with vaginal discharge. Diagnosis is best made microscopically on wet mounts on Pap smear showing fungal hyphae.

Candida Glabrata

Candida glabrata is an opportunistic human fungal infection that causes superficial mucosal and life-threatening bloodstream infections in immunocompromised persons.

Candida Krusei

Candida krusei is a human fungal infection of patients with hematologic malignancies and transplant recipients. The fungus causes invasive candidiasis in hospitalized patients mainly due to its natural resistanceto fluconazole.

DIMORPHIC FUNGI

Some fungi occur in both the yeast and mycelial forms, which are called dimorphic fungi, which cause pneumonia and disseminated infection. Dimorphic fungi causing systemic infections involving multiple organs are given in Table 7.26.

- *Blastomyces dermatitidis* causes disseminated infection that involves skin, bone and genitourinary system.
- *Histoplasma capsulatum* causes systemic infection that involves liver, spleen, spleen and bone marrow demonstrated in macrophages in immunocompromised states such as human immunodeficiency virus (HIV) infection, corticosteroid therapy and immunosuppression therapy.
- *Coccidioides immitis* can involve central nervous system and bone.
- *Paracoccidioides brasiliensis* produces mucosal lesions in mouth and nose.

Blastomyces Dermatitidis

Blastomyces dermatitidis resides in moist soil and decomposed wood and leaves, which causes blastomyosis involving skin, bone and genitourinary system. Patient presents with fever, cough, night sweats, musculoskeletal pain, weight loss, chest pain and fatigue.

Histoplasma Capsulatum

Histoplasma capsulatum infection occurs in immunocompromised states like human immunodeficiency virus (HIV) infection, corticosteroid therapy and immunosuppression therapy. Fungus causes multiple pulmonary lesions with late calcification.

- Disseminated form of *Histoplasma capsulatum* is marked by multisystem involvement (liver, spleen and bone marrow, lymph nodes, skin) with infiltrates of macrophages filled with fungal yeast forms.

Table 7.26 Dimorphic fungi causing systemic infections involving multiple organs

Systemic Fungi	Possible Sites of Infection
Blastomyces dermatitidis	Skin, bone and genitourinary system
Coccidioides immitis	Central nervous system and bone
*Histoplasma capsulatum**	Lungs, Liver, spleen and bone marrow
Paracoccidioides brasiliensis	Lung, mucosa of mouth and nose; dissemination to central nervous system

**Histoplasma capsulatum infection occurs in immunocompromised states like HIV infection, corticosteroid therapy and immunosuppression therapy. Histoplasma capsulatum is demonstrated in reticuloendothelial cells of liver, spleen and bone marrow.*

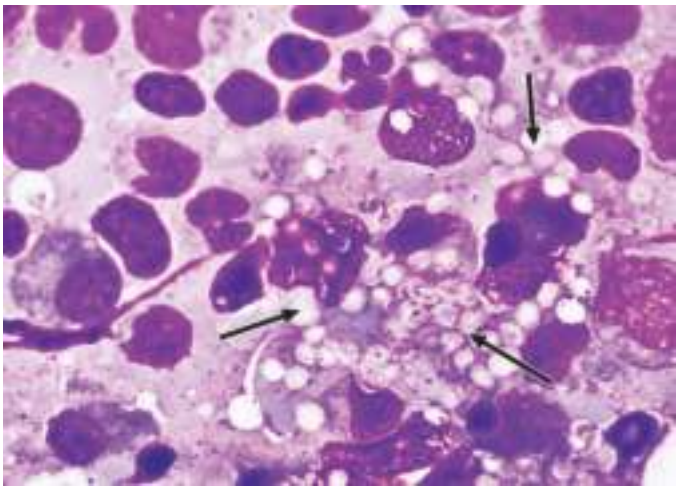


Fig. 7.48: Bone marrow demonstrates *Histoplasma capsulatum* as empty spaces in the reticuloendothelial cells in immunocompromised state (arrows) (1000X).

- *Histoplasma capsulatum* may remain clustered beyond the death of their host cells. It is difficult to identify the fungus on hematoxylin and eosin-stained sections, which appear as retracted artifact.
- Gomori methenamine silver stain and periodic acid–Schiff (PAS) stains are helpful to highlight organisms.
- Bone marrow demonstrates *Histoplasma capsulatum* as small, uniform, oval narrow-based yeasts appear as empty spaces in the reticuloendothelial cells in immunocompromised state is shown in Fig. 7.48.

Coccidioides Immitis

Coccidioides immitis occurs in primary and disseminated forms involving central nervous system and bone. Fungal spherules contain endospores found within granulomas.

Paracoccidioides Brasiliensis

Paracoccidioides brasiliensis is dimorphic human fungus endemic in subtropical regions of Mexico and Central and South America. The fungus infects lungs, oral mucosa and nasal cavity. Initial lesion occurs in lung, that disseminates to involve central nervous system.

BASIDIOMYCOTA

Basidiomycota are filamentous fungi composed of hyphae except its yeast form, which reproduce by sexual reproduction via formation of specialized club-shaped end cells called basidia that normally bear external meiospores (usually four). These specialized spores are called **basidiospores**.

CRYPTOCOCCUS NEOFORMANS

Pneumonia results from the inhalation of spores of *Cryptococcus neoformans*, which has a proteoglycan capsule responsible for its pathogenicity.

- *Cryptococcus neoformans* almost exclusively affects persons with impaired cell-mediated immune response, which has a proteoglycan capsule, which is essential for pathogenicity.
- The fungus primarily affects the meninges and lungs in immunocompromised persons, which appears as faintly stained, basophilic yeast with a clear, 3–5 μm thick mucinous capsule. *Cryptococcus neoformans* stains positively with a mucicarmine stain or Indian ink due to presence of polysaccharides in its capsule.
- Endobronchial ultrasound (EBUS) bronchoscopy demonstrates *Cryptococcus neoformans* is shown in Fig. 7.49. Mucicarmine stain demonstrates *Cryptococcus neoformans* is shown in Fig. 7.50. Silver methenamine stain demonstrates *Cryptococcus neoformans* is shown in Fig. 7.51.

MALASSEZIA FURFUR

Malassezia furfur is a fungus that resides on the superficial layers of the dermis, that causes tinea versicolor, catheter-related fungemia and sometimes pneumonia.

ZYGOMYCOTA

Zygomycota are usually rapid growing fungi characterized by **primitive coenocytic hyphae (mostly aseptate)**.

- Asexual reproduction in the zygomycetes results in nonmotile spores are called sporangiospores contained in sporangia borne on simple or branched sporangiophores.

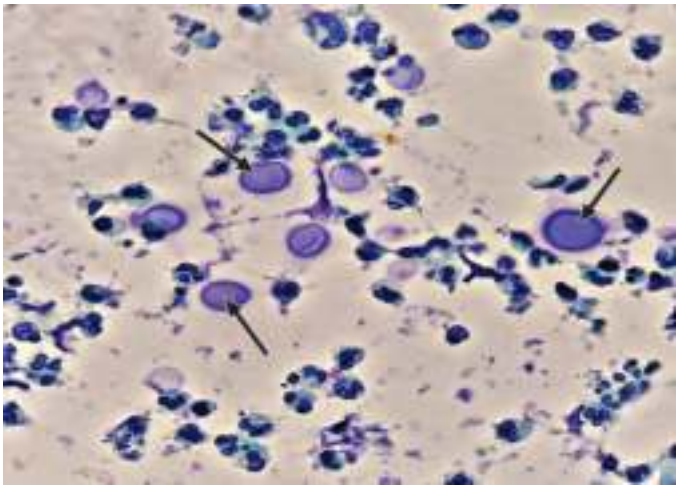


Fig. 7.49: Endobronchial ultrasound (EBUS) bronchoscopy demonstrates *Cryptococcus neoformans* (arrows) (400X).

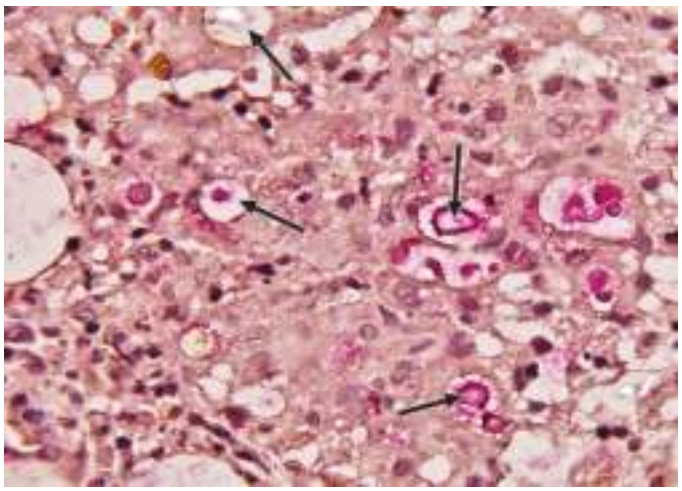


Fig. 7.50: Mucicarmine stain demonstrates *Cryptococcus neoformans* (arrows) (400X). (*Courtesy:* Dr. Vikram Raj Gopinathan, Postgraduate from Maulana Azad Medical College, New Delhi and presently working as Assistant Professor of Pathology, Christian Medical College, Vellore, Tamil Nadu.)

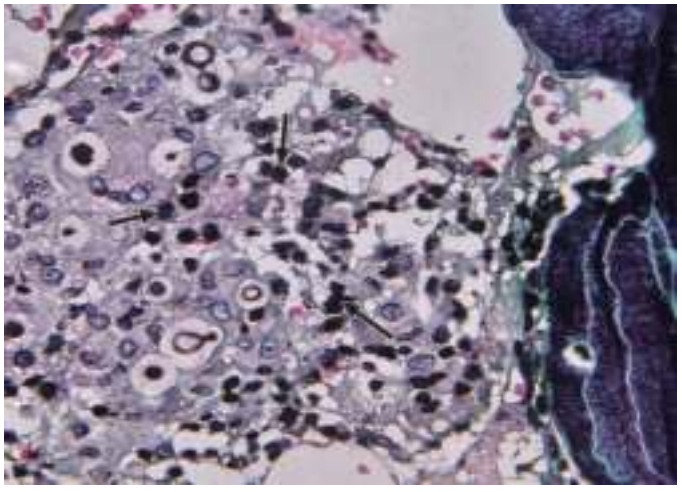


Fig. 7.51: Silver methenamine stain demonstrates *Cryptococcus neoformans* (arrows) (400X). (*Courtesy:* Dr. Vikram Raj Gopinathan, Postgraduate from Maulana Azad Medical College, New Delhi and presently working as Assistant Professor of Pathology, Christian Medical College, Vellore, Tamil Nadu.)

- Sexual spores are produced when two morphologically similar gametangia of opposite mating types fuse.

Pathology Pearls: Zygomycota—Members
Zygomycota members such as *Absidia corymbifera*, *Rhizomucor pusillus* and *Rhizopus oryzae* cause rhinocerebral, thoracic and systemic infections in poorly controlled diabetes mellitus, burns and immunocompromised persons.
Absidia corymbifera
Absidia corymbifera causes rhinocerebral, thoracic and systemic infections in poorly controlled diabetes mellitus, burns and immunocompromised persons.
Rhizomucor pusillus
Rhizomucor pusillus causes disseminated infections in immunocompromised persons.
Rhizopus oryzae
Rhizopus oryzae invades and occlude blood vessels resulting in organ infarcts and hematogenous dissemination in immunocompromised persons.

MUCORMYCOSIS

Mucormycosis has been previously called zygomycosis, which is also known as 'black fungus' which usually adversely affects immunocompromised persons.

- Symptoms depend on where in the body the infection occurs. There are three principal forms of mucormycosis, namely rhinocerebral, pulmonary, and cutaneous.
- Mucormycosis can produce necrotizing opportunistic infections that begin in the nasal sinuses or lungs.
- Histologic examination shows a purulent arteritis with thrombi composed of broad nonseptate hyphae branching at 90° accompanied by numerous neutrophils and histiocytes. Histologic characteristics of mucormycosis are prominent organ infarct, angioinvasion and perineural invasion.
- Mucormycosis should be suspected in patients who present with a paranasal sinusitis unresponsive to antibiotic treatment, particularly those who also have an underlying chronic disease (e.g. diabetes or leukemia). Histopathologic features of mucormycosis are shown in Fig. 7.52.

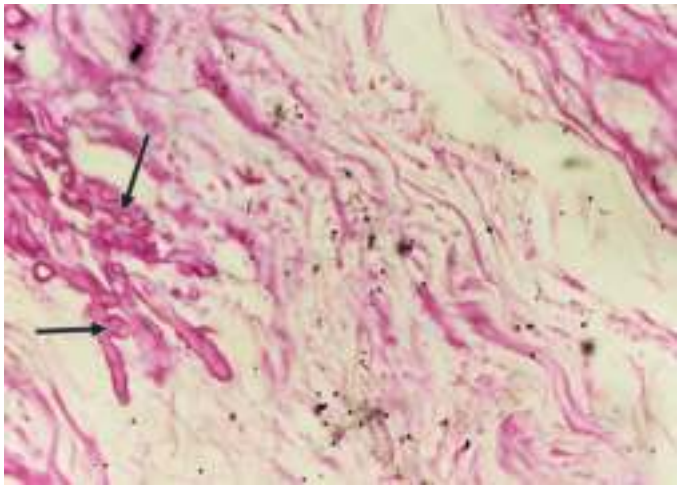


Fig. 7.52: Histopathologic features of mucormycosis. It demonstrates broad nonseptate hyphae branching at 90° accompanied by numerous neutrophils and histiocytes. Histopathologic characteristics of mucormycosis are prominent tissue infarct, angioinvasion and perineural invasion (arrows) (400X).

Pathology Pearls: Mucormycosis—Clinical Manifestations

Rhinocerebral Mucormycosis

- Pulmonary mucormycosis is usually fatal. The hard palate or nasal cavity is typically covered by a black crust, and the underlying tissues become friable and hemorrhagic.
- The fungal hyphae grow into arteries, causing devastating and rapidly progressive septic embolic infarctions also called zygomycosis.
- Rhinocerebral mucormycosis is caused by filamentous fungus that involves the nose, paranasal sinuses and brain in immunocompromised persons.

Pulmonary Mucormycosis

- Pulmonary mucormycosis is usually fatal. The hard palate or nasal cavity is typically covered by a black crust, and the underlying tissues become friable and hemorrhagic.
- The fungal hyphae grow into arteries, causing devastating and rapidly progressive septic embolic infarctions.

Subcutaneous Mucormycosis

- Subcutaneous mucormycosis is caused by opportunistic fungal infection in immunocompromised persons. Infection is acquired by direct inoculation through trauma.
- Patient presents with an indurated, localized, firm, nontender and mobile swelling, that rapidly evolved to necrosis is common pathologic finding.
- Diagnosis is established on histologic examination and culture.

DEMATIACEOUS FUNGI

Dematiaceous fungi are characterized by dark pigmentation of hyphae, which can cause diseases by direct inoculation or spore inhalation in human beings.

- Dematiaceous fungi are unique owing to the presence of melanin pigment in their walls, which imparts the characteristic dark color to their spores and hyphae.
  - Melanin may also be a virulence factor. Melanin production in hyphae scavenges oxygen-derived free radicals and inhibits phagocytosis.
  - Acute inflammatory cells fail to clear fungal infection leading to pyogranulomatous inflammation, which shows neutrophils and mononuclear cells.
  - Pigmentation may be faint on histologic examination; Masson-Fontana stain for melanin may prove helpful.
- Dematiaceous fungi can invade subcutaneous tissue, lungs, central nervous system and systemic or dissemination in immunocompromised patients.

Pathology Pearls: Dematiaceous Fungi

Examples of human diseases caused by dematiaceous fungi include mycetoma, phaeohyphomycosis and chromoblastomycosis. Mycetoma and chromoblastomycosis are prevalent in tropical and subtropical regions.

- **Mycetoma:** It is chronic subcutaneous collection of pigmented hyphae (fungus ball) that expands within the tissues and form tumor-like growth.
- **Phaeohyphomycosis:** It occurs due to trauma characterized by widely disseminated infection in immunocompromised patients. Its pigmented hyphae invade within skin, lung and brain.
- **Chromoblastomycosis:** It is chronic subcutaneous infection characterized by pigmented round structures termed 'copper pennies' within skin but no hyphae present.

Clinical Pearls: CARD19 (Caspase Recruitment Domain Family Member 19) Deficiency

- CARD19 is a protein encoding gene, that regulates apoptosis and inflammation through modulation of signals from pro-inflammatory to anti-inflammatory cytokines.
- Deficiency of CARD19, an autosomal recessive disorder, is associated with increased susceptibility to fungal infections especially phaeohyphomycosis, dermatophytes and *Candida albicans*.

MYCETOMA (MADURA FOOT)

Mycetoma (madura foot) is a chronic granulomatous subcutaneous infection caused by fungi (eumycetoma) or aerobic filamentous actinomycetoma. Mycetoma is commonly prevalent in tropical and subtropical countries like Central America, South America, Africa and India. Men especially agricultural workers are more susceptible.

- Mycetoma involves lower extremities, hands, back and buttocks.
  - Mycetoma starts with as a painless lump under the skin involving underlying fascia, which progresses to open infection, discharging sinuses with 'small grains' containing fungal spores leading to swollen, disfigured body regions such as lower extremities, hands, head and neck, chest, shoulder, arms, back and buttocks. Rare sites for eumycetoma include abdominal wall, face involving bones, paranasal sinuses, orbit or eyelid, and genitalia involving vulva or scrotum.
  - Madura foot shows suboptimal response to a wide range of antibiotics.
  - Untreated cases have no other option but to undergo amputation that consequently results in lifelong disability.

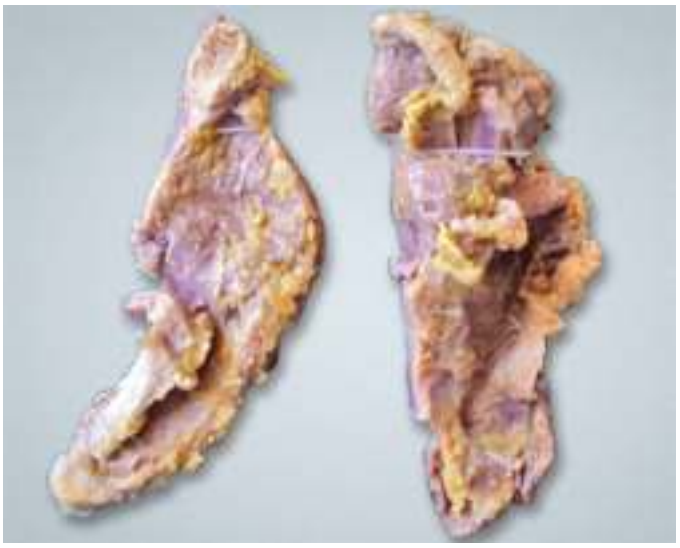


Fig. 7.53: Gross morphology of mycetoma. Cut surface of mycetoma foot demonstrates black granules. (*Courtesy*: Department of Pathology, Sapthagiri Institute of Medical Sciences, Bengaluru, Karnataka.)

- Histologic examination of mycetoma shows black mycotic granules or grains surrounded by dense extracellular matrix. Pigmentation may be faint; **Fontana-Masson stain for melanin** may prove helpful. Gross morphology of mycetoma is shown in Fig. 7.53.

Actinomycetoma Madurae

Actinomadura madurae is one of the most frequent actinomycetes. Actinomycetoma occurs more often on chest or abdominal wall as compared to the extremities and mycetoma involving both limbs also have been reported.

Madurella Grisea

Madurella grisea causes **'black grain' mycetoma** in India, Africa, Central America and South America. Granules are black, round to lobed and soft to firm in consistency, which measure 0.3–0.6 mm in diameter with two distinct zones, hyaline to weakly pigmented central zone and deeply pigmented periphery. Diagnosis is established by examination of tissue sample under light microscope.

PHAEOHYPHOMYCOSIS

Phaeohyphomycosis refers to infections due to dematiaceous fungi, that can invade subcutaneous tissue, lungs, central nervous system and systemic or dissemination in immunocompromised patients.

- Dematiaceous fungal hyphae contain black melanin pigment in their walls, which likely act as virulence factor. Virtually, every person is exposed to dematiaceous fungi through inhalation, as these are ubiquitous in the environment.
- The highly virulent fungi implicated in causing brain abscess include *Cladophialophora bantiana* (neutropic; immunocompetent), *Exophiala dermatitidis* (neutropic; black yeast), *Lomentospora prolificans* (resistant to all antifungal drugs), *Rhinocladiella mackenziei*, *Verruconis gallopava*, *Chaetomium strumarium*, *Fonsecaea monophora*, *Wangiella dermatitidis*, *Bipolaris specifera*, *Curvularia lunata* and *Exserohilum heteropogonicola* (outbreak associated with corticosteroid injections).

Clinical Features

Patients present with invasive sinusitis, subcutaneous nodules or abscess, keratitis, pulmonary masses, brain abscesses, osteomyelitis, mycotic arthritis, endocarditis and disseminated infection.

Laboratory Diagnosis

Phaeohyphomycosis can frequently be discerned in tissue specimens stained with conventional hematoxylin and eosin and examined under light microscope, which appears as brownish, pigmented and septate hyphae, reflecting their high melanin content. Masson-Fontana staining for melanin confirms the diagnosis. Phaeohyphomycosis is distinguished from mycetoma and chromoblastomycosis by the absence of specific histopathologic findings such as sclerotic bodies or grains in the tissue. Culture is required to identify the causative species. Phaeohyphomycosis in brain (pigmented fungus) is shown in Figs 7.54 and 7.55.

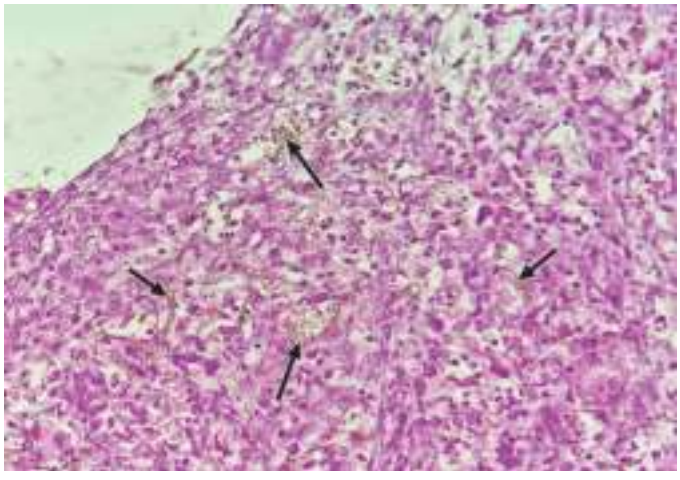


Fig. 7.54: Phaeohyphomycosis in brain (pigmented fungus). Phaeohyphomycosis is a clinical syndrome caused by melanized or dematiaceous fungi characterized by the presence of brown mycelial structures. The fungus has caused black, necrotic brain tissue, black pus, and black cerebrospinal fluid. Histologic examination of lesion demonstrates brown-walled septate hyphae (arrows) (400X). (*Courtesy*: Dr. Tushar Kalonia and Dr. Neha Kumari.)

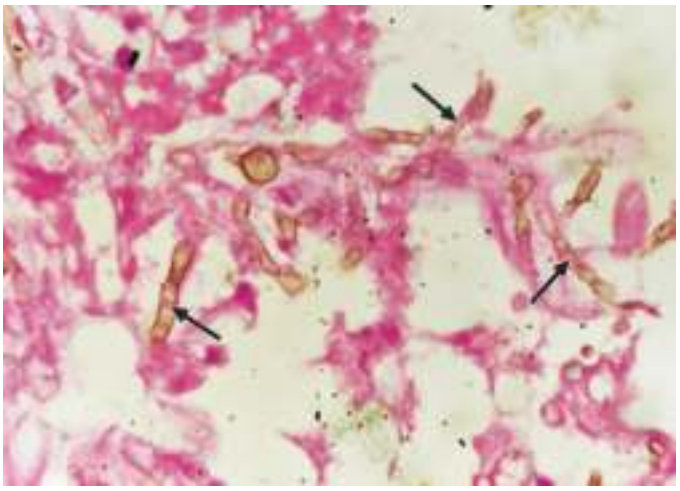


Fig. 7.55: Phaeohyphomycosis in brain (pigmented fungus). The fungus has caused black, necrotic brain tissue, black pus, and black cerebrospinal fluid. Histologic examination of lesion demonstrates brown-walled septate hyphae (arrows) (400X). (*Courtesy*: Dr. Neha Kumari and Dr. Tushar Kalonia.)

CHROMOBLASTOMYCOSIS

Chromoblastomycosis is a chronic fungal infection, which most often affects and subcutaneous tissue of upper and lower limbs at the inoculation site by a minor injury such as a cut with a splinter in barefooted person. Examples of chromoblastomycosis include *Phialophora verrucosa*, *Fonsecaea compacta*, *Fonsecaea pedrosi*, *Rhinocladiella aquaspersa* and *Cladophialophora carrionii*. Chromoblastomycosis is found in soil and plant debris mainly in tropical and subtropical regions. It is transmitted by traumatic inoculation.

Clinical Features

Patient presents with slowly progressive raised hyperkeratotic crusted warty looking lesion within 1.5 years over foot or hands of adult men engaged in outdoor work. The affected limb can enlarge generally that mimics elephantiasis. Satellite new primary lesions can develop. Rarely, squamous cell carcinoma of skin develops within long-standing chromoblastomycosis.

Laboratory Diagnosis

Histologic examination of chromoblastomycosis demonstrates dark typical thick-walled dark brown sclerotic bodies with thick-walled septations. It is dark colored fungus due to presence of melanin in its walls. Culture of fungus in Sabouraud medium with antibiotics at 25–30°C grows olive-green to black colonies after one or two weeks.

OTHER FUNGI CAUSING SKIN AND SUBCUTANEOUS TISSUE INFECTIONS

Sporothrix schenckii and Cladosporium fungi can cause skin and subcutaneous infections by direct inoculation following minor injury or inhalation. Other fungi causing skin and subcutaneous infections through traumatic injury are given in Table 7.27.

SPOROTHRIX SCHENCKII

Sporothrix schenckii causes sporotrichosis, which is a chronic infection of the skin and subcutaneous tissue. The fungus lives in soil, decaying plants and hay; and other living plants. *Sporothrix schenckii* usually enters the body through traumatic implantation of soil, plants and organic matter contaminated with fungus.

CLADOSPORIUM

Cladosporium is a genus of mold that includes more than 40 individual species of fungus, which can be found in indoor (basements, bathrooms, under sinks, carpets, curtains) and outdoor (decaying trees, dead plants and tree trunks) places. Most kinds of Cladosporium are not harmful to human beings. *Cladosporium* species have been reported to cause infections of the skin and toe-nails as well as sinuses and lungs including bronchial asthma.

Table 7.27 Other fungi causing skin and subcutaneous infections through traumatic injury

Fungus	Clinical Manifestations
Sporothrix schenckii	▪ Granulomatous ulcer at punctured site ▪ Secondary lesions along draining lymphatics
Cladosporium species	▪ Wart nodules ▪ Crusty abscess along draining lymphatics

PARASITIC INFESTATIONS

Parasites need another living host to get the nutrients they need to survive. Parasitic diseases are caused by protozoa and helminths, which can be spread through contaminated water, food, waste, soil, and blood. Cysticercosis, malaria, filariasis and hydatid cysts are common in India. Clinically important protozoa are given in Table 7.28. Clinically important metazoa (helminths) are given in Table 7.29.

Table 7.28 Clinically important protozoa

Pathogenic Protozoa	Mode of Locomotion	Site of Infection
Intestinal protozoa		
Entamoeba histolytica (amoeba)	Move by cytoplasmic projections	▪ Colon and secondary liver involvement ▪ Cysts demonstrated in stool
Giardia lamblia (flagellates)	Move by rotating whip-like flagella	▪ Duodenum (infection with drinking contaminated water) ▪ Foul smelling watery diarrhea ▪ Trophozoites and cysts demonstrated in stool
Cryptosporidium parvum (sporozoan)	Nonmotile adult form	▪ Parasite is intracellular in intestinal villus epithelium ▪ Infection by drinking contaminated water ▪ Duodenum involved (foul smelling diarrhea) ▪ Stool examination showing trophozoites and cysts
Urogenital system		
Trichomonas vaginalis (flagellates)	Move by rotating whip-like flagella	▪ Vagina, vulva and cervix in women ▪ Urethra, seminal vesicle and prostate in men ▪ Higher than normal pH favoring growth
Blood and tissues protozoa		
Plasmodium species (sporozoan)	Nonmotile adult form	Sporozoites introduced by mosquito bite
Toxoplasma gondii (sporozoan)	Nonmotile adult form	Sporozoites entering liver becoming merozoites infecting red blood cells
Trypanosoma species (flagellates)	Move by rotating whip-like flagella	▪ Cardiomyopathy in Americans by *Trypanosoma cruzi* ▪ CNS by *Trypanosoma brucei* and *Trypanosoma gambiense*
Leishmania species (flagellates)	Move by rotating whip-like flagella	▪ Phlebotomus (sand fly) bite ▪ Liver, spleen, lymph nodes and bone marrow (parasite infecting macrophages and migrating to these organs) ▪ Leishman-Donovan (LD) bodies demonstrated in tissue

Table 7.29 Clinically important helminths

Groups	Examples
Cestodes (tapeworms)	▪ *Echinococcus granulosus* (dog tapeworm) produces hydatid cyst in liver, lung and brain ▪ *Taenia solium* (pork tapeworm) produces cysticercosis diagnosed by CT and histopathologic examination ▪ *Taenia saginata* (beef tapeworm) ▪ *Diphyllobothrium latum* (broad fish tapeworm)
Trematodes (flukes)	▪ *Schistosoma haematobium* (blood fluke) ▪ *Schistosoma mansoni* (blood fluke) ▪ *Clonorchis sinensis* (Chinese or oriental liver fluke) ▪ *Paragonimus westermani* (lung fluke)
Nematodes (roundworms)	▪ *Ancyclostoma duodenale* (hookworm in intestine) ▪ *Ascaris lumbricoides* (giant roundworm in intestine) ▪ *Enterobium vermicularis* (small roundworm or threadworm in intestine) ▪ *Trichinella spiralis* (worm in intestine) ▪ *Trichuris trichiura* (whipworm in intestine) ▪ *Wuchereria bancrofti* (filarial worm) causing tissue infection ▪ *Oncocerca volvulus* (filarial worm) causing tissue infection ▪ *Loa-loa* (filarial worm or African eye worm) causing tissue infection ▪ *Brugia malayi* causing tissue infection ▪ *Dracunculus medinensis* causing tissue infection ▪ *Toxocara canis* (dog worm) causing tissue infection

PROTOZOA

Protozoa are eukaryotic, unicellular and lacking cell wall. The organelles of protozoa have similar functions to the organelles of higher animals. Protozoa reproduce primarily by asexual means, although some can reproduce by sexual mode.

ENTAMOEBA HISTOLYTICA

Entamoeba histolytica in the colon of infected persons in the form of infective cyst and trophozoites in the vegetative form. *Entamoeba histolytica* is transmitted by fecal–oral contact.

- **Cyst** of *Entamoeba histolytica* is the infective stage of parasite, which is surrounded by specific membrane. Cyst measures 10–20 × 12–50 µm in diameter, which contains 1, 2, 3, or 4 nuclei with a karyosome. Cyst can be demonstrated by **Lugol's iodine** stain or **Gomori-Wheatley stain**.
- **Trophozoite** form of parasite with a diameter of 12–50 µm is surrounded by cell membrane that creates amoeboid pseudopodia that participates in endocytosis of food particles.
  - Trophozoite contains a nucleus in which DNA is concentrated in the form of small dense karyosome and evenly distributed chromatin in the peripheral region.
  - Trophozoite invades submucosal veins of the colon, enters the portal circulation, and gains access to the liver leading to amoebic liver abscess.

Pathophysiology

Entamoeba histolytica can cause two types of clinical forms or amoebiasis: (a) acute or chronic intestinal amoebiasis, and (b) amoebic liver abscess.

Intestinal Amoebiasis

Acute intestinal amoebiasis is caused by trophozoites of *Entamoeba histolytica*. Trophozoites adhere to the epithelial cells of the colon, and synthesize proteolytic enzymes such as hyaluronidase, cysteine proteinase, cathepsin B, which produce a local inflammatory reaction, congestion and degradation.

- Trophozoites further invade the intestinal submucosal tissue and induce cyclooxygenase 2 enzyme leading to increased secretion of prostaglandin E_2 (PGE_2), which contribute to inflammatory process.
- Acute intestinal amoebiasis manifests with persistent diarrhea containing mucus and blood in the stool, and accompanied by abdominal pain, flatulence, vomiting and fever. Severe diarrhea results in hemorrhages and ulcers in the colon, dehydration, electrolyte water imbalance, cardiovascular instability and collapse.
- Untreated cases lead to chronic intestinal amoebiasis, which is characterized by alternating bloodless diarrhea and constipation of varying severity, ulcerative colitis, liver enlargement, low-grade fever and anemia.

Amoebic Hepatitis and Abscess

In some persons, amoeba trophozoites may travel through the bloodstream from the large intestine to the liver.

- Amoebic hepatitis can develop as a consequence of acute intestinal amoebiasis, which is characterized by liver hepatomegally, fever, chills and perspirations. Biochemical analysis reveals elevated liver enzymes ALT and AST.
- Amoebic liver abscess is dangerous complication of acute intestinal amoebiasis, which is filled with a dark brown material that resembles anchovy paste. Patient presents with abrupt onset of fever, anorexia and dull aching abdominal pain in the right upper quadrant or epigastrium, usually lasting less than 10 days. Jaundice is unusual.
- An amoebic liver abscess can rupture and extend into the peritoneal cavity. Trophozoites of *Entamoeba histolytica* in the mucosa and submucosa of large intestine are shown in Fig. 7.56.

Other Organs Involved due to Amoebiasis

Amoebic liver abscess can create an abscess in the various organs such as lungs, pericardium, spleen, brain, kidneys and urinary bladder in immunocompromised persons. Sometimes amoebic abscesses require surgical removal. *Entamoeba histolytica* can cause asymptomatic infestations (subclinical disease) as trophozoites do not cause damage to the mucosa of large intestine.

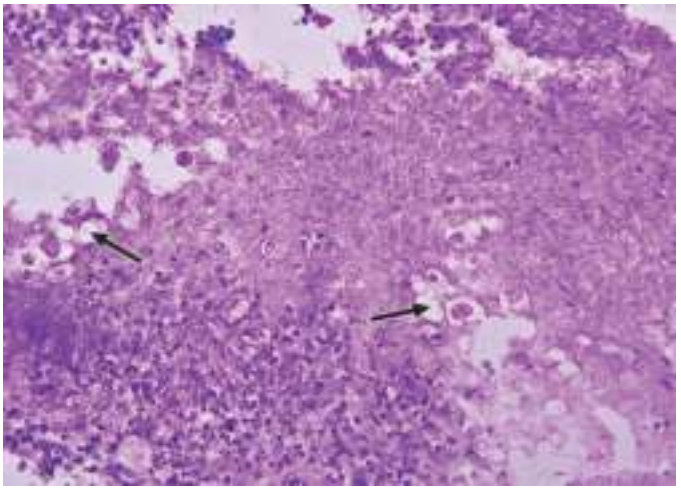


Fig. 7.56: Trophozoites of *Entamoeba histolytica* in the mucosa and submucosa of large intestine. *Entamoeba histolytica* is a protozoan of human large intestine, which is a causative agent of amoebiasis (arrows) (400X).

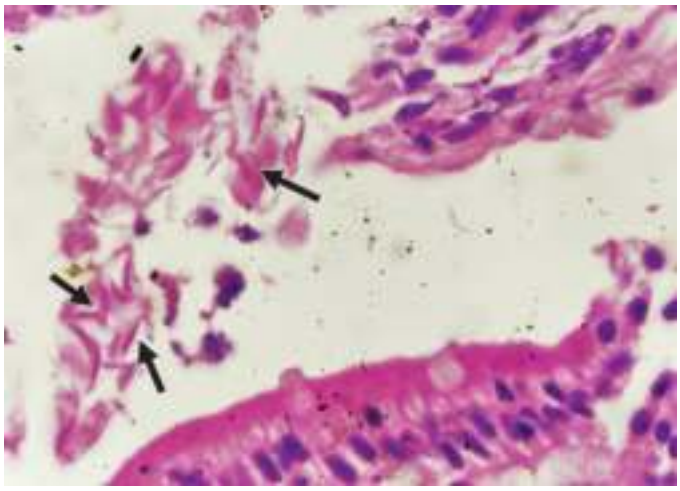


Fig. 7.57: Giardia lamblia parasites are seen on the mucosal side of intestine (arrows) (400X).

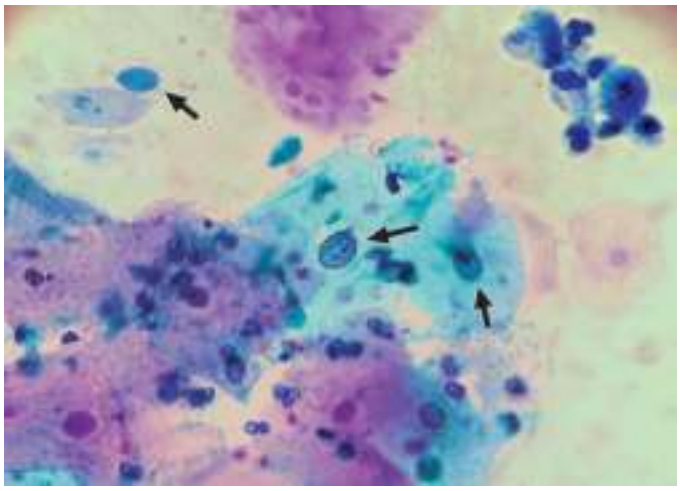


Fig. 7.58: *Trichomonas vaginalis*. Pap smear demonstrates *Trichomonas vaginalis* (arrows) (400X).

GIARDIA LAMBLIA

Giardiasis is an infestation of the small intestine by the flagellated protozoan *Giardia lamblia*. Parasite can be acquired from contaminated water or food.

- Patient presents with abdominal cramping and non-bloody diarrhea. The gastrointestinal symptom usually resolves in 1–4 weeks, but chronic giardiasis may lead to malabsorption, weight loss, and growth retardation. *Giardia lamblia* does not induce significant villus architectural changes.
- The *Giardia lamblia* are recovered from stool specimens, duodenal aspirates, or luminal surface of normal appearing mucosa of intestinal biopsies. The parasite is mistaken as cytoplasmic debris. Giemsa stain and Masson trichrome stain highlight the parasite. *Giardia lamblia* parasites are seen on the mucosal side of intestine are shown in Fig. 7.57.

TRICHOMONAS VAGINALIS

Trichomonas vaginalis is the second most common cause of vaginitis commonly transmitted by sexual contact. Patient presents with a profuse opaque or creamy colored frothy vaginal discharge with fishy smell.

- Vaginal discharge may cause vulvar irritation and burning micturition due to urethral inflammation. *Trichomonas vaginalis* is best diagnosed in freshly prepared wet mounts (i.e. smears of unfixed vaginal discharge in which the flagellated protozoa keep moving).
- *Trichomonas vaginalis* is also demonstrated as flagellated motile organisms in wet mounted smear. *Trichomonas vaginalis* is shown in Fig. 7.58.

MALARIAL PARASITE

Plasmodium is a wide distribution in many tropical or subtropical regions of the world. *Plasmodium* species known to cause malaria in human beings are *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium ovale* and *Plasmodium malariae*. *Plasmodium* species infect erythrocytes. Unlike those of other *Plasmodium* species, gametocytes of *Plasmodium falciparum* are elongated and crescent-shaped by which they are identified.

- *Plasmodium falciparum* causes 'blackwater fever' and hemoglobinuria. Red blood cell hemolysis releases hemoglobin into the urine leading to renal failure. Ischemic injury to the brain leads to range of symptoms such as somnolence, hallucinations, behavioral changes, seizures, and even coma. The liver, spleen, and lymph nodes are darkened by macrophages that are filled with hemosiderin and malaria pigments.
- Timely identification of the infecting *Plasmodium* species is important, as *Plasmodium falciparum* can be fatal and is often resistant to chloroquine treatment. Peripheral blood smear demonstrates infected red blood cells with *Plasmodium falciparum*.

Life Cycle

Asexual life cycle of *Plasmodium falciparum* occurs in human and sexual life cycle in mosquito. Infected mosquito bites and injects sporozoites, which infect the hepatocytes resulting in release of merozoites.

- The **merozoites** infect red blood cells and develop into trophozoites, schizont and gametocytes. The trophozoites multiply and produce merozoites, which infects other red blood cells.

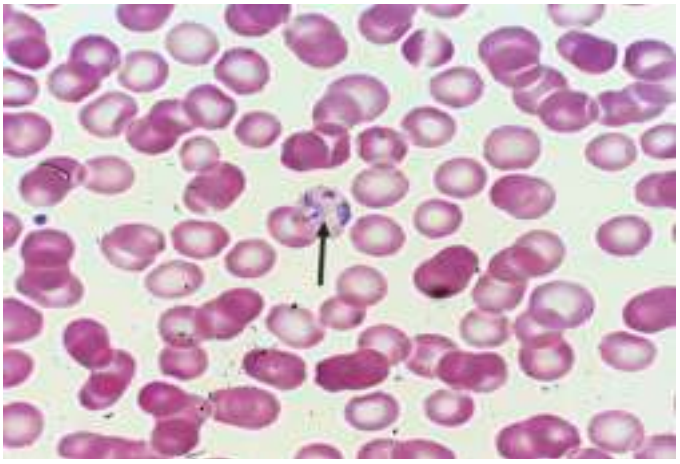


Fig. 7.59: *Plasmodium vivax*. Peripheral blood smear examination shows infected red blood cells and Schuffner's dots and amoeboid trophozoite (arrows) (1000X).

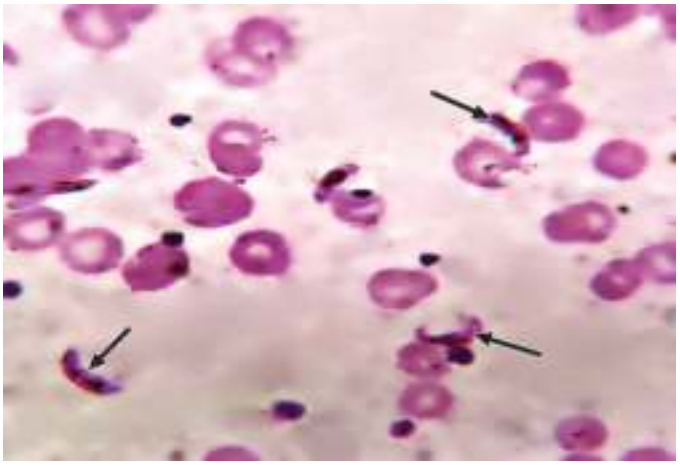


Fig. 7.60: *Plasmodium falciparum* in peripheral blood smear. Unlike those of other *Plasmodium* species, gametocytes of *Plasmodium falciparum* are elongated and crescent-shaped by which they are identified (arrows) (1000X).

- Rupture of infected red blood cells liberate gametocytes. The female mosquito picks up gametocytes from an infected human. Sexual cycle occurs in the mosquito leading to formation of sporozoites.
- *Plasmodium vivax* and **Schuffner's dots** and amoeboid trophozoite in infected red blood cells are shown in Fig. 7.59. *Plasmodium falciparum* in peripheral blood smear is shown in Fig. 7.60.

LEISHMANIASIS

Leishmania parasites are intracellular protozoans, which are transmitted to humans through insect bites of Phlebotomus sandflies, which acquire infections from feeding on infected animals. They cause a spectrum of clinical syndromes, ranging from indolent self-resolving cutaneous ulcers to fatal disseminated disease.

- Amastigote form is present in the human body, which measures 1–2 × 3–6 micron in diameter and ovoid in shape. Amastigote form is composed of intercalated ring of DNA, which serves key metabolic functions and possible drug target.
- Amastigote form is detectable within the reticuloendothelial cells (macrophages/monocytes). Kinetoplast looks like a small nucleus and helpful feature for identification. Culture and polymerase chain reaction are diagnostic modalities.

Clinical Features

Three distinct clinical entities are recognized: localized cutaneous leishmaniasis, mucocutaneous leishmaniasis, and visceral leishmaniasis.

- **Cutaneous leishmaniasis (localized/diffuse):** Cutaneous leishmaniasis is caused by *Leishmania major*, *Leishmania tropica*, *Leishmania guinensis*. Patient with small, erythematous and pruritic papule leading to formation of round and ring-shaped ulcer and local lymphadenopathy, which may regress spontaneously with scar formation although recurrences may occur.
- **Mucocutaneous leishmaniasis:** Mucocutaneous leishmaniasis is caused by *Leishmania brasiliensis* and *Leishmania guinensis*. Patient presents with disfigurement of nasopharynx, pharynx and perioral skin.
- **Visceral leishmaniasis (kala azar):** Visceral leishmaniasis is caused by *Leishmania donovani* (India, East Africa), *Leishmania infantum*, *Leishmania tropica* and *Leishmania chagasi*.
  - Amastigote form of parasite involves reticuloendothelial cells. Patient presents with persistent fever, progressive weight loss, hepatosplenomegaly and hematopoietic suppression (anemia, thrombocytopenia, and leukopenia).
  - Light-skinned persons develop darkening of the skin, which is usually fatal if untreated. Histology of visceral leishmaniasis is shown in Fig. 7.61. Bone marrow involvement by leishmaniasis is shown in Fig. 7.62.

BABESIOSIS

Babesiosis is caused by microscopic parasite 'Babesia' that infects red blood cells. Babesia parasitic infection is transmitted by certain tick especially during warm months in North East and upper Mid-East regions.

- The parasite grows and reproduces inside the red blood cells of the infected persons and often causes intense pain due to red blood cells rupture.
- Many persons infected with Babesia remain asymptomatic. Disease can be prevented if steps are taken to reduce exposure to ticks.

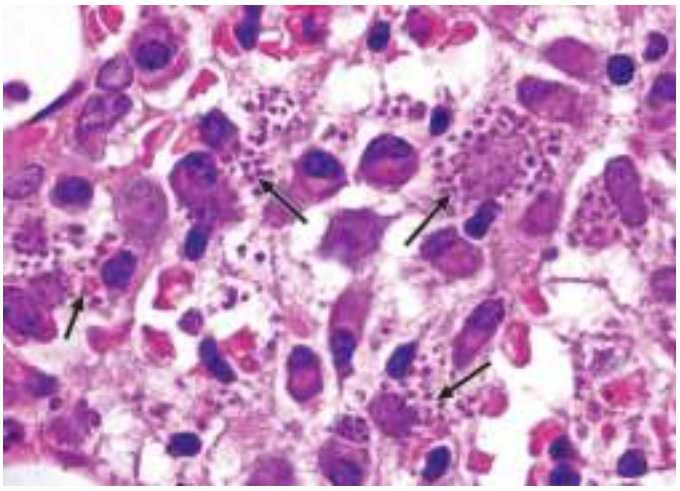


Fig. 7.61: Histology of visceral leishmaniasis demonstrates amastigote stage of *Leishmania donovani* in reticuloendothelial cells (arrows) (400X).

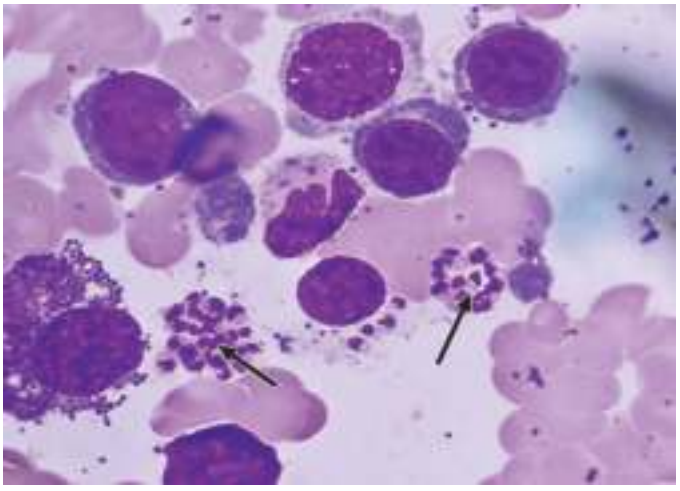


Fig. 7.62: Bone marrow involvement by leishmaniasis demonstrates amastigote stage of *Leishmania donovani* in reticuloendothelial cells (arrows) (1000X).

Clinical Features

Severity of the symptoms of babesiosis can vary from slight flu-like to life-threatening complications.

- Patient presents with high fever, chills, muscular joint aches and fatigue. Less common manifestations include severe headache, abdominal pain, nausea, jaundice and skin bruising.
- Disease progression may cause chest or hip pain, breathlessness and drenching sweats. Patient may develop complications such as hypotension, hemolytic anemia, liver dysfunction, renal failure and heart failure.

AFRICAN TRYPANOSOMIASIS

Human African trypanosomiasis is also known as **sleeping sickness**. It is transmitted by the bite of tsetse fly (Glossina genus) to persons from infected persons with protozoan parasite and residing in sub-Saharan Africa. Trypanosoma can cross the placenta and infect the fetus. Human African trypanosomiasis takes two forms, depending on the subspecies of the parasite involved: (a) *Trypanosoma brucei gambiense* (95%), and (b) *Trypanosoma brucei rhodesiense* (5%). Without treatment, disease is considered fatal.

Clinical Features

- **Hemolymphatic stage of disease:** In the first stage, trypanosomes multiply in the subcutaneous tissues, blood and lymph. It is also called hemolymphatic stage. Patient presents bouts of fever, headache, lymphadenopathy, arthralgia and itching.
- **Meningoencephalic stage of disease:** In the second stage, trypanosomes cross the blood–brain barriers to infect the central nervous system. This is known as meningoencephalic stage. Patient presents with behavioral changes, confusion, sensory disturbances, poor coordination and sleeping sickness.

CHAGAS DISEASE

Chagas disease is known as American trypanosomiasis. When the persons become infected by the parasite *Trypanosoma cruzi*, they can suffer of Chagas disease. The feces of the vector 'Tryptominae', or 'kissing bugs', transmits the parasites to the persons. Other routes of transmission include oral route, blood/blood products transfusion, mother–fetus transmission and organ transplantation. Symptoms change over the course of the infection. *Trypanosoma cruzi* infection is curable if patients are treated.

Clinical Features

Patient of Chagas disease presents itself in two phases: (a) initial acute phase of infestation in Chagas disease, and (b) chronic phase of infestation in Chagas disease patients are at risk for severe acute respiratory syndrome CoV-2 (SARS-CoV-2) manifestions and should be a priority group to be vaccinated.

- **Initial acute phase of infestation** in Chagas disease persists for two months after infection, in which numerous parasites circulate in the bloodstream but persons are usually asymptomatic in 50% of cases. Rest 50% of persons may present with skin lesion, purple swelling of the lids of unilateral eye, fever, headache, lymphadenopathy, pallor, muscular pain, breathlessness, chest pain and abdominal pain.
- **During chronic phase of infestation** in Chagas disease patients have hidden parasites in the cardiac muscle and muscular coat of gastrointestinal tract (i.e. esophagus and colon) and nervous system leading to cardiac arrhythmias, megaesophagus, megacolon and neurological manifestations.

METAZOA (HELMINTHS)

Metazoa (helminths) are multicellular eukaryotic organisms in biological animal kingdom. Metazoa include *Strongyloides stercoralis*, *Wuchereria bancrofti*, *Taenia solium*, *Taenia saginata* and *Taenia asiatica*, *Ancylostoma duodenale*, *Enterobius vermicularis*, Trichinosis and Schistosoma.

STRONGYLOIDIASIS

Strongyloidiasis is a human disease caused by nematode round worm called *Strongyloides stercoralis*, which most often infects small intestine and rarely colon. The parasite is diagnosed by demonstration of larvae, eggs and adult worms embedded in the crypts. Eosinophils, sometimes with Charcot-Leyden crystals may be present. Gastric strongyloidiasis may occur in association with human T-lymphotropic virus causing adult T cell lymphoma. *Ascaris lumbricoides* causing small intestine gangrene is shown in Fig. 7.63.

LYMPHATIC FILARIASIS

Infective form is third stage larva of *Wuchereria bancrofti* responsible for lymphatic filariasis, which enters through skin inoculation by mosquito bite and localizes in the lymphatic system especially inguinoscrotal region. Microfilariae are demonstrated in small number in circulating blood during the day and peak density at night between 10 pm and 2–4 am due to feeding habit of mosquitoes at night. In subperiodic form, microfilariae are demonstrated between noon and 8 pm due to feeding habit of mosquitoes during daytime.

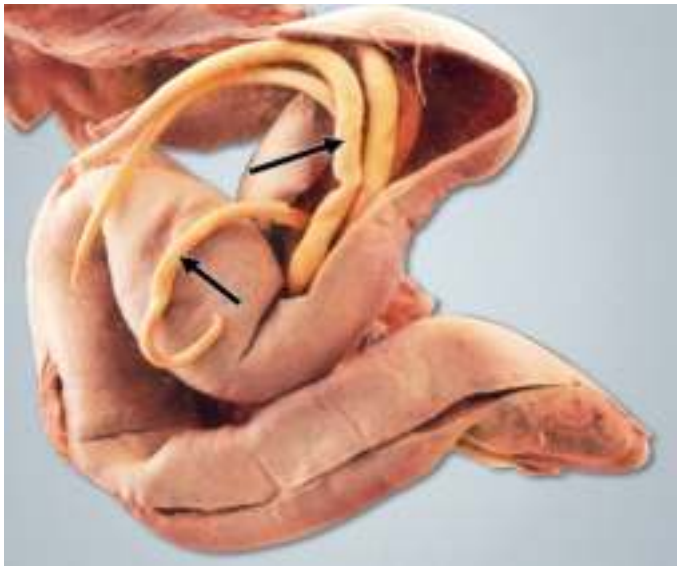


Fig. 7.63: *Ascaris lumbricoides* causing small intestinal gangrene. *Strongyloides stercoralis* (roundworm nematode) causing gangrene of small intestine (arrows).

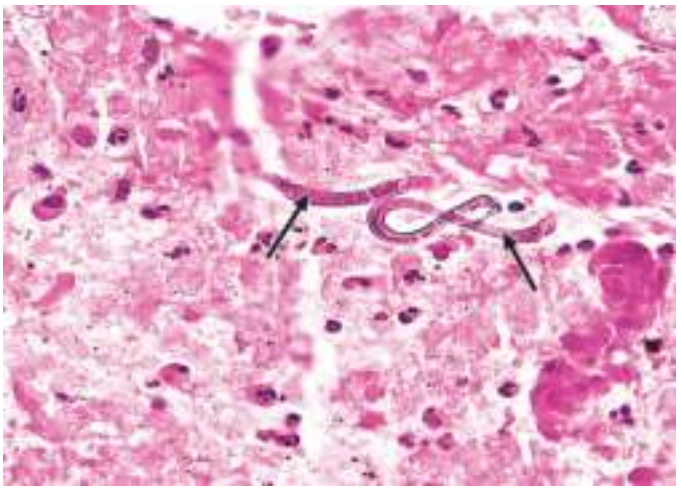


Fig. 7.64: *Wuchereria bancrofti* adult worms in endometrial curettings. Histologic examination of endometrial curettings shows adult *Wuchereria bancrofti* adult worms, necrotic endometrial fragments and inflammatory cells (arrows) (400X). (*Courtesy*: Dr. Tushar Kalonia.)

Pathogenesis

Adult worm and developing larva incite an inflammatory reaction in the lymphatic system lymphangitis. The reaction products of growing larvae are highly allergenic producing urticaria, 'fugitive swellings' and lymphedema. Intensity and type of host immune response may reflect range of clinical manifestations. Immune response varies by stage of infection.

- Elephantiasis is relatively a late complication of filariasis and characterized by swelling of limbs, scrotum, breasts or vulva with dermal hypertrophy. Impairment of the circulation leads to secondary bacterial and fungal infections in the regions.
- Chronic lymphedema leads to hyperplasia of connective tissue, infiltration by eosinophils, macrophages and plasma cells. *Wuchereria bancrofti* adult worms in endometrial curettings are shown in Fig. 7.64.

Clinical Features

Initially, patient is asymptomatic. Later patient presents with fever and symptoms due to inflammation and obstruction of lymphatic channels (limbs, breasts, and scrotum), lymphadenitis (femoral, inguinal, axillary and epitrochlear nodes), orchitis, lymphocele, hydrocele and **elephantiasis**.

Laboratory Diagnosis

Diagnosis of filariasis is frequently made on clinical grounds in endemic regions but demonstration of microfilariae in circulating blood is the key. Where more than one species of filarial infection occurs, it needs well stained slides for morphological identification of microfilariae.

- **Conventional method:** Thick stained peripheral blood smear is examined to demonstrate microfilariae.
- **Concentration techniques:** It is done by nucleopore filtration or Knott's concentration, detection of circulating filarial antigen by rapid format card test/immunochromatographic card test (ICT).
- **Serodiagnosis:** Filariasis is diagnosed by polymerase chain reaction (PCR) based assays for DNA.
- **Imaging studies:** High frequency ultrasound, lymphoscintigraphy is performed to diagnose filariasis.
- **Light microscopy:** Adult worm lodges in the lymph node leading to lymphangitis and lymph varices. The vascular endothelium of the vessels is occupied by the parasite leading to obliterative endolymphangitis and occlusion of the lymphatic channels. There is infiltration of monocyte, macrophage and giant cells killing the worm and replacing the lymph node with fibrous tissue eventually.

CYSTICERCOSIS—TAPEWORM (CESTODE)

Three species of cestodes in human include: *Taenia solium, Taenia saginata* and *Taenia asiatica*. Out of these Taenia solium causes major health problems, *Taenia solium* taeniasis is acquired by human beings through the ingestion of the parasite's larval cysts (cysticerci) in undercooked and infected pork.

- **Tissue infection** caused by ingestion of contaminated food, water or faces with larva cysts of the cestode *Taenia solium* (cysticercus cellulose). In cysticercosis, the human represents an intermediate host and the parasite develops cysticerci in various organs.
- The term **taeniasis** refers to intestinal infection with cestodes (adult tapeworms), which occurs from ingestion of larvae in undercooked pork. Cysticerci are larva forms or tapeworms found within a fluid-filled cyst.

Life Cycle

- Human beings are carriers of cestode (tapeworm), who excrete eggs in their feces and contaminate the environment when they defecate in open areas.
- Human beings can also become infected with *Taenia solium* eggs due to poor hygiene (via the fecal–oral route) or ingestion of contaminated water and food. Ingested *Taenia solium* eggs develop to larvae (called **cysticerci**) in nervous system, heart, skeletal muscle, subcutaneous tissue and eyes of the human body. When eggs enter the central nervous system and produce **neurocysticercosis**, which can cause neurological symptoms such as severe headache, blindness, convulsions and epileptic seizures.
- *Taenia solium* is the cause of epilepsy in 30–70% of population in endemic areas where people live in proximity to roaming pigs.

Laboratory Diagnosis

Clinical history, biopsy, serology, cerebrospinal fluid examination, cytological examination and imaging can aid in the proper diagnosis of cysticercosis.

- Ultrasonography can reveal cystic lesions. Computed tomography scan can reveal hyperdense lesions in the tissue with or without calcification.
- Cytological examination reveals fibrillary stroma interspersed nuclei and a honeycomb pattern infiltrated by mixed inflammatory infiltrate.
- The treatment of taeniasis by *Taenia solium* is important to prevent neurocysticercosis and as a tool to assist in controlling the parasite transmission cycle.

Surgical Pathology: Cysticercosis

Gross Morphology

Gross morphology of lesion reveals circumscribed, white to tan, cystic nodules measuring 1 mm to 2 cm in diameter containing clear fluid. Larva forms are identified within the cyst cavity.

Light Microscopy

- Histologic examination of cysticercosis lesion reveals larva form in the cystic cavity, scolex with hooklets and two pairs of suckers.
- The larva form is composed of duct-like invaginations and lined by a double-layered eosinophilic structure. Its body exhibits a myxoid matrix and calcified bodies. Birefringent hooklets may be identified.
- There is presence of granulomatous reaction, inflammatory infiltrate with lymphocytes and eosinophils, fibrosis and calcification. Histopathologic examination of cysticercosis lesion is shown in Fig. 7.65.

HYDATID CYST—TAPEWORM (CESTODE)

Hydatid cyst is caused by *Echinococcus granulosus*, a tapeworm (cestode). Adult parasites are found in dogs and sheep. Humans are infected after ingestion of eggs. Right lobe of liver is the commonest site of hydatid cyst followed by lung.

- When the lungs are affected, protoscolices might be found in sputum or bronchial washings.
- Liver contains multiple cysts of variable size invading surrounding tissue. Color of the cysts resembles white of boiled egg.
- Echinococcosis is diagnosed mainly with imaging techniques such as ultrasonography, radiology, magnetic resonance imaging (MRI) or CT scanning, supported by serology. Hydatid cyst of spleen is shown in Fig. 7.66.

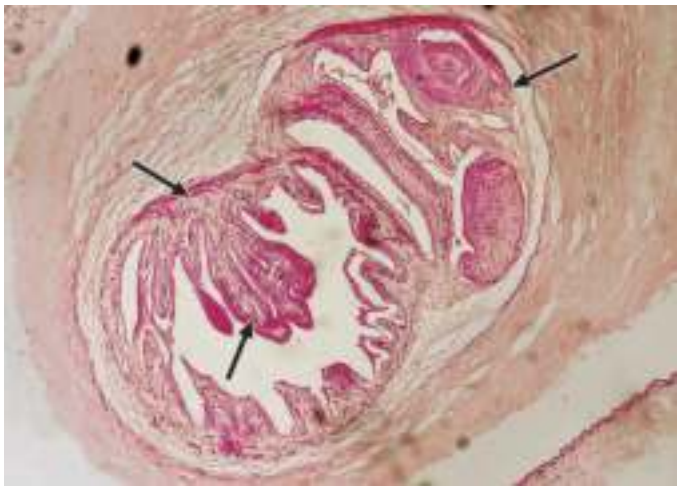


Fig. 7.65: Histopathologic examination of cysticercosis lesion reveals larva form in the cystic cavity, scolex with hooklets and two pairs of suckers. The larva form is composed of duct-like invaginations and lined by a double-layered eosinophilic structure. Its body exhibits a myxoid matrix and calcified bodies. There may be presence of granulomatous reaction, inflammatory infiltrate with lymphocytes and eosinophils, fibrosis and calcification (arrows) (400X).

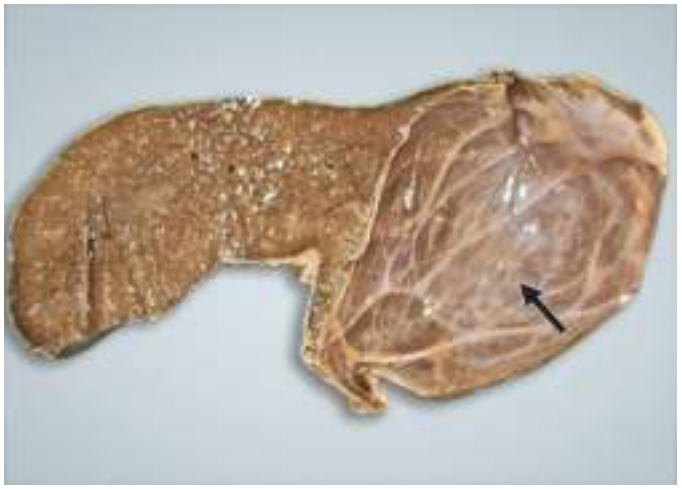


Fig. 7.66: Hydatid cyst of spleen. The outer, thick fibrotic wall of the cyst is clearly seen and the cyst is filled with multiple daughter cysts of varying sizes (arrow).

Cytological Smear Examination

Cytological smear examination shows laminated membranous structures resembling ectocyst of hydatid cyst.

- Periodic acid–Schiff (PAS) stain highlights the laminated membranous structures as magenta-colored structures.
- Gomori methenamine stain stains laminated membranous structures black.
- Claw-like refractile hooklets are demonstrated in the background by Ziehl-Neelsen stain as bright purple. Accidental spillage during fine needle aspiration may cause anaphylactic shock.

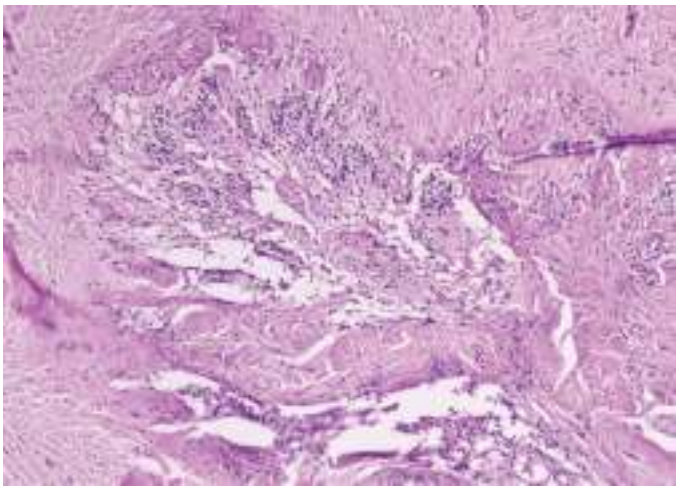


Fig. 7.67: Histology of hydatid cyst involving liver. The cyst wall shows an inner germinal layer (endocyst), outer chitinous (fibrous lamina) ectocyst layer surrounded by either granulation tissue or a fibrous capsule (pericyst) (400X).

Histologic Examination

On histologic examination, hydatid cyst wall consists of endocyst and ectocyst surrounded granulation tissue (pericyst). Serological tests used in humans include enzyme-linked immunosorbent assays (ELISAs), indirect immunofluorescence, indirect hemagglutination, immunoblotting and latex agglutination. Histology of hydatid cyst involving liver is shown in Fig. 7.67.

ANCYLOSTOMA DUODENALE (NEMATODE)

The adult worm of *Ancylostoma duodenale* (hookworm) lives in the small intestine of human beings in the jejunum less often in the duodenum and rarely in the ileum. Freshly passed adult worm is small greyish white, cylindrical and curved shape and reddish-brown color due to the ingested blood in the intestinal tract. Life cycle of *Ancylostoma duodenale* is completed in human beings. No intermediate host is required.

Mode of Transmission

Human feces are the only source of infection. Infective filariform larva is transmitted either by penetration of skin or fecal–oral route or rarely via breastfeeding by mothers to infants.

Clinical Features

The infective filariform larvae at the site of the penetration of the skin, produce a local reaction called **ground itch**.

- The migration of large number of filariform larvae, through the lung produces minute hemorrhages and infiltration of leukocytes resulting in the entrapment of the larva in the lung tissue associated with

low-grade fever, mild cough, dizziness, pneumonia with pulmonary consolidation. Both eosinophilia and leukocytosis occur at this stage.
- Intestinal infection manifests as abdominal pain, nausea, vomiting, hemorrhagic diarrhea, blood loss and iron deficiency anemia associated with pallor, fatigue, and breathlessness on exertion. Hypoproteinemia associated edema.

ENTEROBIUS VERMICULARIS ('PINWORM') NEMATODE

Enterobius vermicularis ('pinworm') is an intestinal nematode encountered worldwide and more common in temperate zones. Individuals can be infected at any age, but parasitism is more common in children. Most people complain of pruritus due to migration of *Enterobius vermicularis*.

TRICHINOSIS

Trichinosis is a type of roundworm infestation, which primarily induce disease in bears, foxes and pigs. The infestation is acquired eating trichinella roundworm larvae in raw or undercooked meat. Trichinella larvae mature into adult worms in the small intestine over several weeks. The adult roundworms then produce larvae that travel through various tissues including skeletal muscle. Trichinosis is most widespread in rural regions across world.

Clinical Features

Severity of clinical manifestations of trichinosis infection depend on the number of larvae consumed in the infected meat. Patient presents with diarrhea, abdominal pain, fatigue, nausea and vomiting within 1–2 days after infestation. Other symptoms usually start 3–8 weeks after infestation such as high-grade fever, skeletal muscle pain and tenderness, swelling of eyelids and face, weakness, headache, photophobia and conjunctivitis.

SCHISTOSOMIASIS

Schistosomiasis, also called as bilharzia, or snail fever or Katayama fever is an acute and chronic disease caused by trematode parasitic flatworms of the genus Schistosoma. The parasite may infect intestine or urinary tract. Lack of proper hygiene, swimming or fishing in infested water make the persons vulnerable to infection. Parasite species and geographical distribution of schistosomiasis are given in Table 7.30.

Life Cycle

Person becomes infected, when larva form of the parasite is released by fresh water snails. The larva form of parasite penetrates skin during contact with infected water.
- Transmission occurs when persons suffering from schistosomiasis contaminate fresh water sources with their excreta containing larva form of parasite, which hatch in water. The larva then develops into adult schistosomiasis in the body.
- Adult worms reside in the blood vessels, where the female adult worms release eggs. Some of the eggs are excreted in the feces or urine to continue the parasite's life cycle. Other eggs become trapped in body tissues, causing immune reactions and progressive damage to organs.

Clinical Features

Within 1–2 months of infestation, patient presents with fever, chills, cough and muscular aches. Without treatment, schistosomiasis can persist for years and the patients present with abdominal pain, hepatomegaly, diarrhea, bloody stool or blood in the urine.

ONCHOCERCIASIS

Onchocerciasis is also known as river blindness, because the blackflies that transmit *Onchocerca volvulus*, which breed in rapidly flowing streams mostly near remote rural villages. It is the second most cause of blindness after trachoma.

Table 7.30 Parasite species and geographical distribution of schistosomiasis

Species	Geographical Distribution
Intestinal schistosomiasis	
Schistosoma mansoni	Africa, the Middle East, the Caribbean, Brazil, Venezuela and Suriname
Schistosoma japonicum	China, Indonesia, Philippines
Schistosoma mekongi	Columbia and Lao People's Democratic Republic
Schistosoma guineensis and related *Schistosoma intercalatum*	Rain forest regions of Central Africa
Urinary bladder	
Schistosoma haematobium	Africa, Middle East, Corsica (France)

- Onchoerciasis is caused by parasitic filarial worm *Onchocerca volvulus* transmitted through repeated bites by female blackflies of the genus Simulium of an infected person.
- These female blackflies exist mainly in Africa and some parts of Central and South America. In the human body, adult worms produce embryonic larvae (microfilariae) that migrate to the skin, subcutaneous tissue, eyes and other organs.

Life Cycle

The life cycle of *Onchocerca volvulus* begins inside human host, which consists of the following four stages.

- **Stage I:** Young parasitic worms called microfilariae move toward the skin of human host. Female blackfly bites the human host, and ingest the microfilariae.
- **Stage II:** While inside the female blackfly, the microfilariae develop into larvae within a week.
- **Stage III:** The infected female blackfly bites a person, transferring the worm larvae into the human host. Here the larvae develop into adult worms in a process that takes about 6–12 months. Swollen nodules are formed around the adult worms.
- **Stage IV:** Adult worms reside inside the human body for up to 15 years. Most of the microfilariae inside the human body, trigger the inflammatory immune response. Depending on the location of the worms within human body, the inflammatory response may cause damage to skin or eye.

Clinical Features

Patient with *Onchocerca volvulus* infestation presents with severe itching, skin rashes over hands, feet, buttocks and shoulders, lumps under the skin undergoing into blisters due to presence of worms, and eye damage. Untreated onchocerciasis may also result in permanent skin damage and blondness.

Section II

HEMATOLOGY

8. Red Blood Cell Disorders
9. White Blood Cell Disorders
10. Platelet Disorders and Bleeding Diathesis
11. Coagulation Disorders and Diagnostic Approach of Bleeding Diathesis
12. Blood Banking and Transfusion Practices
13. Lymph Node, Spleen and Thymus Gland Disorders

CHAPTER

8

Red Blood Cell Disorders

Vinay Kamal, Anubhav and Vigyat

LEARNING OBJECTIVES

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- Hematopoiesis: overview
  - Site of hematopoiesis
    - Hematopoiesis during intrauterine life
    - Hematopoiesis during postnatal period
  - Hematopoietic microenvironment
    - Hematopoietic cells
    - Nonhematopoietic cells
- Regulation of hematopoiesis
  - Hematopoietic transcription factors
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PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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- Cardiac hemolytic anemia
- Microangiopathic hemolytic anemia
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- Thrombotic thrombocytopenic purpura
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DIAGNOSTIC APPROACH OF HEMOLYTIC ANEMIA

BONE MARROW FAILURE SYNDROMES

- Aplastic anemia
- Pure red cell aplasia (erythroblastopenia)
- Sideroblastic anemia
- Congenital dyserythropoietic anemia
- Anemia of chronic disease
- Myelophthisic anemia
- Fanconi anemia

HEMATOPOIESIS

HEMATOPOIESIS: OVERVIEW

Process of formation of blood cells is called hematopoiesis, which occurs within bone marrow microenvironment (niche), where hematopoietic stem cells (HSCs) come in contact with many other cells.

- Cell-to-cell communication occurs in bone marrow by binding via cell surface receptors, adhesion molecules, cytokines and hematopoietic growth factors. Binding of hematopoietic growth factor to its receptor activates the JAK/STAT. MAPK and phosphatidylinositol 3-kinase (PI3K) pathways, which l ead to transcriptional activation of specific genes resulting in cell proliferation, differentiation and inhibition or activation of apoptosis.
- Apoptosis occurs by activation of caspases by DNA damage, release of 'cytochrome c' from mitochondria and by FAS ligand.
  - Apoptosis is inhibited by BCL-2. E2F is a transcription factor needed for cell cycle transition from G1/S phase. ET2 is inhibited by tumor suppressor retinoblastoma protein (pRB), which can be indirectly activated by p53 tumor suppressor protein.
  - The synthesis and degradation of different cyclins stimulate the cell to pass through the different phases of cell cycle.
  - The growth factors may also suppress apoptosis by activating AKT (protein kinase B). Hematopoiesis during intrauterine life and postnatal life is shown in Fig. 8.1 and Table 8.1.

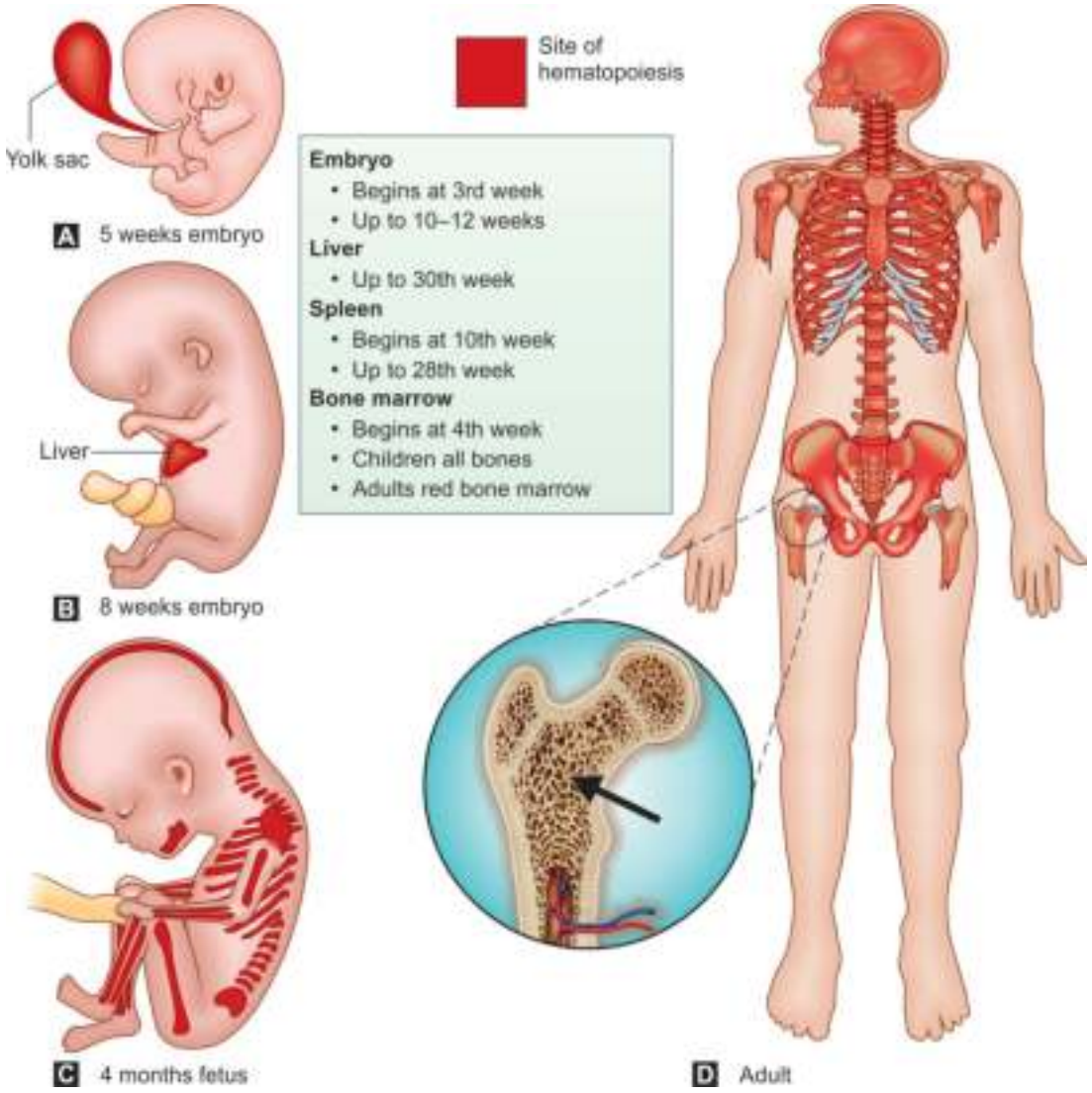


Fig. 8.1: Hematopoiesis during intrauterine life and postnatal period.

Table 8.1 Sites of hematopoiesis and bone marrow cellularity during intrauterine and postnatal life

Age Group	Sites of Hematopoiesis	Type of Hemoglobin and Globin Chains	Bone Marrow Cellularity
Embryonic development			
2–8 weeks	Aorta gonad mesonephros (AGM)/yolk sac	▪ Hemoglobin Gower I ($\zeta_2\varepsilon_2$) ▪ Hemoglobin Gower II ($\alpha_2\gamma_2$) ▪ Hemoglobin Portland ($\zeta_2\gamma_2$)	Nil
Fetus			
2–7 months	Liver, spleen, bone marrow, thymus, lymph nodes during 2–7 months of age	▪ HbA ($\alpha_2\beta_2$) small quantity ▪ HbA_2 ($\alpha_2\delta_2$) small quantity	100%
5–9 months	Bone marrow (main site during 5–9 months)	▪ HbF ($\alpha_2\gamma_2$) 90–95% just before birth	100%
Infants to childhood age group			
Newborn to 3 months and children	Bone marrow (all bones rich in red bone marrow in newborn)	▪ HbA ($\alpha_2\beta_2$) 25% ▪ HbA_2 ($\alpha_2\delta_2$) 1% ▪ HbF ($\alpha_2\gamma_2$) 75%	100% and then decreased to 60–80% during childhood
Adults age group			
22–40 years, 40–70 years and >70 years (bone marrow cellularity decrease as age advances)	▪ Red bone marrow in vertebrae, ribs, sternum, skull, sacrum, pelvis; proximal end of femur and humerus. ▪ Red bone marrow is gradually replaced by yellow bone marrow in most bones except bones mentioned as site of hematopoiesis	▪ HbA ($\alpha_2\beta_2$) >95% ▪ HbA_2 ($\alpha_2\delta_2$) <3.5% ▪ HbF ($\alpha_2\gamma_2$) 1%	▪ 60–70% ▪ 40–50% ▪ 30–40%

SITE OF HEMATOPOIESIS

Hematopoiesis during Intrauterine Life

During embryonic life, hematopoiesis begins in the 2 weeks embryo. Liver takes over hematopoiesis function in 8 weeks embryo and continues until a few weeks before birth. Spleen starts hematopoiesis during 3–7 months of intrauterine period. Thymus gland and lymph nodes participate in production of lymphocytes. Bone marrow also participates in hematopoiesis during intrauterine life.

Hematopoiesis during Postnatal Period

At birth until first 2–3 years after birth, all bones contain red marrow involved in hematopoiesis. During adult life by the age of 18–22 years, hematopoiesis occurs in cranial bones, vertebrae, sternum and ribs, iliac bones and upper ends of femur and humerus.

HEMATOPOIETIC MICROENVIRONMENT

Hematopoietic microenvironment comprises two types of cells: hematopoietic stem cells and nonhematopoietic cells. Hematopoietic microenvironment is shown in Fig. 8.2 and Table 8.2.

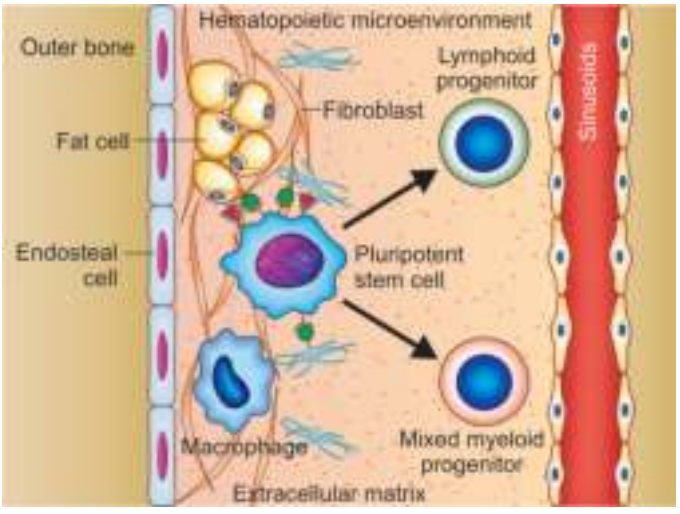


Fig. 8.2: Hematopoietic microenvironment in bone marrow: Adipocytes, endothelial cells, fibroblasts, osteoblasts, T cells and macrophages participate in expression of homing receptors and synthesis of soluble growth factors, soluble differentiation factors, extracellular matrix components; and integral membrane proteins that function as juxtacrine regulators (SCF, FL, SCI). Cytokines and growth factors synthesized by extracellular component participate in regulation of hematopoietic stem cell/progenitor cell differentiation and expansion. Collagen gives structural strength. Extracellular components such as glycosaminoglycans participate in cell-to-cell interactions and localization of growth factors; while cytoadhesion molecules participate in adhesion of hematopoietic precursors to ECM proteins.

Table 8.2 Hematopoiesis depends on hematopoietic microenvironment (i.e. stromal cells and extracellular matrix)

Hematopoietic Components	Functions
Hematopoietic cells	
Hematopoietic stem cells (HSCs)	CD34+ (potential to self-replicate and differentiate to progenitor cells)
Progenitor cells (committed cells)	Myeloid progenitor cells, lymphoid progenitor cells, i.e. colony forming units resulting in production of red blood cells, white blood cells and platelets
Cellular stroma	
Macrophages, fibroblasts, reticulum cells, adipocytes, endothelial cells, and T cells	▪ Expression of homing receptors ▪ Synthesis of soluble growth and differentiating factors ▪ Synthesis of integral membrane proteins that function as juxtacrine regulators (stem cell factor) ▪ Synthesis of extracellular matrix components
Extracellular microenvironment	
Soluble factors (cytokines and growth factors)	Regulation of hematopoietic stem cell/progenitor cell differentiation and expansion
Extracellular matrix (ECM) and collagen fibers	Structural support
ECM glycosaminoglycans (heparan, chondroitin, dermatan sulfate)	Cell-cell interactions; localization of growth factors
Cytoadhesion molecules	Adhesion of hematopoietic precursors to extracellular matrix proteins

The hematopoietic stem cell (HSC) attaches to bone marrow stromal cells via specific receptors and ligands. The HSC is then influenced by both positive and negative regulatory growth factors.

Hematopoietic Cells

Hematopoietic cells are composed of hematopoietic stem cells (CD34+), progenitor cells (committed cells) and mature cells (RBCs, WBCs and platelets). Self-renewal is an important property of hematopoietic stem cells. Some of the hematopoietic stem cell disorders are given in Table 8.3.

Hematopoietic Stem Cell Lineages

Hematopoietic stem cells (HSCs) have the potential to self-replicate and differentiate into committed progenitors of trilineage myeloid and lymphoid cells (B cells, T cells and NK cells).

- Trilineage myeloid cell gives rise to erythroid/megakaryocytic, eosinophilic, and granulocytic/macrophage.
- Progenitor cells are multipotent stem cells derived from hematopoietic stem cells. These are committed to one cell lineage such as myeloid, erythroid, megakaryocyte or lymphoid cells.
- Late progenitor cells differentiate and undergo maturation forming red blood cells (RBCs), white blood cells (WBCs), platelets and lymphocytes.

Colony Forming Units

Committed stem cells have been grown *in vitro* by cell culture techniques on semisolid media of agar or methylcellulose, with the production of colonies of differentiated progeny. Colony consists of 40 to a few hundred cells.

- Thus, the committed stem cells have been called colony forming units (CFU), e.g. CFU-G/M, CFU-Eo and CFU-E/M. Cluster consists of 3–40 cells.

Table 8.3 Hematopoietic stem cell disorders

Hematopoietic Stem Cell (HSC)	Hematopoietic Stem Cell Disorders
Proliferation and differentiation of HSC	▪ Chronic myelogenous leukemia ▪ Essential thrombocythemia ▪ Polycythemia vera
Proliferation with nil or minimal differentiation of HSC	▪ Acute myelogenous leukemia ▪ Acute lymphoblastic leukemia
Failure of proliferation with somatic mutation of HSC	▪ Paroxysmal nocturnal hemoglobinuria
Proliferation with abnormal differentiation and apoptosis of HSC	▪ Myelodysplastic syndrome
Failure of proliferation of HSC	▪ Aplastic anemia

- During bone marrow transplantation, administration of G-CSF, GM-CSF and IL-1 acts on bone marrow microenvironment and causes migration of bone marrow stem cells to peripheral blood. Colony forming units are shown in Fig. 8.3 and Table 8.4.

Nonhematopoietic Cells

Nonhematopoietic cells comprise stromal cells, macrophages, fibroblasts and vascular endothelial cells. These cells synthesize growth factors, which stimulate hematopoiesis.

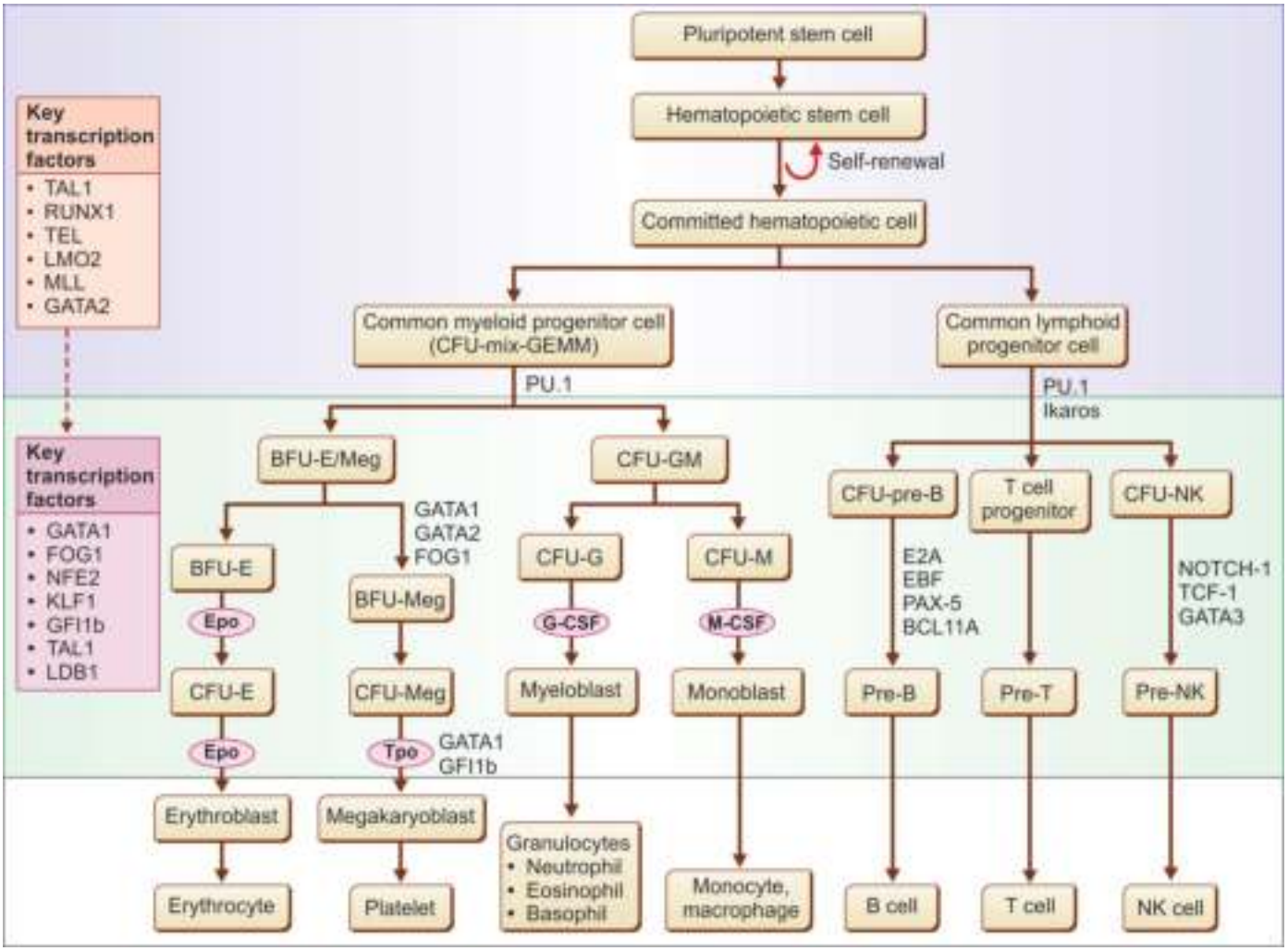


Fig. 8.3: Hematopoiesis shows stages of development of blood cells.

Table 8.4 Colony forming units in bone marrow microenvironment

Colony Forming Unit	Production of Cells in Bone Marrow
CFU-GEMM or CFU-MIX	Earliest units giving rise to granulocytic, erythroid, monocytic and megakaryocytic cells
CFU-GM	Unit of progenitor cells giving rise to granulocytic–monocytic series
CFU-Eo	Colonies of eosinophilic myeloid cells
CFU-Baso	Colonies of basophilic myeloid cells
CFU-MG	Colonies of megakaryocytic myeloid cells
BFU-E (burst-forming unit-erythroid)	Earliest progenitor erythroid cells
CFU-E	More differentiated cells of erythroid progenitors

REGULATION OF HEMATOPOIESIS

- Hematopoiesis is regulated by hematopoietic growth factors, inhibitory mechanisms and apoptosis. Negative regulators of hematopoiesis include TGF-β, TNF-α, interferons, PGEs, lactoferrin, CD8+ cytotoxic T cells and natural killer cells, vitamin D_3 (1,25-dihydroxyvitamin D_3), acidic isoferritins and macrophage inflammatory protein-1α (MIP-1α).
- Kit-ligand and FLT-3 ligand and vascular endothelial growth factor (VEGF) stimulate pluripotent hematopoietic stem cells.
- Erythropoietin (EPO) regulates eythropoiesis and production of erythroid cells, their differentiation and survival by inhibiting apoptosis.
- Thrombopoietin (TPO) is synthesized by liver and kidneys, that stimulates megakaryopoiesis.
- Hematopoietic growth factors are the cytokines that stimulate bone marrow stromal cells to synthesize colony-forming units. GM-CSF stimulates granulocytes, monocytes, eosinophils, erythroid cells, megakaryocytes, hematopoietic progenitor cells (HPCs) and dendritic cells. G-CSF regulates granulocytes and hematopoietic progenitor cells (HPCs). M-CSF regulates monocyte/macrophage and osteoclast cells.
- IL-3 is multi-CSF growth factor for the trilineage myeloid stem cells; thus, stimulate the more committed precursor cells. Many interleukins (IL-1, IL-2, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13) regulate lymphopoiesis. Hematopoietic growth factors (HGFs) regulating hematopoiesis are given in Table 8.5. Selected hematopoietic growth factors and their source are given in Table 8.6.

Table 8.5 Hematopoietic growth factors (HGFs) regulating hematopoiesis

Hematopoietic Growth Factor	Chromosome	Source	Major Target Cells/Actions
Kit-ligand	–	Bone marrow stromal cells	Pluripotent hematopoietic stem cell
FLT-3 ligand	–	T cells	Pluripotent hematopoietic stem cell
Erythropoietin (EPO)	7	Kidney (liver)	Erythroid cells, their differentiation and survival by inhibiting apoptosis
Thrombopoietin (TPO)	3	Bone marrow stromal cells, hepatocytes, kidney	Megakaryopoiesis, hematopoietic stem cells
IL-3 (multi-CSF)	5	Activated T cells, mast cells	Hematopoietic trilineage progenitor cells and more committed cells, mast cells
GM-CSF	5	Activated T cells, bone marrow stromal cells, endothelial cells	Granulocytes, monocytes, eosinophils, erythroid cells, megakaryocytes, hematopoietic progenitor cells (HPCs), dendritic cells
G-CSF	17	Monocytes, macrophages, bone marrow stromal cells	Granulocytes, hematopoietic progenitor cells (HPCs)
M-CSF	1	Monocytes, macrophages, bone marrow stromal cells	Monocytes, macrophages, osteoclasts
IL-1	2	Monocytes, macrophages, dendritic cells	Monocytes, endothelial cells, fibroblasts, lymphocytes, polymorphonuclear cells and early hematopoietic progenitor cells
IL-2	4	Activated Th1 cells	Activation and proliferation of T cells, B cells and natural killer cells
IL-4	5	Activated Th2 cells	Stimulates Th2 cells, suppresses Th1, mast cells, basophils
IL-5	5	Activated Th2 cells, mast cells	Eosinophil, B cells, cytotoxic T cells
IL-6	7	Macrophages, Th2 cells, fibroblasts	Early hematopoietic progenitor cells, B cells, T cells, megakaryocytes and myeloma cells
IL-7	8	Bone marrow stromal cells, dendritic cells, hepatocytes, keratinocytes, neurons, epithelial cells and thymus stromal cells	Pre-T cells, pre-B cells, natural killer cells
IL-8	4	Monocytes, macrophages, endothelial cells	Chemotaxis of granulocytes (chemokine)

Contd...

Table 8.5 Hematopoietic growth factors (HGFs) regulating hematopoiesis (*Contd...*)

Hematopoietic Growth Factor	Chromosome	Source	Major Target Cells/Actions
IL-9	5	Activated Th2 cells	T cells, B cells, early erythroid cells, mast cells
IL-10	1	Activated Th2 cells, monocytes, macrophages, B cells	B cells, mast cells, Th2 and inhibits Th1
IL-11	19	Bone marrow stromal cells	B cells, megakaryocytes and natural killer cells
IL-12	3.5	Monocytes, macrophages, B cells, T cells	Th1 cells, natural killer cells
IL-13	5	Activated Th2 cells, basophils	▪ Isotype switching of B cells ▪ Inhibition of cytotoxic and inflammatory functions of monocytes and macrophages
IL-14	16	T cells	Activated B cells
IL-15	4	Monocytes, macrophages, endothelial cells, fibroblasts	▪ CD8+ cytotoxic T cells ▪ Natural killer cells ▪ Costimulator of B cells
IL-16	15	T cells, eosinophil, epithelial cells	Chemotactic for CD4+ helper T cells
IL-17	2	Activated Th17 cells	Induces cytokine production by bone marrow stromal cells
IL-18	7	Macrophages, keratinocytes	Induces interferon production by Th1 cells, natural killer cells
Stem cell factor/KL (SCF/KL)	12	Fibroblasts, monocytes, macrophage, T cells	▪ Stem cells ▪ Early hematopoietic progenitor cells ▪ Basophils, mast cells ▪ Melanocytes ▪ Germ cells
FLT3 (fetal liver tissue 3) ligand	19	Bone marrow stromal cells, hepatocytes, kidney	▪ Hematopoietic stem cells ▪ Hematopoietic progenitor cells ▪ Dendritic cells

Table 8.6 Selected hematopoietic growth factors and their source

Source of Hematopoietic Growth Factors	Hematopoietic Growth Factors
Kidney, liver	Erythropoietin, thrombopoietin
Monocytes/macrophage	IL-1, IL-6, G-CSF, M-CSF
Lymphocytes	IL-2, IL-3, IL-4, IL-5, IL-6, GM-CSF
Endothelial cells	IL-6, GM-CSF, M-CSF

G-CSF—granulocyte-colony stimulating factor; GM-CSF—granulocyte-macrophage colony stimulating factor; and M-CSF—macrophage-colony stimulating factor.

ERYTHROPOIETIN

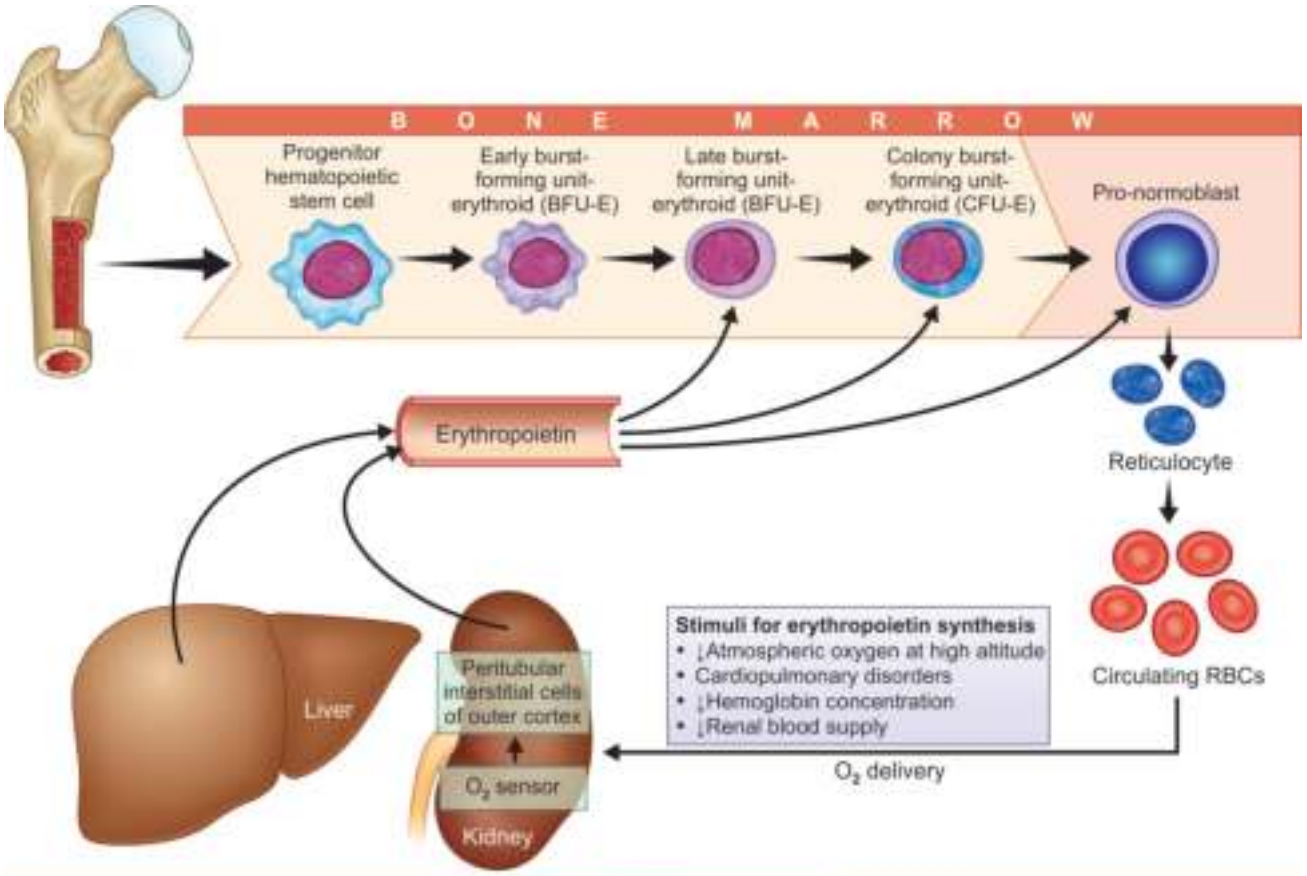
Erythropoietin (EPO) is glycoprotein synthesized by peritubular cells of **kidney** and **liver** regulates erythroid cells, their differentiation and survival by inhibiting default apoptosis. Hematopoietic stem cells are sensitive to oxygen tension in the blood.

- A heme containing protein senses oxygen need and triggers the synthesis of erythropoietin and its release in the bloodstream.
- Erythropoietin interacts with receptor-bearing cells in the bone marrow, where physiologic oxygen demands are translated into increased red blood cell production.
- Under normal conditions, the plasma erythropoietin level is maintained between 2 and 25 IU/L. Red blood cell production is also regulated by serum erythropoietin levels (Fig. 8.4).
- Characteristics and functions of erythropoietin are given in Table 8.7. Conditions associated with increased erythropoietin production are given in Table 8.8.

HEMATOPOIETIC TRANSCRIPTION FACTORS

Hematopoietic transcription factors play major role in the regulation of formation, survival, proliferation and differentiation of multipotent stem cells, as they undergo the transition to erythroid cells/megakaryocytes.

- Key hematopoietic transcription factors known to be involved in the specification and maintenance of hematopoietic stem cell (HSC) include GATA binding protein 2 (GATA 2), runt-related transcription factor 1 (RUNX1), T cell acute



Characteristics of erythropoietin

General characteristics	Functions	Response to anemia
• Erythropoietin is a glycoprotein • Cellular hypoxia stimulates erythropoietin synthesis • Erythropoietin synthesis occurs in kidneys (80–90%) and liver (<15%) • Normal range of plasma erythropoietin is 2–25 IU/L	• Stimulates BFU-E and CFU-E to divide and mature • Increases rate of mRNA and protein (hemoglobin synthesis) • Decreases normoblast maturation time • Increases rate of enucleation (extrusion of nucleus) • Stimulates early release of bone marrow reticulocytes (shift)	Generally increased except in anemia of renal disease

Fig. 8.4: Red blood cell production is also regulated by serum erythropoietin levels. Erythropoietin is synthesized by the kidney (85%) and liver (15%), which senses blood oxygen levels. Stimuli for erythropoietin synthesis include decreased atmospheric oxygen at high altitude, cardiopulmonary disorders, decreased hemoglobin concentration, and decreased renal blood supply. Erythropoietin promotes survival of BFU-E, CFU-E, and normoblasts in the bone marrow resulting in formation of reticulocytes and mature red blood cells.

lymphoblastic leukemia 1 (TAL1), translocation Ets leukemia (TEL), LIM domain only 2 (LMO2) and mixed lineage leukemia (MLL).

- Later on, other transcription factors known to be involved in hematopoiesis include GATA binding protein 1 (GATA 1), friend of GATA1 (FOG1), nuclear factor erythroid 2 (NFE2), Kruppel-like factor 1 (KLF1), growth factor independent 1B (GFI1B) and T cell acute lymphoblastic leukemia 1 (TAL1).
- During normal development of erythroid lineage, GATA2 initiates the programming of erythroid lineage and plays key role in the expansion and maintenance of hematopoietic progenitors, it is replaced by GATA1 during the terminal maturation of erythroid cells. At this stage, the level of GATA2 keeps on declining and GATA1 keeps on increasing. GATA1 is first expressed in megakaryocyte-erythroid progenitor cells (MEPs), and thus essential for the terminal differentiation of both erythroid and megakaryocytes. Transcription factors in hematopoietic lineage differentiation are given in Table 8.9. Molecular regulators of hematopoietic stem cell (HSC) fate are given in Table 8.10.

HEMATOPOIETIC GROWTH FACTORS IN CLINICAL PRACTICE

Hematopoietic growth factors are essential components for hematopoiesis. Recombinant DNA technology and availability of hematopoietic growth factors have

Table 8.7 Characteristics and functions of erythropoietin

Characteristics of Erythropoietin	
Composition	Glycoprotein
Stimulus for synthesis	Cellular hypoxia
Site of synthesis	▪ Kidneys (80–90%) ▪ Liver (<15%)
Normal range	Plasma 2–25 IU/L
Functions of Erythropoietin	
▪ The production of erythropoietin by kidney (80–90%) and liver (<15%) in response to its oxygen supply. Erythropoietin stimulates erythropoiesis and so increases oxygen delivery. Hypoxia induces hypoxia-inducible factors (HIFs) α and β, which stimulate erythropoietin production. von Hippel-Lindau (vHL) protein breaks down HIFs. Prolyl hydroxylase 2 (PHD2) hydroxylates HIFs-2α allowing vHL binding to HIFs. ▪ Stimulates BFU-E and CFU-E to divide and mature cells ▪ Increases rate of mRNA and protein (hemoglobin) synthesis ▪ Decreases maturation time of normoblasts ▪ Increases rate of extrusion of nucleus from normoblasts ▪ Stimulates early release reticulocytes from bone marrow	
Erythropoietin Synthesis Response to Anemia	
Erythropoietin synthesis occurs in anemias except anemia of chronic renal disease	

Table 8.8 Conditions associated with increased erythropoietin production

Chronic Hypoxia	
▪ Pulmonary disease ▪ Congenital heart disease	▪ Heavy tobacco smokers ▪ High altitude
Tumors	
▪ Renal cell carcinoma ▪ Hepatocellular carcinoma ▪ Cerebellar hemangioma	▪ Pheochromocytoma ▪ Adrenal adenoma with Cushing's syndrome
Tumor-like Disorder	
Adult polycystic disease of kidney	
Therapeutic Administration	
Androgens therapy	

significantly altered care of patients with bone marrow disorders and hematologic malignancies.

Table 8.9 Transcription factors in hematopoietic lineage differentiation

Hematopoietic Lineage	Transcription factors
Erythroid/megakaryocytic lineage	▪ GATA1 ▪ FOG1 ▪ GFI1B ▪ FLI1 (friend leukemia integration 1 also known as ERGB)
Myeloid lineage	▪ PU-1 ▪ C/EBPα ▪ C/EBPε_2 ▪ GFI1 ▪ EGR1 (early growth response 1) ▪ RARα
Lymphoid lineage	▪ PU-1 ▪ Ikaros family of hematopoietic-specific zinc finger proteins ▪ E2A ▪ EBF ▪ PAX-5 ▪ NOTCH-1 ▪ GATA3

- Erythropoietin (EPO) administration stimulates erythropoiesis in chronic renal disease. G-CSF, GM-CSF, IL-3, Flt3 ligand (FL) are administered for priming of bone marrow for donation used for bone marrow transplantation.
- G-CSF, GM-CSF, erythropoietin, IL-13 are administered to treat myelodysplastic syndrome and stimulate bone marrow recovery in bone marrow transplantation. IL-2 enhances killing of CSCs. IL-2 and IL-15 are administered to enhance immune system. Clinical application of hematopoietic growth factors is given in Table 8.11. Dietary requirements for red blood cell production are given in Table 8.12.

ROLE OF HORMONES IN HEMATOPOIESIS

TSH, thyroid hormones, ACTH, corticosteroids and human growth hormone also stimulate erythropoietin synthesis. Polycythemia is often a feature of Cushing's syndrome. However, very high doses of steroid

Table 8.10 Molecular regulators of hematopoietic stem cell (HSC) fate

HSC Receptor Proteins	Osteoblast Ligands	Functions
NOTCH proteins	Jagged, delta proteins	Promote HSC self-renewal and blockade of differentiation
Frizzled proteins (Wnt receptors)	Wnt proteins	Promote HSC self-renewal and expansion
Patched proteins (Shh receptors)	Sonic hedgehog (Shh)	Promote mitosis and initiation of differentiation
Tie-2	Angioprotein 1	Promote HSC quiescence
CXCR4	SDF1/CXCL12	Promote survival and proliferation of HSC

Table 8.11 Clinical application of hematopoietic growth factors

Hematopoietic Growth Factors	Indications in Clinical Practice
Erythropoietin (EPO)	Stimulation of erythropoiesis in chronic renal disease
G-CSF and GM-CSF	Recovery from treatment-induced myelosuppression
GM-CSF, erythropoietin, IL-3	Treatment of myelodysplastic syndromes
IL-2	Enhanced killing of cancer stem cells (CSCs)
G-CSF, GM-CSF, IL-3, FL	Priming of bone marrow for donation used for transplantation
IL-1, IL-6	Enhancement of the acute phase response
IL-2, IL-15	Enhancement of the immune system
G-CSF, GM-CSF, erythropoietin, IL-13	Stimulation of bone marrow recovery in bone marrow transplantation
G-CSF, GM-CSF, IL-3	Treatment of bone marrow failure

G-CSF—granulocyte-colony stimulating factor, GM-CSF—granulocyte-macrophage colony stimulating factor.

Table 8.12 Dietary requirements for red blood cell production

Dietary Element	Role in Red Blood Cell Production
Amino acids	Synthesis of chains of globin
Iron	Hemoglobin synthesis
Vitamin B_{12} and folic acid	Nucleic acid synthesis of erythroid precursors
Vitamin B_6	Functions as coenzyme in amino acid metabolism and synthesis of ALA in heme synthesis
Vitamin C	Required for folate metabolism and to facilitate the absorption of iron
Vitamin E	Maintenance of red blood cell integrity
Copper	Participates in transfer of iron for maintenance of red blood cell integrity
Thyroid hormones, corticosteroids, human growth hormone and androgens	Stimulate erythropoietin synthesis

hormones seem to inhibit erythropoiesis. Androgens stimulate estrogen production, which depress the erythropoiesis.

PATHWAYS INVOLVED IN HEMATOPOIESIS

Bone marrow hematopoietic stem cells give rise to myeloid, lymphoid and erythroid precursor cells, which differentiate to form mature cells under the influence of colony stimulating factors, cytokines and erythropoietin.

- Bone marrow stromal cells can synthesize local hormones participating in hemopoiesis.
- Erythrocytes normally lose their nuclear material prior to entering the blood circulation. Platelets are formed from megakaryocytes. Pathways involved in hematopoiesis are shown in Fig. 8.5.
- Human blood is composed of plasma (55%) and formed elements (45%). Blood formed elements include RBCs, WBCs and platelets.
- Plasma contains water and solutes such as plasma proteins, nutrients, vitamins, hormones, glucose, respiratory gases O_2 and CO_2, bilirubin, inorganic constituents such as sodium, potassium, calcium chloride, carbonate, and bicarbonate; and waste products like urea and creatinine (Fig. 8.6).

ERYTHROPOIESIS

The production of RBCs is referred to as erythropoiesis. Hemocytoblasts give rise to erythrocytes. Erythropoietin synthesized by juxtaglomerular cells of kidneys participates in erythropoiesis, which is essential for the differentiation of erythroid precursors. Erythropoietin is released in response to hypoxia in renal arterial blood supply. Patients suffering from erythropoietin secreting tumors, e.g. leiomyoma, renal cell carcinoma, hepatocellular carcinoma, and cerebral hemangioblastoma develop secondary polycythemia.

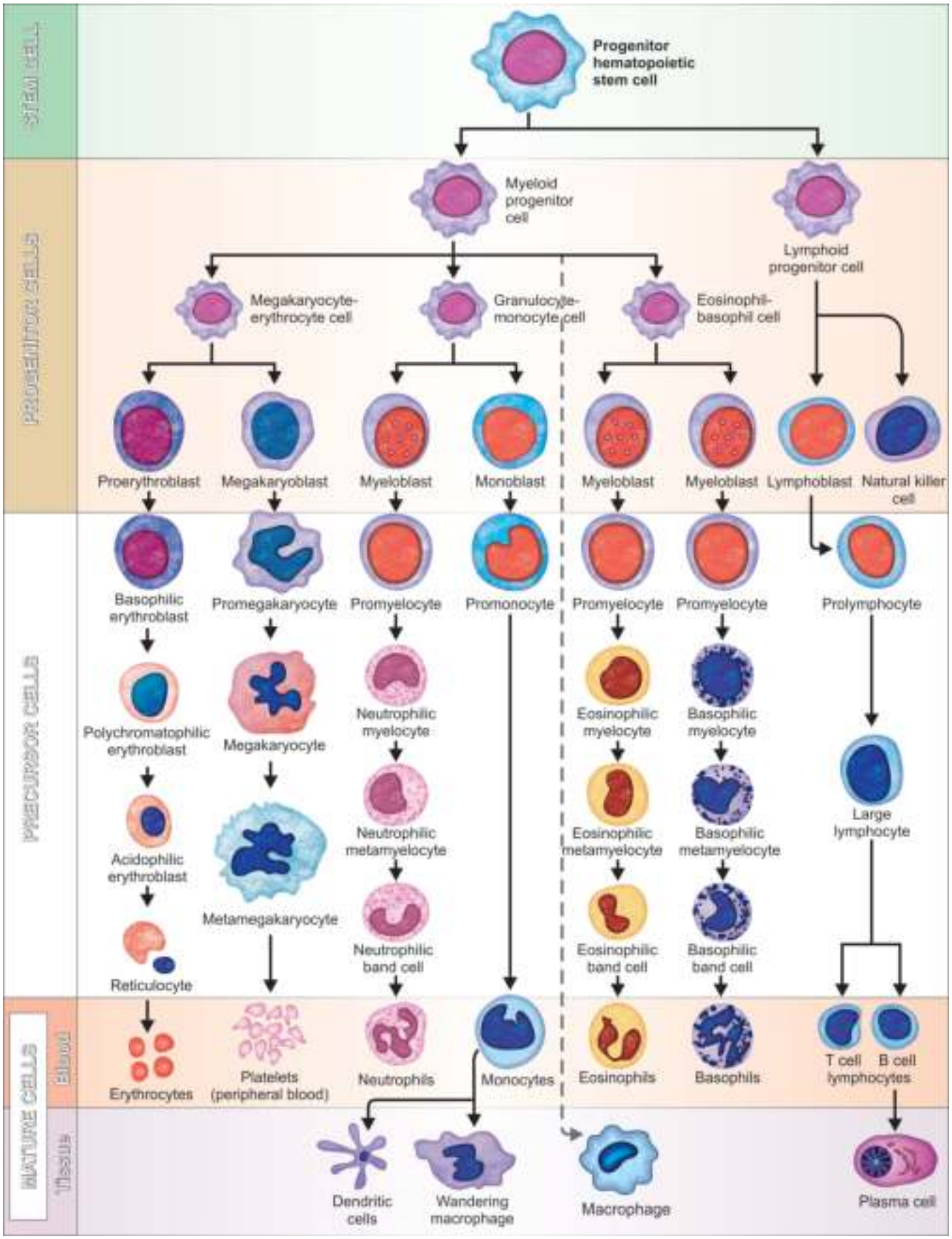


Fig. 8.5: Pathways involved in hematopoiesis.

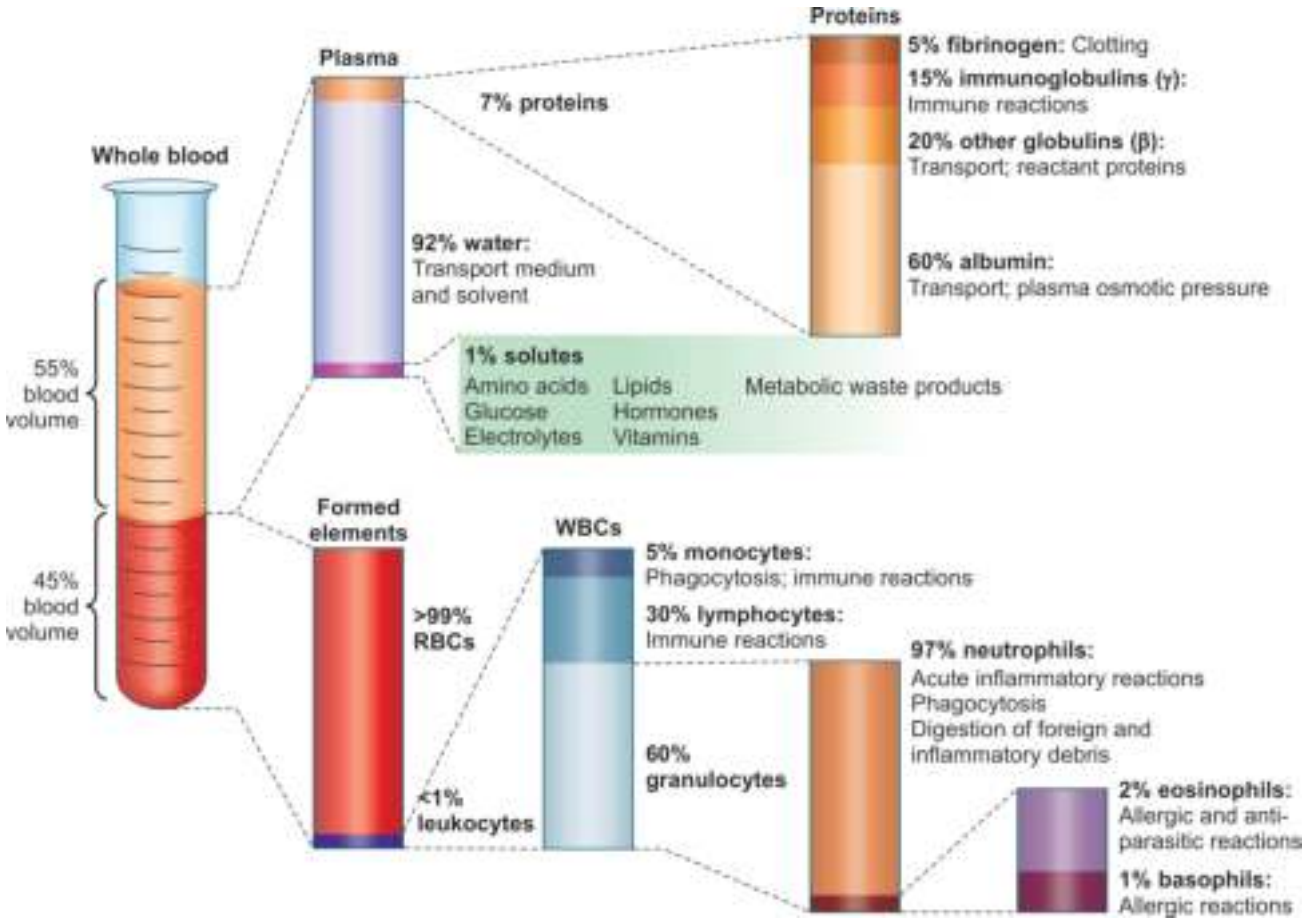


Fig. 8.6: Composition of blood shows plasma and formed elements.

Dyserythropoiesis and Ineffective Erythropoiesis: Terms

Dyserythropoiesis

- The term dyserythropoiesis refers to production of abnormal cells resulting in their destruction in the bone marrow prior to their release into blood circulation.
- The developing normoblasts may show nuclear budding, abnormal mitosis, fragmentation of nuclei, basophilic stippling and Howell-Jolly bodies.
- Howell-Jolly bodies may also be demonstrated in the red blood cells.

Ineffective Erythropoiesis

- The term ineffective erythropoiesis refers to erythroid hyperplasia in the bone marrow, but the patient is anemic. Ineffective erythropoiesis occurs in megaloblastic anemia and myelodysplastic syndrome.
- Reticulocyte count is normal or low in these cases.
- Erythroid hyperplasia in hemolytic anemia is associated with increased reticulocyte count.

Development of Red Blood Cells

During red blood cells (RBCs) development, there is gradual reduction in size of cells, loss of mitochondria, disappearance of ribonucleic acid (RNA), extrusion of nuclei and gradual appearance of hemoglobin. Stages of development of RBCs are given in Table 8.13.

Red Blood Cells: Stages of Development

- Proerythroblasts undergo 3–4 mitotic cell divisions, so that each stem cell gives rise to 8 or 16 cells of basophilic, polychromatic and orthochromatic erythroblasts in about 7 days. Iron is required for heme synthesis.
- Vitamin B_{12} and folic acid are needed for maturation of nucleus of erythroid precursors.
- Comparison of the DNA and RNA content, their distribution in the bone marrow and peripheral blood of the erythroblast (normoblast) reticulocyte and mature red blood cell is given in Table 8.14.

Reticulocyte

- Reticulocyte, a young RBC contains network of ribonucleic acid (reticulum) in cytoplasm. It normally takes about 4 days to mature into an erythrocyte in blood circulation.
- Supravital stains used for reticulocyte count include new methylene blue, and acridine orange. When stained with new methylene blue, the RNA precipitates as bluish filaments and their cytoplasm.
- Newborn baby has 2–6% circulating reticulocytes. Reticulocyte count is 0.5–1.5% in adults.

Mature Red Blood Cells

- RBCs are biconcave discs, anucleate, and essentially without organelles, and filled with water and 97% hemoglobin (Hb), a protein that functions in O_2 and CO_2 transport. Biconcave shape of RBCs possesses huge surface area to volume, and thus contributes to its gas transport function.
- Cytoskeleton proteins present in RBC membrane give erythrocytes, their flexibility, and allows them to change shape as necessary.
- ATP is generated anaerobically, so the erythrocytes do not consume the oxygen, during transport. RBCs contain carbonic anhydrase enzyme, which catalyzes reaction between CO_2 and H_2O and helps in transport of CO_2 from tissues to the lungs. The life span of an erythrocyte is 100–120 days.

Metabolic Pathways in the Red Blood Cells

The metabolic pathways in the red blood cells (RBCs) are given in Table 8.15.

- Glycolytic metabolic signaling pathway in phosphofructokinase and pyruvate kinase (PK) enzymes participate in production of ATP accounting for 90% of glucose consumption in RBC.
- Hexose-monophosphate shunt signaling pathway provides NADPH and glutathione to reduce oxidants that would shift the balance of oxyhemoglobin to methemoglobin.
- Rapoport-Luebering metabolic signaling pathway in mature red blood cells of 2,3-bisphosphoglycerate (2,3-BPG) by 2,3-bisphosphoglyceric acid synthase, which regulates oxygen release from hemoglobin and delivery to tissues.
- Methemoglobin reductase metabolic signaling pathway protects hemoglobin from oxidation via NADPH (from glycolytic pathway) and methemoglobin reductase.

LEUKOPOIESIS

White blood cells are composed of granulocytes and agranulocytes. Granulocyte is the collective name designated to neutrophils, eosinophils and basophils derived from myeloblast. Agranulocytes comprise monocytes and lymphocytes. Characteristics of leukocytes are given in Table 8.16.

Granulocytes

Granulocytes are derived from myeloblast and progenitor committed cells. During development through various stages such as myeloblast, promyelocyte, myelocyte, metamyelocyte, band form and mature granulocyte (Table 8.17).

- There is progressive condensation and lobulation of nuclei, development of cytoplasmic granules, loss of RNA and mitochondria. Red bone marrow also contains a large reserve of mature granulocytes.

Table 8.13 Stages of development of red blood cells (RBCs)

Cell	Size (μm)	Nucleus	Cytoplasm
Proerythroblast	15–20	Large with immature chromatin, usually a single nucleolus	Blue
Early erythroblast	12–16	Fine chromatin clumps, nucleolus barely visible	Deep blue due to high RNA
Intermediate erythroblast	12–15	Smaller size, chromatin clumps	Polychromatic due to beginning of hemoglobinization
Late erythroblast	8–12	Small, dense, pyknotic and eccentric	Polychromatic
Reticulocytes	8–10	No nucleus	Polychromatic, remnants of RNA visible as a network on supravital staining
Erythrocyte	7–8	No nucleus	Pink

Table 8.14 Comparison of the DNA and RNA content, their distribution in the bone marrow and peripheral blood of the erythroblast (normoblast), reticulocyte and mature red blood cell

Parameters	Erythroblast (Normoblast)	Reticulocyte	Mature Red Blood Cell
Nuclear DNA	Present	Absent	Absent
RNA in cytoplasm	Present	Present	Absent
Bone marrow	Present	Present	Absent
Peripheral blood	Absent	Present	Present

Table 8.15 Metabolic pathways in the red blood cells

Metabolic Pathways in RBC	Key Enzymes in Metabolic Pathway	Functions	Abnormality in Metabolic Pathways and Associated Hematologic Disorders
Glycolytic pathway	▪ Phosphofructokinase ▪ Pyruvate kinase (PK)	This pathway participates in production of ATP accounting for 90% of glucose consumption in red blood cell (RBC)	Hereditary pyruvate kinase deficiency (hemolytic anemia)
Hexose-monophosphate shunt pathway	▪ Glucose-6-phosphate dehydrogenase (G6PD) ▪ Glutathione reductase	This pathway provides NADPH and glutathione to reduce oxidants that would shift the balance of oxyhemoglobin to methemoglobin	▪ Hereditary G6PD deficiency (hemolytic anemia) ▪ Glutathione reductase deficiency (hemoglobinopathies)
Rapoport-Luebering metabolic pathway	2,3-Bisphosphoglyceric acid synthase	The pathway controls the amount of 2,3-bisphosphoglycerate (2,3-BPG), which in turn affects the oxygen affinity of hemoglobin	Tissue hypoxia
Methemoglobin reductase metabolic pathway	Methemoglobin reductase	This pathway protects hemoglobin from oxidation via NADPH (from glycolytic pathway) and methemoglobin reductase	▪ Tissue hypoxia ▪ Hemolytic anemia

Table 8.16 Characteristics of leukocytes

Cell Type	Prevalence	Morphology	Primary Function	Comments
Neutrophil (12–15 µm)	50–70%	▪ Nucleus 2–5 lobed ▪ Cytoplasm contains small purple granules rich in digestive enzymes	Essential blood phagocytes, engulf and kill bacteria	Life span of 2 days with only 4–10 hours in the circulation
Eosinophil (12–15 µm)	1–4%	Bilobed nucleus with large coarse orange-colored granules containing toxic proteins, inflammatory mediators and digestive enzymes	▪ Destroys worms and fungi ▪ Participates in allergies and other inflammatory reactions	Found in much large numbers in the spleen and bone marrow
Basophil (10–12 µm)	0–1%	Constricted nuclei with dark blue to black granules in cytoplasm	Participates in inflammatory and allergic reactions	Cytoplasmic granules contain histamine, prostaglandins and other chemical mediators of allergic response
Monocyte (12–20 µm)	2–8%	Largest size cells with large nuclei often indented. Granules are not visible on light microscopy	▪ Participates in phagocytosis followed by final differentiation into macrophages and dendritic cells ▪ Dendritic cells are relatives of macrophages responsible for processing foreign matter and presenting to lymphocytes	Monocytes also secrete several chemical mediators that moderate the functions of the immune system
Lymphocyte (7–10 µm) or (10–14 µm)	20–40%	Small spherical cells with uniformly staining dark round nuclei	Participates in specific acquired immunity	▪ T cells are responsible for cell-mediated immunity and assisting B cells ▪ B cells differentiate into plasma cells and participate in humoral immunity ▪ Natural killer cells are related to T cells but display no antigen specificity. These cells are active against cancer cells and virally infected cells

Table 8.17 Stages of maturation of myeloid cells

Cell	Size (μm)	Nucleus	Cytoplasm
Myeloblast	15–20	Nucleus is large, immature, fine dispersed chromatin with 2–5 nucleoli	Cytoplasm is scanty, light blue
Promyelocyte	16–20	Similar to myeloblast	Azurophil granules appear
Myelocyte	14–16	Chromatin condensed, no nucleoli	▪ Specific and azurophil granules present ▪ Specific granules predominate
Metamyelocyte	14–18	Indented or kidney-shaped; peripheral clumping of chromatin	Specific numerous pink granules and fine faint pink azurophil granules
Band form	14–16	U-shaped or band-like nucleus with heavily clumped chromatin	Specific numerous pink granules and fine faint pink azurophil granules
Neutrophil	12–15	2–5 lobes joined by chromatin strands	Specific numerous pink granules and fine faint pink azurophil granules

- Mature granulocytes pass actively across endothelial lining of bone marrow sinusoids. Turn out of granulocytes is very high. Stages of maturation of myeloid cells are shown in Fig. 8.7. Myelocytes in the bone marrow differentiate to form neutrophils, eosinophils and basophils.

Neutrophils

Neutrophils measure 12–15 μm in diameter, which constitute 50–70% of leukocytes. These cells have 2–5 lobed nucleus. Cytoplasm contains small purple granules rich in digestive enzymes. Neutrophils remain in circulation for 4–10 hours. Life span of neutrophils is 2 days. Neutrophils are recruited to the site of injury within first 24 hours of inflammation.

Eosinophils

Eosinophils measure 12–15 μm in diameter, which constitute 1–4% of leukocytes. These cells have bilobed nucleus. Cytoplasm contains large coarse orange-colored granules. Eosinophilic granules contain highly cationic major basic protein (MBP) that kills only invasive

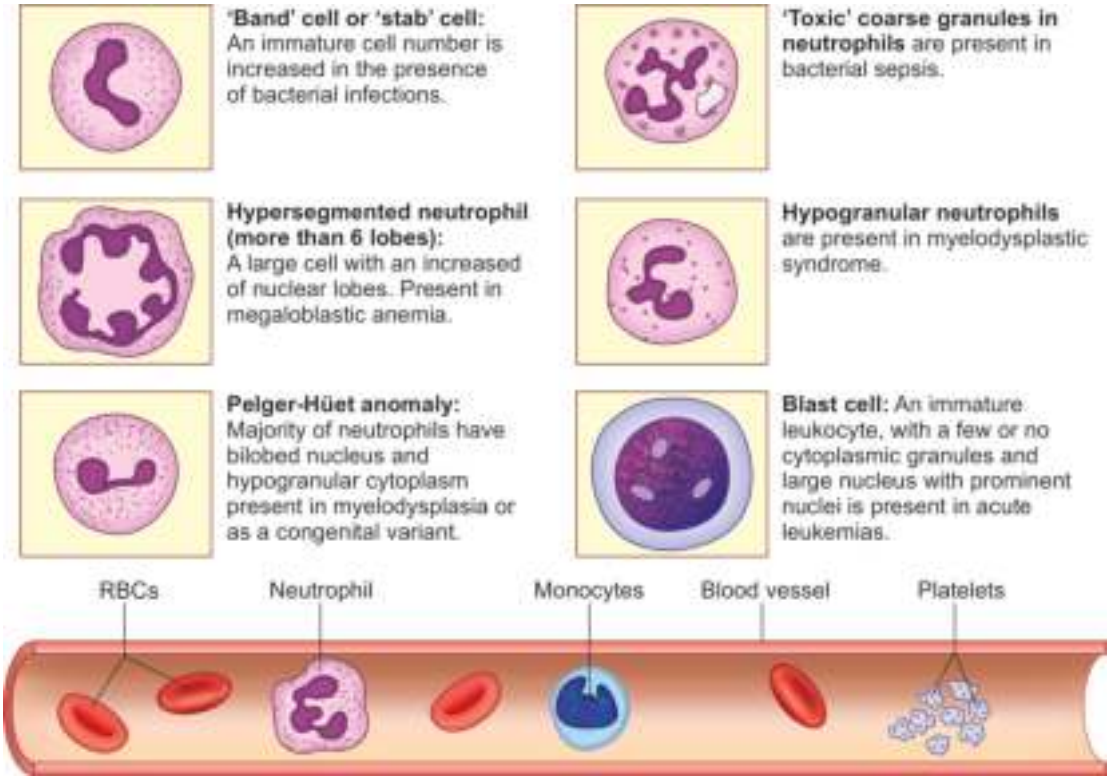


Fig. 8.7: WBCs qualitative anomalies.

helminthes. Eosinophils are recruited by chemokine (eotaxin) at injury site in IgE-mediated allergic reactions. Eosinophilic red granules contain crystalline material in cytoplasm, which becomes Charcot-Leyden crystals in the sputum of bronchial asthmatic patients.

Basophils

Basophils measure 10–12 µm in diameter, which constitute 0–4% of leukocytes. These cells have pale staining constricted nucleus. Cytoplasm contains dark blue to black granules. Basophils possess coarse granules rich in histamine.

White Blood Cells: Qualitative Anomalies

- **May-Hegglin anomaly:** May-Hegglin anomaly is autosomal disorder due to mutation of MYH9 gene. The disorder is characterized by basophilic inclusions (Döhle body-like) in neutrophils, anemia, thrombocytopenia and giant platelets.
- **Alder-Reilly anomaly:** It is autosomal recessive disorder associated with several genetic mucopolysaccharidoses. There is deficiency of lysosomal enzymes required forbreakdown of mucopolysaccharides. There is presence of liliac inclusions with clear halo in neutrophils stained with Giemsa stain. These granules are also demonstrated with toluidine blue.
- **Pelger-Hüet anomaly:** Pelger-Hüet anomaly is autosomal dominant disorder due to mutation of lamin B receptor (LBR) gene. It is characterized by lack of segmentation of neutrophils. Nuclei are dumbbell-shaped in >70% of neutrophils. Chromatin is coarse. Two lobes of the nuclei are joined by thin chromatin bridge.

Agranulocytes

Agranulocytes comprise monocytes and lymphocytes. Monocytes differentiate into tissue macrophages. Bone marrow participates in production of B and T cells. B cells mature in the bone marrow. T cells migrate to thymus gland for maturation. Multiple subtypes of lymphocytes are given in Table 8.18. Comparison between B cells and T cells is given in Table 8.19.

Monocytes

Monocytes are derived from monoblasts in the bone marrow. These have life span of 12 hours. Monocyte contains reinform, lobulated or indented nuclei with open chromatin without nucleoli. Cytoplasm is pale blue. Monocytopoiesis is regulated by GM-CSF.

Lymphocytes

Markers of B cell, T cell and natural killer cells are given in Table 8.20.

- **B cells:** B cells, when stimulated with antigen, become plasma cells and secrete immunoglobulins (e.g. IgG, IgA, IgM, IgD and IgE).
  - Plasma cells participate in humoral immunity. Plasma cell has amphophilic cytoplasm and an eccentric nucleus with heterochromatin in a characteristic cartwheel or clockface arrangement.
  - On electron microscopy, cytoplasm of plasma cell contains a pale zone containing extensive Golgi apparatus, centrioles and abundant rough endoplasmic reticulum, ribosomes, mitochondria, lysosomes and the plasma membrane.
- **T cells:** T cells participate in cell-mediated immunity. Activated macrophages display antigens to T cells. Macrophages synthesize IL-12 that stimulates T cell responses. Activated T cells recruit monocytes from the circulation with IFN-γ, a powerful activator of macrophages.
- **Natural killer cells:** Natural killer cells (NK cells) kill tumor cells and virus infected cells by lysing or damaging plasma membranes.

THROMBOPOIESIS

Platelets are produced in the bone marrow by a process termed thrombopoiesis. Platelets production is regulated by thrombopoietin synthesized from **liver**. Megakaryocytes are derived from the bone marrow stem cells known as megakaryoblasts, which mature in about 10 days.

Table 8.18 Multiple subtypes of lymphocytes

Lymphocytes	Subtype	Actions
B cell	Plasma cell	Synthesis of antibodies either against persistent antigen to injury site or against altered tissue components in chronic inflammation
T cell	▪ Effector T cells	▪ Delayed hypersensitivity ▪ Showing mixed lymphocytic reactivity ▪ Cytotoxic killer cells (K cells)
	▪ Regulatory T cells	▪ Helper T cells ▪ Suppressor T cells
Natural killer cell (NK cell)	Cytotoxic cells	▪ Kill tumor cells ▪ Virus infected cells by lysing or damaging plasma membranes

Table 8.19 Comparison between B cells and T cells

Parameters	B Cells	T Cells
Origin	Stem cells in bone marrow	Stem cells in bone marrow
Site of maturation	Bone marrow and lymphoid cells	Thymus gland
Distribution in lymph node	Cortex (germinal follicles and medullary cords) of lymph nodes	Paracortical areas of lymph nodes
Distribution in spleen	Germinal centers of follicles	Periarteriolar lymphoid sheaths
Differentiation (product of antigenic stimulation)	Plasma cells and memory cells	CD4+ helper T cells, CD4+ regulatory T cells, CD8+ cytotoxic T cells and memory T cells
Circulation in blood	Low numbers (10–20%)	High numbers (60–70%)
Life span	Short-lived (weeks to a few months)	Long lived (2–4 years)
Fc receptors	Present	Absent
Electron microscopy	Microvilli present	Smooth surface
Requires antigen presenting cells with MHC	No	Yes required APCs with MHC
General functions	Production of antibodies to inactivate, neutralize target antigens	Cell function in regulating immune functions killing foreign cells, hypersensitivity, synthesize cytokines
MHC molecules	MHC I and MHC II	MHC I and MHC II
Immune surface marker	Immunoglobulin	T cell receptor
Immunological markers	CD19 to CD22	CD1 to CD8

Table 8.20 Markers of B cell, T cell and natural killer cells

B Cell Markers	
▪ CD19 (pan B cell marker)	▪ κ light chain
▪ CD20 (pan B cell marker)	▪ λ light chain
▪ CD22	
T Cell Markers	
▪ CD2	▪ CD5
▪ CD3 (pan T cell marker)	▪ CD7
▪ CD4 (helper T cell marker)	▪ CD8 (cytotoxic T cell marker)
Natural Killer Cell Marker	
CD56	

Megakaryoblast

Megakaryoblast measures 15–30 µm in diameter. It has high nucleus to cytoplasmic ratio. It contains basophilic cytoplasm with granules.

Promegakaryocyte

Promegakaryocyte measures 20–30 µm in diameter, which contains oval lobulated and elongated nucleus with basophilic cytoplasm.

Platelets

Platelets are 3–4.5 microns in diameter, which possess 2 membrane glycoproteins. Normal platelet count is 150,000–400,000/cu mm.

- Platelets are formed by pinching off cytoplasm of megakaryocytes situated close to bone marrow sinusoids circulation. It is known as **platelet budding**.
- Platelets have normal life span of 8–10 days. Platelets are destroyed by macrophages, mainly in the spleen and also in the liver.
- Platelet consists of dense granules and α-granules. These contain primary inflammatory chemical mediators. Platelets regulate vascular endothelial permeability and proliferation of mesenchymal cells in chronic inflammation.
- Platelets participate in surveillance of blood vessel continuity, formation of primary and secondary hemostatic plugs, and healing of injured tissue. Structure and functions of platelets are shown in Fig. 8.8 and Table 8.21.

BONE MARROW ASPIRATION

Bone marrow is composed of network of blood vessels, hematopoietic stem cells, stroma, nerves and reticulo-endothelial cells.

- Bone marrow venous sinusoids are lined by endothelial cells. Bone marrow stroma is composed of cells such as fibroblasts, stromal cells, fat cells, endothelial cells and macrophages and extracellular matrix.
- Extracellular matrix comprises collagen, laminin, fibronectin and proteoglycans.
- Bone marrow examination gives more information and morphologic characteristics are better defined in stained blood films. Indications of bone marrow aspiration are given in Table 8.22.

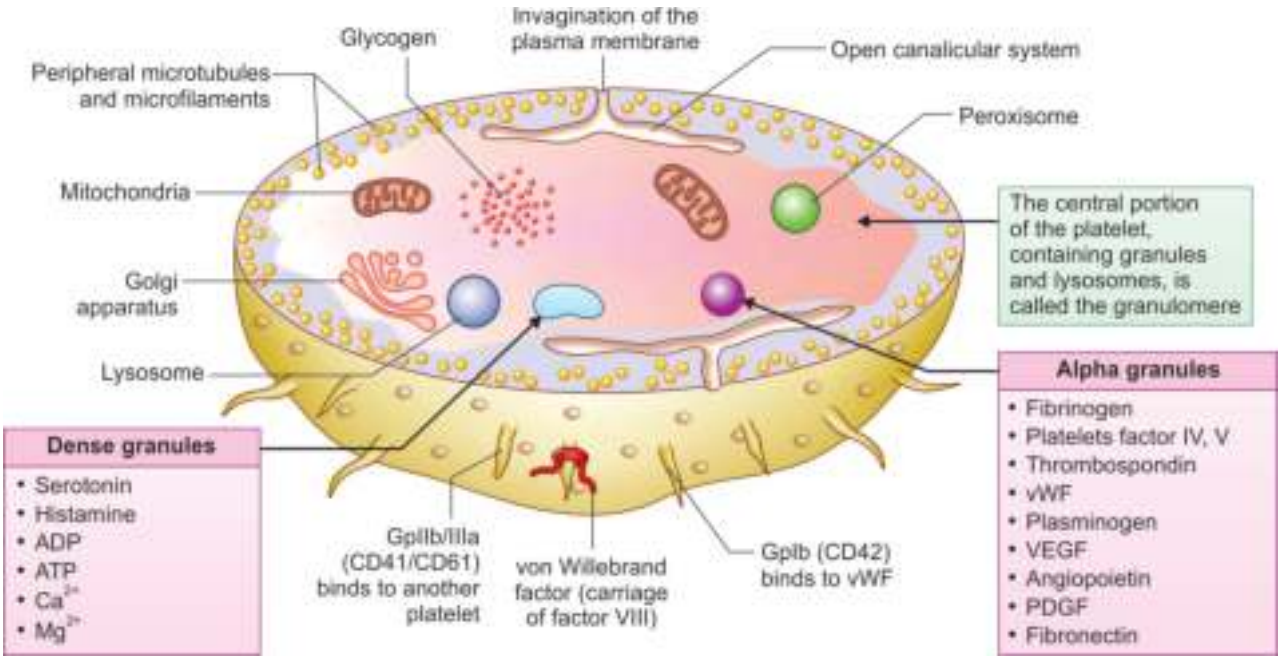


Fig. 8.8: Structure of platelet. Platelet consists of outer glycocalyx layer and inner plasma membrane. Cytoskeleton maintains discoid shape. It consists of dense and α-granules.

Table 8.21 Structure and functions of platelets

Parameters	Functions
Platelet membrane	
▪ Glycocalyx ▪ Plasma membrane	▪ Outermost coat comprising glycolipids, glycoproteins and mucopolysaccharide ▪ Negative charge due to sialic acid residue of proteins and lipids ▪ Composed of glycolipids, cholesterol and glycoproteins ▪ Lipoprotein layer containing platelet factor III involved in blood coagulation
Membrane glycoproteins (acting as receptors for cell-cell and ligand-cell interaction)	
▪ Glycoprotein IIb/IIIa ▪ Glycoprotein Ib–IX	▪ Cross-linking of IIb/IIIa to vWF and fibrinogen leading to platelets aggregation ▪ Deficiency of GpIb–IX results in Bernard-Soulier syndrome (bleeding diathesis)
Cytoskeleton	
▪ Short actin filament ▪ Actin microfilament network ▪ Microtubules	▪ Present under plasma membrane involved in maintaining discoid shape ▪ Present in cytoplasm ▪ Present in peripheral part of cytoplasm involved in maintaining discoid shape
Dense granules	
▪ ADP ▪ ATP ▪ Calcium ▪ Serotonin	▪ Recruits platelets and activates new platelets result in aggregation of platelets ▪ Agonist for cells other than platelets ▪ Extracellular source for hemostatic reactions ▪ Vasoconstrictor
α-Granules	
▪ Fibrinogen ▪ Platelets factor IV ▪ Thrombospondin ▪ Factor V ▪ vWF binds factor VIII ▪ Plasminogen ▪ PDGF	▪ Causes aggregation of platelets and itself gets converted to fibrin ▪ Binds with heparin ▪ Promotes aggregation of platelets ▪ Promotes aggregation of platelets ▪ Participates in adhesion of platelets and aggregation of platelets ▪ Inhibits fibrinolysin ▪ Plasminogen gets converted to plasmin. Plasmin participates in fibrinolysis ▪ Promotes repair of smooth muscle cells

Table 8.22 Indications of bone marrow aspiration

Diagnostic Purposes

- Unexplained leukopenia, thrombocytopenia, undefined anemia, monoclonal hypergammaglobulinemia, lasts or other abnormal cells in the blood suggestive of bone marrow pathology
- Diagnosis of hematopoietic neoplasms
- Evaluation of storage diseases (Gaucher's disease, Niemann-Pick disease), amyloidosis and mastocytosis
- Work up of fever of unknown etiology, monoclonal gammopathy, splenomegaly or other organomegaly

Staging for Hematopoietic Malignant Disorders

- Diagnosis of metastatic tumor to bone marrow, solitary metastatic bone disease (lung carcinoma, renal cell carcinoma, well-differentiated thyroid carcinoma) or multifocal metastatic bone disease (mammary adenocarcinoma, gastric carcinoma, colorectal carcinoma, neuroblastoma)
- Staging of lymphomas

Monitoring the Follow-up Cases

- Follow-up after induction of chemotherapy for acute leukemia and maintenance of chemotherapy
- Monitoring toxicity and antineoplastic effects of antineoplastic therapy
- Restaging of malignant lymphoma after treatment
- Follow-up after hematopoietic stem cell transplantation
- Follow-up of patients with aplastic anemia, paroxysmal nocturnal hemoglobinuria and Fanconi's anemia for the development of myelodysplastic syndrome

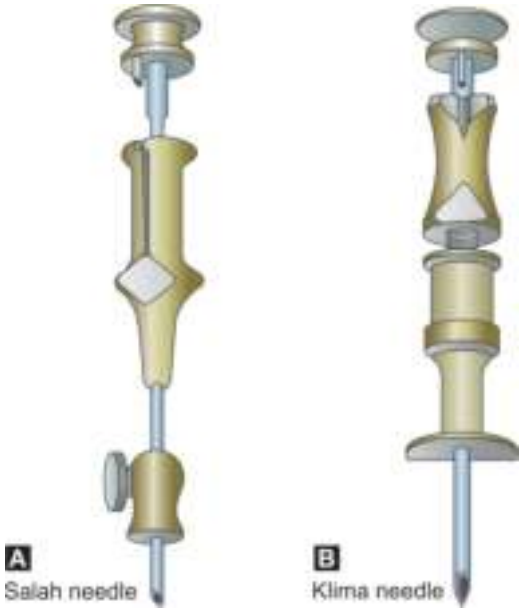


Fig. 8.9A and B: Bone marrow aspiration needles.

BONE MARROW ASPIRATION NEEDLES

Klima needle and **Salah needle** are most often used to aspirate bone marrow. **Islam needle** is used in patients either with osteosclerotic bone, or osteoporotic bone. The needles are stout wide bore, short bevel, stellate and adjustable guard that prevents overpenetration. Bone marrow aspiration needles must be sterilized in a hot air oven or autoclaved before being used (Fig. 8.9A and B). Diagnostic significance of bone marrow examination is given in Table 8.23.

Table 8.23 Diagnostic significance of bone marrow aspiration

Disorders	Examples
Red blood cell disorders	▪ Megaloblastic anemia ▪ Iron deficiency anemia to assess iron stores in bone marrow ▪ Pure red cell aplasia ▪ Sideroblastic anemia to assess iron in sideroblasts
White blood cell disorders	▪ Subleukemic leukemia ▪ Acute myelogenous leukemia ▪ Acute lymphoblastic leukemia
Myeloproliferative disorders	▪ Chronic myelogenous leukemia ▪ Polycythemia rubra vera ▪ Idiopathic thrombocythemia
Megakaryocytic disorders	▪ Idiopathic thrombocytopenic purpura ▪ Other thrombocytopenic purpura
Plasma cell disorders	▪ Multiple myeloma ▪ Waldenström's macroglobulinemia
Storage disorders	▪ Gaucher's disease ▪ Niemann-Pick disease
Metastases in bone marrow	▪ **Adults:** Cancers of prostate, urinary bladder, breast, lung (small cell type), kidney, gastrointestinal tract and thyroid gland. Hodgkin's disease involves bone marrow in stage IV. Non-Hodgkin's lymphoma also involves bone marrow ▪ **Children:** Neuroblastoma, embryonal rhabdomyosarcoma, and retinoblastoma
Parasites	▪ *Leishmania donovani* bodies in kala-azar ▪ Malarial parasites
Histiocytic disorders	▪ Langerhans' histiocytosis ▪ Hemophagocytic syndrome

BONE MARROW ASPIRATION SITES

Posterior superior iliac crest site is preferred in adults and older children. **Calcaneum bone** is site of choice in infants. In children under 2 years of age, bone marrow aspiration may be done from medial aspect of tibia below tibial tubercle.

- **Midline of sternum** at the height of the second intercostal space provides best representative material in adults. But the patient is more apprehensive when bone marrow aspiration is done from this site.
- No attempt should be made to aspirate bone marrow from below the second intercostal space, because if the inner cortical layer is perforated, the great vessels and the right atrium located below this space may be damaged.

BONE MARROW ASPIRATION PROCEDURE

Under aseptic conditions and local anesthesia by adjusting guard the needle is inserted with a boring and slight rotating motion in bone marrow by applying a strong vacuum pressure via an attached syringe.

BONE MARROW ASPIRATE SMEAR PREPARATION

Bone marrow fragments are aspirated and smears are prepared, stained with Romanowsky stains and examined under low power.

- The ideal area for examination of bone marrow aspirate smear is one in which the hematopoietic stem cells constitute a monocellular layer and the red blood cells exhibit pink stained morphology.
- The cellularity of bone marrow varies with the age of the patient. In early childhood, the bone marrow contains red marrow and little fat. In adults, bone marrow has about 50% of fat cells, while 75% of fat cells are present in elderly persons.

BONE MARROW ASPIRATE SMEAR STAINING

Bone marrow aspirate smears are stained with Romanowsky stain. One bone marrow aspirate smear is used for iron stain. CD markers used for hematolymphoid neoplasms are given in Table 8.24. Special tests on bone marrow cells to diagnose hematologic malignant disorders are given in Table 8.25.

Table 8.24 CD markers used for hematolymphoid neoplasms

Hematology Cells	Immunophenotyping Markers on Flow Cytometry
RBCs	Glycophorin A
Megakaryocytes	CD41, CD61
WBCs	CD45 (leukocyte common antigen)
Blasts	CD34, TdT, HLA-DR
Myeloid cells	Anti-MPO, CD13, CD33, CD14, CD117
B cells	CD19, CD20, CD10, FMC7, CD23, CD79a, Ig, IgM

Table 8.25 Special tests on bone marrow cells to diagnose hematologic malignant disorders

Special Tests on Bone Marrow Cells	Comments
Conventional chromosomal analysis	Diagnosis and classification of leukemia and myelodysplastic syndrome
Fluorescence *in situ* hybridization (FISH)	Analysis of chromosomal deletion, duplication, translocation, inversion
DNA analysis	Diagnosis and classification of leukemias, myeloproliferative disease, lymphoproliferative disease and detection of minimal residual disease
Immunophenotype analysis	Diagnosis and classification of leukemias, lymphoproliferative disease and detection of minimal residual disease
Flow cytometry	Analysis and quantification of cells in leukemia, i.e. diagnosis and minimal residual disease
Polymerase chain reaction (PCR)	▪ Performed by amplification of a DNA segment digested by a restriction enzyme and fractioned by size using gel electrophoresis. ▪ Performed to detect point mutation of JAK2 in hematologic disorders, minimal residual disease, and diagnosis of inherited mutations of hemoglobin and coagulant proteins
Second generation sequencing technique of whole genome	To detect new clonal point mutations in hematologic malignancies, e.g. acute myelogenous leukemia (AML), myelodysplastic syndrome (MDS), chronic lymphocytic leukemia (CLL)
Northern blot technique	▪ Gene expression studied by analyzing RNA extracted from fresh cells by gel electrophoresis ▪ Gene can be semiquantified by using the enzyme reverse transcriptase to generate a DNA copy and then applying a modified PCR technique
DNA microarray analysis	DNA microarray analysis used to analyze expression of multiple cellular genes. Microarrays can also be used to detect small deletions or gains in DNA

Bone Marrow Aspirate Smears: Evaluation

Bone marrow smears are screened for bone marrow fragments under scanner objective. Most of the bone marrow particles are present in the tail of smears.

- **Cellularity:** Cellularity of bone marrow is assessed by visualization of bone marrow particles. Number, cellularity of fragments and cellular details are observed. Bone marrow is more cellular in children than adults. Bone marrow is reported as hypercellular, hypocellular or normocellular. Bone marrow cellularity ranges for various groups are given in Table 8.26.
- **Erythropoiesis:** Type of erythropoiesis, normoblastic or megaloblastic. Activity and maturity of erythroid precursors are observed. Largest number of bone marrow cells is erythroid cells.
- **Leukopoiesis:** Maturity, abnormal granules, atypical cells, lymphocytes and plasma cells are observed.
- **Myeloid to erythroid ratio:** At least 500 bone marrow cells are counted. Ratio of normal fat cells to hematopoietic cells is 1:1 in adults. Ratio of normal myeloid cells to erythroid cells is 3:1 in adults. Ratio of normal fat cells to erythroid cells is 4:1 in adults.
- **Megakaryopoiesis:** Megakaryocytes are examined in bone marrow particles near tail of bone marrow smears. It is important to look for budding of platelets from megakaryocytes. Relative number of promegakaryoblasts and megakaryocytes is also observed.
- **Other bone marrow cells:** Plasma cells, lymphocytes and reticulum cells are observed. Differential cell count in adult bone marrow aspirate is given in Table 8.27.
- **Abnormal cells:** One must look for metastases, LD bodies, malarial parasite and *Histoplasma capsulatum*. Granulomas are demonstrated in bone marrow due to tuberculosis, sarcoidosis, *Histoplasma capsulatum* and *Cryptococcus neoformans*, systemic lupus erythematosus and infectious mononucleosis. Microorganisms demonstrated in bone marrow smear are given in Table 8.28.

Bone Marrow Iron Content

By means of a cytochemical stain, the amount of stainable iron in the bone marrow can be visualized and quantitatively assessed. It has diagnostic role in diagnosing iron deficiency anemia, β-thalassemia, and sideroblastic anemia.

Perls Prussian Blue Staining: Principle

The bone marrow iron content test is based on the Perls Prussian blue reaction. Ionic iron reacts with acid potassium ferricyanide solution (2% potassium ferricyanide + N/5 HCl) to produce a blue color. Bone marrow aspirate smears are counterstained by 0.33% neutral red to stain nuclei as red.

Normal Bone Marrow Iron Stores: Interpretation

- Iron granules appear as bright blue or blue-green aggregates not exceeding 1 µm in diameter in the cytoplasm of nucleated (sideroblasts) or non-nucleated (siderocytes) precursors contrasting sharply with the pink stained background.
- Normally, 25–50% of normoblasts contain 1–4 small iron granules. Often, iron granules can also be seen in the reticuloendothelial cells of the bone marrow, and they may be spherical or irregularly shaped.
- When the granules surround the erythroid nuclei, they constitute the so-called ring sideroblasts.
- Grading of iron stores on bone marrow aspirate is given in Table 8.29.

Bone Marrow Iron Stores in Pathologic Disorders

- Bone marrow is deficient in iron deficiency anemia. Bone marrow iron is increased in thalassemia. Iron granules scattered throughout cytoplasm surrounding nuclei occur in sideroblastic anemia.
- Excess of iron is deposited in reticuloendothelial cells in dyserythropoietic anemia and anemia of chronic inflammation.
- Demonstration of iron granules in normoblasts under oil immersion lens is given in Table 8.30.

Table 8.26 Bone marrow cellularity ranges for various groups

Age Group	Bone Marrow Cellularity
Newborn to 3 months	80–100%
Children	60–80%
Adults (20–40 years)	60–70%
Adults (>40–70 years)	40–50%
Adults (>70 years)	30–40%

Table 8.27 Differential cell count in adult bone marrow aspirate

Bone Marrow Cells	Percentage of Cells
Myeloid cells*	
Myeloblasts	0–3
Promyelocytes	3–10
Neutrophilic myelocytes	4–10
Metamyelocytes	3–8
Neutrophils including band form	25–45
Erythroid cells	
Proerythroblasts	0–1
Early/basophilic normoblasts	1–5
Intermediate/polychromatic normoblasts	5–20
Late/orthochromatic normoblasts	5–15
Lymphoid cells	
Lymphocytes	5–10
Plasma cells	0–2
Monocyte/macrophages	
Monocytes	1–3
Macrophages	0–1
Megakaryocytic cells	
Megakaryocytes	0–2

**Normal myeloid to erythroid ratio ranges 3:1–15:1.*

Table 8.28 Microorganisms demonstrated in bone marrow

Microorganisms Demonstrated in Bone Marrow in Immunocompromised Persons		
Parasites	▪ *Leishmania donovani* bodies (LD bodies)	▪ Malarial parasite
Bacteria	▪ *Mycobacterium tuberculosis* bacilli	▪ *Mycobacterium leprae* bacilli
Fungi	▪ *Cryptococcus neoformans*	▪ *Histoplasma capsulatum*
Virus	Cytomegalovirus	

Table 8.29 Grading of iron stores on bone marrow aspirate

Grade	Interpretation	Iron Content in Bone Marrow Cells
Grade 0	▪ Iron stores—nil	▪ Absence of iron granules
Grade 1	▪ Diminished iron stores	▪ Small granules in reticulum cells observed under oil immersion lens
Grade 2	▪ Normal iron stores	▪ Presence of iron granules with low power lens
Grade 3	▪ Normal iron stores	▪ Numerous small granules present in all marrow particles
Grade 4	▪ Increased iron stores	▪ Presence of large granules in clumps
Grade 5	▪ Increased iron stores	▪ Presence of dense clumps of granules
Grade 6	▪ Increased iron stores	▪ Very large granules present obscuring marrow cells

Table 8.30 Demonstration of iron granules in normoblasts under oil immersion lens

Erythroid Cells	Iron Granules
Late normoblasts	1–3 pinpoint granules
Abnormal sideroblasts (late and intermediate normoblasts) seen in megaloblastic anemia	1–10 pinhead granules
Ring sideroblasts (sideroblastic anemia)	>5 coarse granules surrounding nucleus

BONE MARROW TREPHINE BIOPSY

Bone marrow trephine biopsy should be performed when the bone marrow obtained by bone marrow aspiration is inadequate for examination in cases of aplastic anemia or myelofibrosis. Comparison between bone marrow aspiration and bone marrow trephine biopsy is given in Table 8.31.

BONE MARROW TREPHINE BIOPSY NEEDLES

Bone marrow trephine biopsy procedure is done with a biopsy needle such as Jamshidi-Swaim needle or Islam needle or Osgood biopsy needle. The preferred site is the posterior superior iliac spine. Sternal site is always avoided due to danger of penetrating heart.

BONE MARROW TREPHINE BIOPSY PROCEDURE

Under aseptic conditions and anesthetizing the area, the sterile trephine biopsy needle is inserted into bone marrow by to and fro rotation clockwise and anticlockwise at least 10 times. Bone marrow trephine biopsy processing technique is given in Table 8.32.

BONE MARROW TREPHINE BIOPSY FIXATION

The material is aspirated by bone marrow trephine biopsy and put in 2 ml of **Helly's fluid for fixation**. Helly's fluid consists of potassium dichromate (2.5 g); mercuric chloride (5.0 g); 40% formaldehyde (5 ml); dissolved in 100 ml of water. The section is stained with hematoxylin and eosin. The preparation allows for optimal evaluation of bone marrow cells with bony trabeculae. It is helpful in evaluation of metastatic tumor or bone marrow fibrosis.

Bone Marrow Trephine Biopsy: Indications

Bone marrow trephine biopsy is performed in diagnosing of following disorders:

- Aplastic anemia
- Metastases in bone marrow
- Myelofibrosis
- Aleukemic leukemia
- Hypoplastic acute myelogenous leukemia M7 (AML-M7)
- Miliary tuberculosis involving bone marrow
- Malignant lymphomas
- Myelodysplastic syndrome
- Chronic lymphoproliferative disorders

Table 8.31 Comparison between bone marrow aspiration and bone marrow trephine biopsy

Parameters	Bone Marrow Aspiration	Bone Marrow Trephine Biopsy
Site of procedure	Posterior iliac crest, sternum in adults and tibia in infants	Posterior iliac crest in adults
Staining techniques	▪ Romanowsky stains ▪ Perls Prussian blue for iron	▪ Hematoxylin and eosin ▪ Silver methenamine stain for demonstration of reticulin
Advantages/ disadvantages	▪ Bone marrow aspiration is simple, reliable, rapid method providing cytologic features of bone marrow cells suited for cytogenetics, molecular and flow cytometric analysis provides excellent appreciation of spatial relationship between cells and overall bone marrow structure ▪ Bone marrow aspiration has low sensitivity in detection of granulomas, solid malignant tumor metastases and lymphoma involvement	▪ Bone marrow trephine biopsy provides excellent appreciation of spatial relationship between cells and overall bone marrow structure ▪ Bone marrow trephine biopsy is required in inadequate or failed bone marrow aspirate, assessment of cellularity, bone marrow architecture, suspected granulomas, detection of micrometastases, lymphoma deposits and bone marrow fibrosis
Results available	Within 1–2 hours	Within 1–7 days depending on decalcification method used for specimen
Indications	▪ Unexplained pancytopenia (anemia, leukopenia, thrombocytopenia) ▪ Suspected bone marrow failure (leukemia, myelodysplastic syndrome, lymphoma, multiple myeloma, metastatic bone disease, Gaucher's disease, Niemann-Pick disease) ▪ Suspected infection (leishmaniasis, tuberculosis)	▪ Aplastic anemia ▪ Malignant disorders (multiple myeloma, lymphoma, metastatic bone disease) ▪ Dry tap on bone marrow aspiration ▪ Miscellaneous (splenomegaly, pyrexia of unknown origin, amyloid deposit)
Special investigations	▪ Cytogenetic analysis ▪ Fluorescence *in situ* hybridization (FISH) ▪ Flow cytometry ▪ DNA, RNA analysis ▪ Microarrays ▪ Progenitor cell culture ▪ Microbiological culture	Immunocytochemistry (*refer* to Table 8.35)

Bone marrow trephine biopsy is required for unexplained pancytopenia or suspected bone marrow infiltration by malignant tumors, storage disorders and infections.

Table 8.32 Bone marrow trephine biopsy processing technique

Fixatives for Bone Marrow Trephine Biopsy

- Neutral buffered formalin (18–24 hours)
- Bouin's fluid
- Buffered formalin
- Zinc formalin
- Schaffer's solution

Decalcification of Bone Marrow Trephine Biopsy

- Ethylenediaminetetra-acetic acid (EDTA) (14%): Excellent preservation of cell morphology, antigens and nucleic acid, but time-consuming process
- Nitric acid (time 6–8 hours): Routinely used in laboratory
- Formic acid (time 12–24 hours): Routinely used in laboratory
- Rapid decalcifier: TBD-1 Shandon decalcifier (15–60 minutes)

- **Combined Fixatives and Decalcifier Agents**
  - **Acetic acid zinc formaldehyde** (rapid technique with excellent preservation of cell morphology and antigens, but poor preservation of nucleic acid)
- **Microwave Assisted Fixation and Decalcification**
  - Microwave assisted fixation and decalcification (rapid process): Bone marrow trephine biopsy specimen should be fixed in 10% neutral formalin and then washed in water before decalcification in 5% nitric acid
- **Paraffin-embedding of Bone Marrow Trephine Biopsy**
  - Histologic sections are obtained by microtome and stained with hematoxylin and eosin stain: Technique is routinely done in histopathologic laboratories. Immunohistochemistry, fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR) are easily feasible.

BONE MARROW TREPHINE BIOPSY SECTION STAINING TECHNIQUE

Bone marrow trephine biopsy sections are stained by hematoxylin and eosin, Perls Prussian blue stain for iron, reticulin stain and Masson trichrome stain. Stains performed on bone marrow and bone marrow trephine biopsy are given in Table 8.33. Galle's iron-grading system in bone marrow trephine biopsy is given in Table 8.34. Lineage associated CD antigens and cellular expression commonly analyzed in bone marrow trephine biopsy by routine flow cytometry are given in Table 8.35.

Table 8.33 Stains performed on bone marrow and bone marrow trephine biopsy

Stain	Comments on Stain Used
Stains on bone marrow smears	
Romanowsky stain	Study of bone marrow cellularity, myelopoiesis, erythropoiesis and myeloid to erythroid ratio; megakaryopoiesis, Gaucher's cells, Niemann-Pick cells, malarial parasite
Perls Prussian blue stain for iron	Evaluation of storage iron and ring sideroblasts
Periodic acid–Schiff (PAS) stain	Visualization of megakaryocytes and fungi
Ziehl-Neelsen stain	Demonstration of acid-fast bacilli
Bone marrow trephine biopsy	
Hematoxylin and eosin stain	Evaluation of histologic features
Perls Prussian blue stain for iron	▪ Evaluation of storage iron but less sensitive than performed on bone marrow aspirate ▪ Ring sideroblasts may be demonstrated in non-decalcified bone sections
Reticulin stain	Evaluation of reticulin fibers in the bone marrow in a case of myelodysplastic syndrome
Masson trichrome stain	Evaluation of collagen fibrosis in bone marrow indicating an advanced disease
Immunohistochemistry	Immunohistochemistry results may be different from non-decalcified bone sections

Table 8.34 Galle's iron-grading system in bone marrow trephine biopsy

Galle's Iron-grading System	Iron Content	Iron Demonstration under Light Microscope
Grade 0	None	No visible iron high-power magnification (X1000)
Grade 1	Very slight	Small iron particles just visible in few reticulum cells under high-power magnification (X1000)
Grade 2	Slight	Small spersely distributed iron granules just visible under low-power magnification (X100)
Grade 3	Moderate	Numerous small iron granules present in the reticulum cells throughout the bone marrow fragments visible under low power magnification (X100)
Grade 4	Moderate heavy	Large iron granules throughout the bone marrow fragments with tendency to aggregate into clumps visible under low-power magnification (X100)
Grade 5	Heavy	Dense large clumps of iron granules visible under low-power magnification (X100)
Grade 6	Very heavy	Very large deposits of iron granules both intracellular and extracellular regions obscuring cellular details in the bone marrow fragments visible under low-power magnification (X100)

Table 8.35 Lineage associated CD antigens and cellular expression commonly analyzed in bone marrow trephine biopsy by routine flow cytometry

Lineage	Immunophenotype Markers on Flow Cytometry	
Hematopoietic stem cells	▪ CD34 (hematopoietic stem cells) ▪ CD117 (c-KIT) (hematopoietic stem cells) ▪ Terminal deoxynucleotidyl transferase (TdT)	▪ CD10 ▪ CD3 ▪ CD19
Myeloid lineage	▪ MPO ▪ CD13 ▪ CD33	▪ CD10 ▪ HLA-DR
Erythroid lineage	▪ CD71 ▪ Glycophorin A (encoded by GYPA gene) ▪ Glycophorin C	▪ Hemoglobin ▪ Spectrin (cytoskeleton protein)
Megakaryocytic lineage	▪ CD41 ▪ CD42b	▪ CD61 ▪ von Willebrand factor (factor VIII RA)
Monocytic lineage	▪ CD14 ▪ CD68 (KP-1 and PGM-1)	▪ CD163

Fluorescently tagged antibodies can be used to determine cell lineage in characterizing patient immunophenotype by flow cytometry. CD55 and CD59: Decay-accelerating factor (DAF) and membrane inhibitor of reactive lysis (MIRL).

RED BLOOD CELL, HEMOGLOBIN, ANTICOAGULANTS AND ANEMIAS

RED BLOOD CELL

Red blood cell measures 7–8 µm in diameter with life span of 100–120 days, which contains hemoglobin. Normal red blood cell membrane cytoskeleton proteins of RBCs comprise spectrin, ankyrin, actin, protein 4.1 and protein 3. Together, these cytoskeleton proteins maintain the normal biconcave shape of RBCs. Patient develops hereditary spherocytosis due to deficiency of any of these RBCs membrane cytoskeleton proteins.

RED BLOOD CELL INDICES

Hematocrit values are basically the measures of red blood cells size and their hemoglobin content, which give valuable information in diagnosing anemia. Automated cell counters provide these data on each sample. These values vary slightly in different laboratories. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) are calculated as discussed under.

Mean Corpuscular Volume

Mean corpuscular volume (MCV) is the index used to measure the average volume of red blood cell. MCV categorizes RBCs according to their size. RBCs with normal size are called normocytic cells. Smaller RBCs are termed microcytes, and larger red blood cells are referred to as macrocytes. Normal MCV is 82–96 femtoliters. MCV is decreased in iron deficiency anemia (<80 femtoliters). **MCV** is increased in megaloblastic anemia (>100 femtoliters).

Mean Corpuscular Hemoglobin

Mean corpuscular hemoglobin (MCH) is the index used to measure average hemoglobin content per red blood cell. MCH is calculated by dividing hemoglobin concentration (g/dl) by red blood cells concentration/liter. Normal value of MCH is 26–33 pg. MCH is decreased in iron deficiency anemia and increased in megaloblastic anemia.

Mean Corpuscular Hemoglobin Concentration

Mean corpuscular hemoglobin concentration (MCHC) measures the average concentration of hemoglobin in a given packed cell volume of red cells. Normal MCHC value is 33–37 g/dl. MCHC is decreased in iron deficiency anemia and increased in hereditary spherocytosis. MCHC remains normal in megaloblastic anemia.

Red Blood Cell Distribution Width

Red blood cell distribution width (RDW) is the index to measure degree of anisocytosis (various in size of red blood cells). Normal value of RDW is 11.5–14.5%. RDW is increased in iron deficiency anemia, megaloblastic anemia and immune-mediated hemolytic anemia. RDW remains within normal range in thalassemia.

Hematocrit or Packed Cell Volume

Packed cell volume (PCV) is the index to measure blood volume proportion occupied by red blood cells. Normal PCV range in males is 40–52% and 36–48% in females.

- Increase in number of red blood cells will increase hematocrit or packed cell volume except in thalassemia and megaloblastic anemia.
- Hemolysis in thalassemia increases production of number of red blood cells, but with small size red blood cells and decreased hematocrit value.
- Patient of megaloblastic anemia shows macrocytic red blood cells size but decreased hematocrit value. Cut off points for anemia according to WHO are given in Table 8.36.

RETICULOCYTE COUNT

Approximately 50,000 reticulocytes are produced daily, which circulate for about one day to form mature red blood cells. Reticulocyte count is used to assess the capacity of the bone marrow to increase RBC production in response to increased demand.

Supravital New Methylene Blue Stain

Reticulocytes are demonstrated by supravital stains such as new methylene blue (best stain) and brilliant cresyl blue. Heinz bodies are demonstrated by supravital stains (Table 8.37).

Conditions Associated with Increased or Decreased Reticulocyte Count

Reticulocyte count is increased following massive bleeding, acute hemolysis, and response to specific therapy in nutritional anemia and even after voluntary blood donation. Peripheral blood smear examination under these conditions shows red blood cell polychromasia and increased reticulocyte count. Reticulocyte count is decreased in aplastic anemia, pernicious anemia, bone marrow metastases and congenital dyserythropoietic anemia. Factors impairing the normal reticulocyte response are given in Table 8.38.

Table 8.36 Cut off points for anemia according to WHO

Subject	Hemoglobin (%)	MCHC
Children (6 months to 6 years)	11 g/dl	34%
Children (>6–14 years)	12 g/dl	34%
Women (during pregnancy)	11 g/dl	34%
Women (nonpregnant)	12 g/dl	34%
Men	13 g/dl	34%

Table 8.37 Composition of new methylene blue reagent

Stain Constituents	Quantity
New methylene blue	0.5 g
Potassium oxalate	0.6 g
Distilled water	100 ml

Table 8.38 Factors impairing the normal reticulocyte response

Bone Marrow Disorders	
▪ Hypoplasia/Aplasia of bone marrow	▪ Lymphoma
▪ Metastatic bone disease	▪ Tuberculosis
▪ Acute leukemia	
Lack of Erythropoietin Synthesis	
Chronic renal disease	
Ineffective Erythropoiesis	
▪ Thalassemia major	▪ Megaloblastic anemia
▪ Myelodysplastic syndrome (MDS)	▪ Myelofibrosis
Reduced Tissue Oxygen Consumption	
▪ Myxedema	▪ Protein malnutrition
Miscellaneous Disorders	
▪ Iron deficiency anemia	▪ Malignant tumors
▪ Chronic inflammatory disease	

HEMOGLOBIN

Normal adult hemoglobin is a tetramer protein composed of four globin (peptide) chains (α_1, α_2, β_1, β_2), each with its own heme molecule. These peptide chains undergo conformational change and move with respect to each other when binding of O_2, CO_2, and 2,3-diphosphoglycerate (2,3-DPG) between β-chains reduce affinity for O_2 and permit O_2 release to the tissues.

- Hemoglobin synthesis occurs in the developing red blood cell. The mitochondria are the primary sites of porphyrin synthesis. Iron is supplied from circulating transferrin; globin chains are synthesized on ribosomes.
  - Embryonic hemoglobin exists up to 8 weeks of intrauterine life.
  - Fetal hemoglobin (HbF) is synthesized after 8 weeks of intrauterine life. Fetal hemoglobin level is attained <2% by 7 months of intrauterine life. HbA_2 synthesis begins by 35 weeks of intrauterine life. Switch over from fetal to adult hemoglobin starts at 30 weeks of intrauterine life.
  - Adult hemoglobin synthesis (HbA) is completed by 38 weeks at the time of birth of newborn. Significant time for switching over synthesis from fetal hemoglobin to adult hemoglobin starts at 30 weeks of intrauterine life. Adult hemoglobin synthesis is completed by 38 weeks at the time of birth of newborn. After one year of life and in adults, normal fetal hemoglobin (HbF) is <1% and HbA_2 is <3%. Adult hemoglobin (HbA) constitutes >95%. Steps of heme synthesis are shown in

Fig. 8.10. Steps of hemoglobin synthesis are shown in Fig. 8.11.

Nomenclature of Hemoglobin

The various forms of hemoglobin are named according to the types of globin chains present. Some of these hemoglobins are present in adults, while others may be found only *in utero* or early in infancy.

- Embryonic hemoglobin exists up to 8 weeks of intrauterine life.
- Fetal hemoglobin is synthesized after 8 weeks of intrauterine life. Normal types of hemoglobin according to developmental stage are given in Table 8.39.

Genetic Control

Hemoglobin consists of pairs of globin chains. A variety of globin chains can be present. Normally, α- and β- and to a lesser extent δ-globin chains form adult hemoglobin. In fetal life (γ, ε, and ζ) chains are present. The globin chains of hemoglobin are synthesized independently under separate genetic control. The α-chain and ζ-chain are under genetic control on chromosome 16. Rest globin chains (β-chain, γ-chain, δ-chain, and ε-chain) are under genetic control on chromosome 11. Globin chains and their locus on chromosomes are given in Table 8.40.

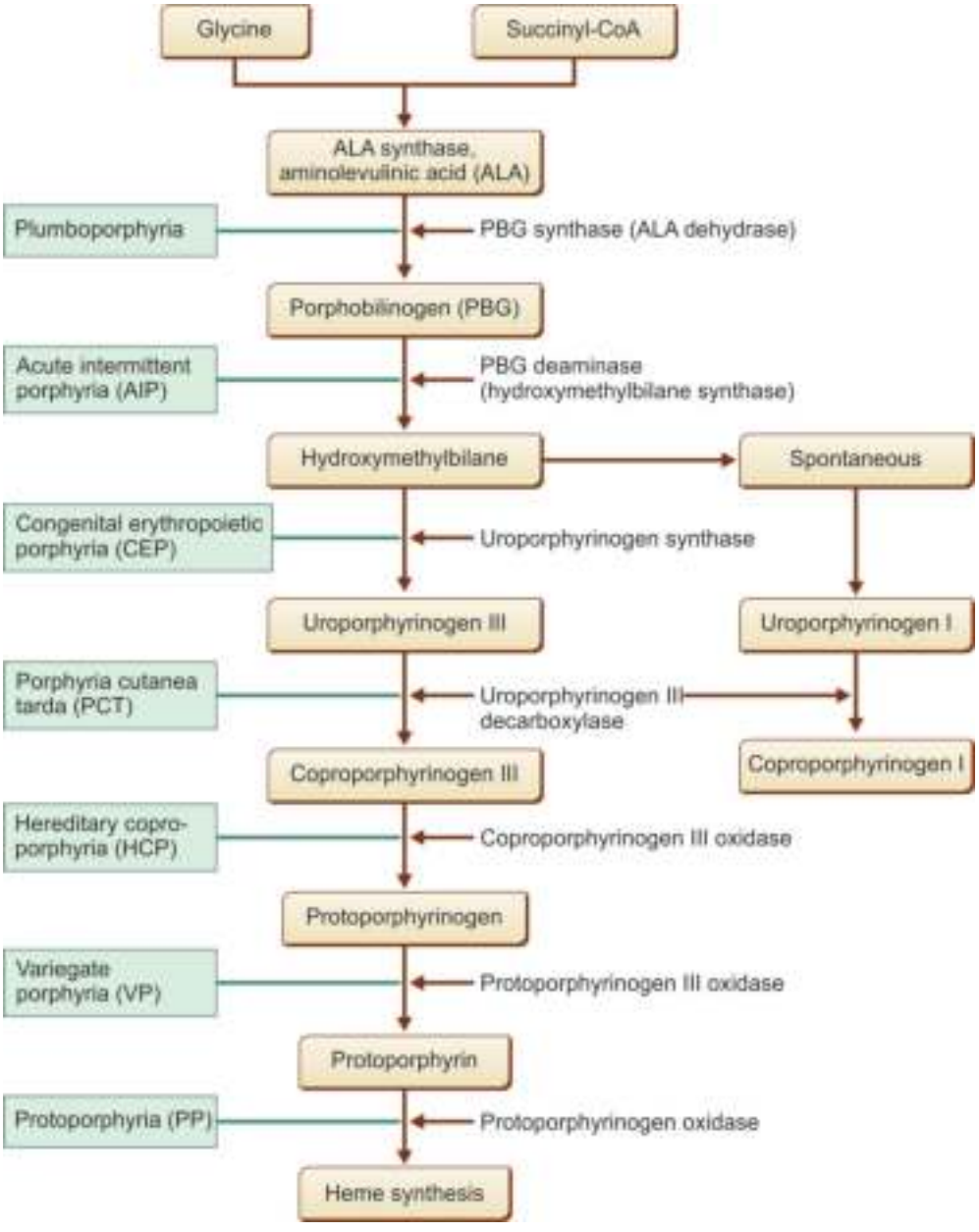


Fig. 8.10: Steps in heme synthesis.

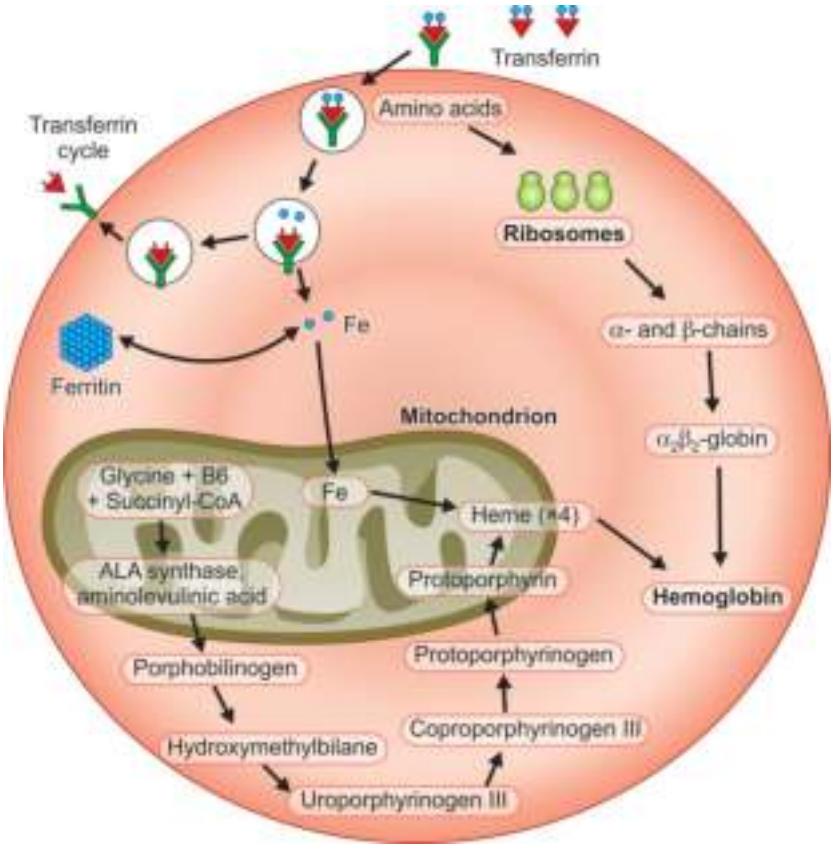


Fig. 8.11: Steps of hemoglobin synthesis

Table 8.39 Normal types of hemoglobin according to developmental stage

Developmental Stage	Site of Hematopoiesis	Type of Hemoglobin	Globin Chains	Reference Interval
Embryonic stage	2–8 weeks	▪ Gower I ▪ Gower II ▪ Portland	▪ $\zeta_2\varepsilon_2$ ▪ $\alpha_2\varepsilon_2$ ▪ $\zeta_2\gamma_2$	▪ Small quantity ▪ Small quantity ▪ Small quantity
Fetal stage	Liver, spleen, bone marrow	▪ HbA ▪ HbA_3 ▪ HbF	▪ $\alpha_2\beta_2$ ▪ $\alpha_2\delta_2$ ▪ $\alpha_2\gamma_2$	▪ Small quantity ▪ Small quantity ▪ 90–95% before birth
Newborn >1-year-old stage	Bone marrow (all bones containing red marrow), liver, spleen	▪ HbA ▪ HbA_2 ▪ HbF	▪ $\alpha_2\beta_2$ ▪ $\alpha_2\delta_2$ ▪ $\alpha_2\gamma_2$	▪ 25% ▪ 1% ▪ 75%
Adult stage	Cranial bone, vertebrae, sternum, ribs, upper end of femur and humerus	▪ HbA ▪ HbA_2 ▪ HbF	▪ $\alpha_2\beta_2$ ▪ $\alpha_2\delta_2$ ▪ $\alpha_2\gamma_2$	▪ >95% ▪ <3.5% ▪ 1%

Hemoglobin Disorders

Hemoglobin disorders are of two types—qualitative and quantitative hemoglobinopathies. These hemoglobin disorders lead to hemolytic anemias.

- Qualitative hemoglobinopathies occur due to defect in abnormal structure of hemoglobin without change in their quantity of synthesis (HbS, HbC, HbD and HbE).
- Quantitative hemoglobinopathies occur due to decreased synthesis of hemoglobin affecting quantity of chains without defect in structure of hemoglobin. Examples are α-thalassemia and β-thalassemia. Hemoglobinopathies associated with microcytosis are given in Table 8.41.

Acquired Abnormal Hemoglobins

Abnormal acquired hemoglobins include methemoglobin, sulfhemoglobin and carboxyhemoglobin. Characteristics of acquired abnormal hemoglobins are given in Table 8.42.

Table 8.40 Globin chains and their locus on chromosomes

Globin Chain	Location of Gene
α-Chain	Chromosome 16
β-Chain	Chromosome 11
γ-Chain	Chromosome 11
δ-Chain	Chromosome 11
ε-Chain	Chromosome 11
ζ-Chain	Chromosome 16

Hemoglobin α-chain and ζ-chain are under genetic control on chromosome 16. Rest hemoglobin globin chains (β-, γ-, δ-, ε-chains) are under genetic control on chromosome 11.

ANTICOAGULANTS USED IN LABORATORY

Most often blood is collected by venepuncture into collection tubes containing anticoagulant. Most commonly used anticoagulants are tripotassium and trisodium salts of ethylenediaminetetra-acetic acid (EDTA), trisodium citrate and heparin. Anticoagulants, mechanism of action and uses are given in Table 8.43.

Table 8.41 Hemoglobinopathies associated with microcytosis

Hemoglobinopathies Associated with Microcytosis
β-Thalassemia major (homozygous)
β-Thalassemia trait (heterozygous)
α-Thalassemia trait
α-Thalassemia trait and hemoglobin constant spring
HbC homozygous and heterozygous
HbD disease
HbO Arab disease
Hb Lepore homozygous and heterozygous
δβ-Thalassemia homozygous and heterozygous
γδβ-Thalassemia homozygous and heterozygous
Hereditary persistence of fetal hemoglobin (HPFH) homozygous
Hereditary persistence of fetal hemoglobin (HPFH); special types of heterozygous

ETHYLENEDIAMINETETRA-ACETIC ACID AND TRISODIUM CITRATE

Ethylenediaminetetra-acetic acid (EDTA) and trisodium citrate remove calcium, which is essential for the

Table 8.42 Characteristics of acquired abnormal hemoglobins

Abnormal Acquired Hemoglobin	Acquired Change	Abnormal Function	Laboratory Detection
Methemoglobin	Hemoglobin iron (Fe^{+++}) oxidized to ferric (Fe^{++}) state in methemoglobin	Methemoglobin cannot combine with oxygen	▪ Maximal absorption band at 630 nm ▪ Chocolate brown-colored blood
Sulfhemoglobin	Sulfur combined with hemoglobin	Sulfhemoglobin has 1/100 oxygen affinity of HbA	▪ Maximal absorption band at 620 nm ▪ Sulfhemoglobin, only hemoglobin not analyzed by cyanmethemoglobin method
Carboxyhemoglobin	Normal hemoglobin combines with carbon monoxide to form carboxyhemoglobin	Carboxyhemoglobin has higher affinity for oxygen	Absorption band at 541 nm

Table 8.43 Anticoagulants, mechanism of action and uses

Anticoagulant	Mechanism of Action	Uses
EDTA	Chelates calcium and removes thus prevents blood coagulation	Hb estimation, platelet count, RBCs count, absolute eosinophil count, estimation of HbF, Hb electrophoresis
Trisodium citrate	Binds with calcium and forms complex	▪ ESR estimation by Westergren's pipette method, blood to anticogulant ratio (4:1) ▪ Prothrombin time done to assess coagulation study, blood to anticoagulant ratio (9:1)
Heparin	Acts against thrombin	Assess osmotic fragility for spherocytosis, RBC enzymes, G6PD and pyruvate kinase
Ammonium–potassium mixture (2:3)	Ammonium causes swelling of RBCs, whereas potassium leads to shrinkage of RBCs resulting in maintaining size of RBCs	Used instead of EDTA
Sodium fluoride	Inhibits glycolysis	Best anticoagulant to estimate blood sugar

initiation of coagulation. EDTA is often used for blood counts, as it causes minimal morphologic and physical effects on the blood cells. Trisodium citrate is the preferred anticoagulant for erythrocyte sedimentation rate (ESR), packed cell volume (PVC), platelets and coagulation studies.

HEPARIN

Heparin acts by forming complex with antithrombin III in the plasma to prevent thrombin formation. Heparin causes a bluish coloration of the background when a smear is stained with Wright-Giemsa stain. Heparin is often used for red blood cell testing, osmotic fragility testing and functional or morphologic analysis of leukocytes.

Hematology Pearls: Romanowsky Stains

Routine stain for peripheral smear and bone marrow examination is a family of Romanowsky stains: May-Grunwald stain, Leishman stain, Giemsa stain, Wright's stain and Jenner's stain. Romanowsky stains also highlight Howell-Jolly bodies, basophilic stippling and Cabot's ring. Romanowsky stain cannot stain Heinz bodies. Comparison between Leishman and Giemsa stains is given in Table 8.44. Composition of buffered water (Sorenson's phosphate buffer) is given in Table 8.45.

ANEMIAS

The criteria for anemia depend upon age, gender, ethnicity, and pregnancy status. World Health Organization has defined anemia as the hemoglobin level below 13 g/dl (hematocrit <39%) in adult males and below 12 g/dl (hematocrit <36%) in adult females. In newborns, hemoglobin level averages 16.5 g/dl (anemia <12.0 g/dl). Hemoglobin level in the age group between six months to two years average 12 g/dl (anemia <10 g/dl). Hemoglobin must be analyzed with a reliable method such as cyanmethemoglobin method. Maintenance of normal level of hemoglobin requires good nutrition, normal gastrointestinal function and absence of chronic diseases. World Health Organization definition of anemia is given in Table 8.46.

Table 8.44 Comparison between Leishman and Giemsa stains

Parameters	Leishman Stain	Giemsa Stain
Stain powder	Leishman powder: 1 g	Giemsa stain powder: 1 g
Methanol	Methanol (acetone-free): 500 ml	Methanol (acetone-free): 66 ml
Glycerol	Nil	Glycerol: 66 ml

Table 8.45 Composition of buffered water (Sorenson's phosphate buffer)

Solution	Salt
Solution A	Potassium dihydrogen phosphate: 9.08 g/L
Solution B	Dibasic hydrogen phosphate: 9.47 g/L

Preparation of buffer water with 6.8 pH: Solution A 50.8 ml + Solution B 49.2 ml = 100 ml.

Table 8.46 World Health Organization definition of anemia

Group	Hemoglobin Level
Adult males	<13.0 gm/dl
Adult nonpregnant females	<12.0 gm/dl
Pregnant females	<11.0 gm/dl
Infants and children (6 months to 6 years)	<12.0 gm/dl
Children (>6 to 14 years)	<11.0 gm/dl

- Clinical manifestations of anemia depend on the rate of onset and degree of anemia. Patient presents with weakness, pallor, exertional breathlessness, dizziness and tachycardia. Diminished tissue oxygenation may provoke the compensatory mechanisms of increased heart rate and force of ventricular contraction.
- Physical examination reveals pallor of conjunctiva, face and palms. Flat spoon-shaped nails (**koilonychia**) are observed in severe iron deficiency anemia. Smooth painful tongue is seen in macrocytic anemia. **Jaundice** and **anemia** are seen in hemolytic anemia.
- Anemias are classified based on morphologic features (size and shape of red blood cells) or mechanism of reduction of hemoglobin (i.e. decreased red blood cells production or increased red blood cells destruction).
- Normal red blood cells are biconcave in shape with a little variation in size and shape. Any deviation from normal morphology is associated with reduced life span of red blood cells.
- Morphologic scheme divides anemia into three groups, based on mean corpuscular volume of red blood cells into microcytic anemia (MCV 50–82 fl), normocytic normochromic anemia (MCV >82–98 fl) and macrocytic anemia (MCV >100 fl). Classification of anemia based on mean corpuscular volume (MCV) is given in Table 8.47.

Table 8.47 Classification of anemias based on mean corpuscular volume (MCV)

Microcytic Anemias (MCV 50–82 fl)		
Disorders of iron metabolism	▪ Iron deficiency anemia ▪ Anemia of chronic disease ▪ Hereditary atransferrinemia	▪ Shahidi-Nathan-Diamond syndrome characterized by congenital microcytic hypochromic anemia with iron overload
Disorders of porphyrin and heme synthesis	▪ Acquired sideroblastic anemias ▪ Hereditary sideroblastic anemia ▪ X chromosome-linked sideroblastic anemia	▪ Idiopathic refractory sideroblastic anemia, complicating other diseases associated with drug (isoniazid) or toxins (ethanol, lead)
Disorders of globin synthesis	▪ Thalassemia (α or β) ▪ Hemoglobinopathies (HbS, HbC, HbD, HbE, HbG)	
Normocytic Normochromic Anemias (MCV >82–98 fl)		
Anemia with appropriate bone marrow response	▪ Acute post-hemorrhagic anemia ▪ Hemolytic anemia with pronounced reticulocytosis (hereditary spherocytosis, hereditary ovalocytosis, paroxysmal nocturnal hemoglobinuria, G6PD deficiency, pyruvate kinase deficiency, hemolytic disease of newborn, blood transfusion reactions, autoimmune hemolytic anemia)	
Anemia with impaired bone marrow response	▪ Bone marrow aplasia (aplastic anemia, pure red cell anemia) ▪ Bone marrow failure (metastatic bone disease, myelofibrosis, Gaucher's disease, Niemann-Pick disease) ▪ Decreased erythropoietin production due to renal disease, liver disease, endocrinal deficiency, malnutrition, anemia of chronic disease	
Macrocytic Anemias (MCV >100 fl)		
Vitamin B_{12} (cobalamin) deficiency	▪ Inadequate dietary intake ▪ Impaired absorption (pernicious anemia, ileal resection, gastric partial or total resection, celiac disease, malabsorption syndrome, intestinal lymphoma, scleroderma) ▪ Competitive consumption of vitamin B_{12} (*Diphyllobothrium latum*—fish tapeworm) ▪ Increased requirement of vitamin B_{12} (pregnancy, neoplasms, hyperthyroidism, chronic pancreatic disease) ▪ Impaired utilization of vitamin B_{12} (transcobalamin II deficiency, abnormal serum cobalamin-binding protein, enzyme deficiencies, nitrous oxide administration)	
Folate deficiency	▪ Decreased dietary intake ▪ Impaired absorption (steatorrhea, celiac disease, anticonvulsant drugs, tropical sprue, intrinsic intestinal disease) ▪ Increased requirement (pregnancy, infancy, neoplasms, hypothyroidism, hyperactive hematopoiesis)	
Unresponsive to vitamin B_{12} and folate in cases of metabolic inhibitors	▪ Purine synthesis metabolic inhibitors (6-mercaptopurine, 6-thioguanine, azathioprine) ▪ Pyrimidine synthesis metabolic inhibitors (6-azauridine) ▪ Thymidylate synthesis metabolic inhibitor (methotrexate, 5-fluorouracil) ▪ Deoxyribonucleotide synthesis metabolic inhibitor (hydroxyurea, cytarabine, severe iron deficiency anemia)	
Inborn errors of cobalamin and folate	▪ Cobalamin deficiency, e.g. Imeslund-Gräsbeck disease, congential deficiency of intrinsic factor, transcobalamin deficiency, cobalamin mutant syndromes with homocysteinemia and or methylmalonic acidemia ▪ Folate deficiency, e.g. congenital folate malabsorption, dihydrofolate reductase deficiency, N5-methyltetrahydrofolate homocysteine methyltransferase deficiency	
Inborn errors related disorders	▪ Lesch-Nyhan syndrome ▪ Hereditary otitic aciduria ▪ Deficiency of formiminotransferase ▪ Deficiency of methyltransferase	▪ Congenital dyserythropoietic anemia ▪ Refractory megaloblastic anemia ▪ Erythroleukemia

CAUSES OF ANEMIA

Anemia may be caused by two major mechanisms: decreased production and destruction of red blood cells (hemolytic anemia). Iron plays role in heme synthesis. Vitamin B_{12} and folic acid are essential for DNA synthesis during hematopoiesis. Decreased RBC production results from damage to hematopoietic stem cells or deficiency of nutrients such as iron, vitamin B_{12} and folic acid. Classification of anemia is shown in Figs 8.12 and 8.13.

DIAGNOSTIC APPROACH OF ANEMIA

Anemia is diagnosed by incorporating information obtained from the clinical history, physical examination and laboratory evaluation.

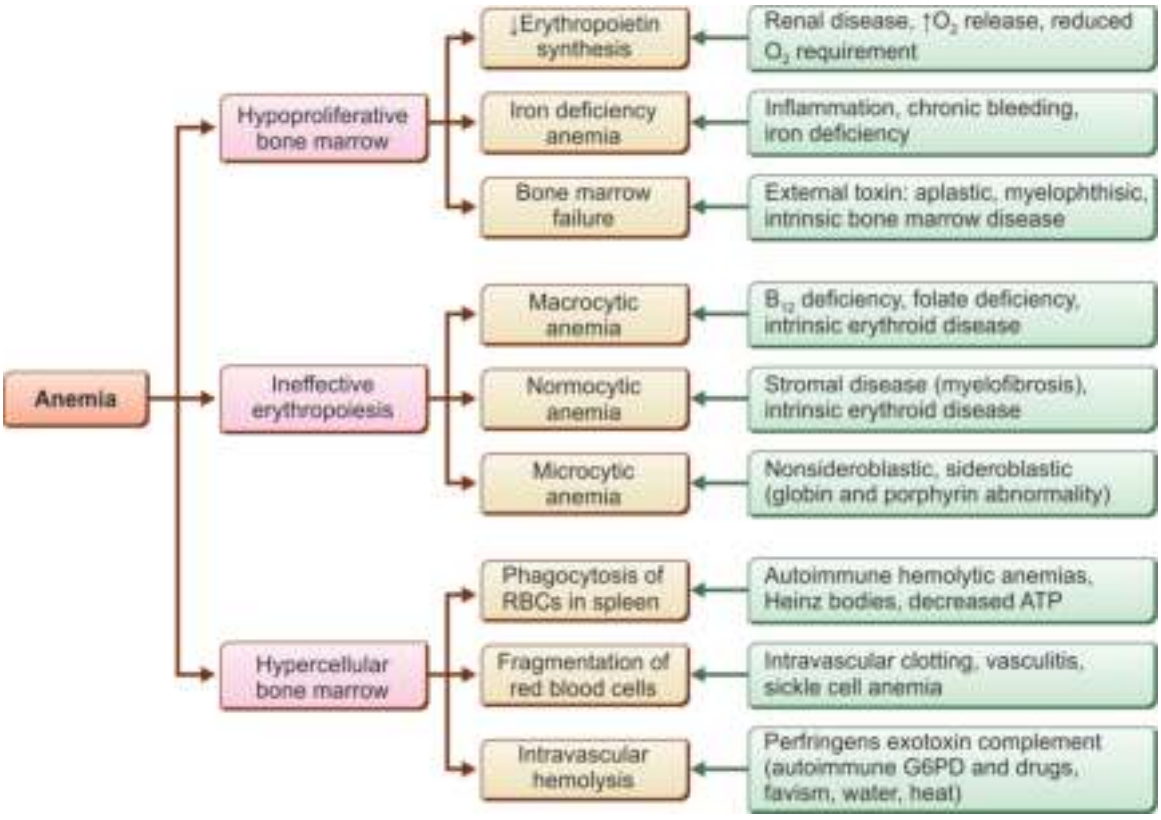


Fig. 8.12: Classification of anemia according to hypoproliferative, ineffective or hypercellular (hemolytic process) erythropoiesis.

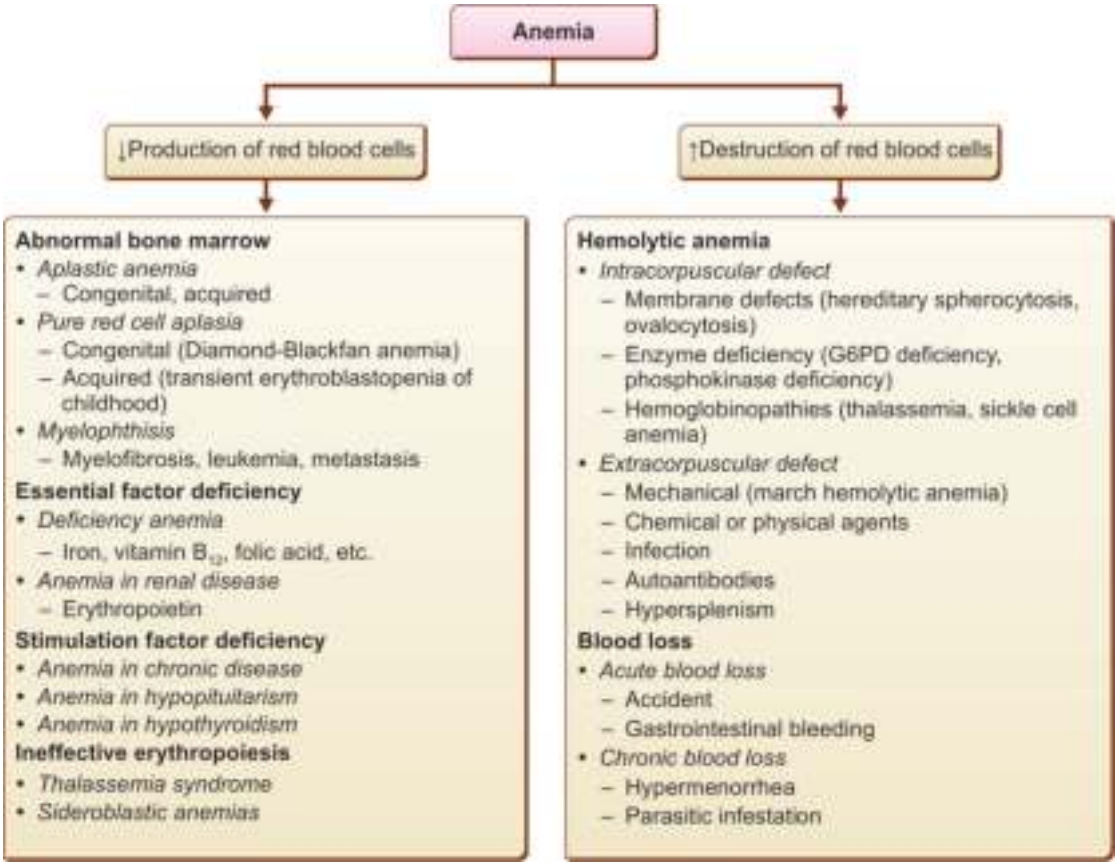


Fig. 8.13: Classification of anemia according to decreased production or increased destruction of red blood cells.

- Diseases with clinical presentations similar to anemia include hypoxia, carbon monoxide poisoning, methemoglobinemia and ischemic heart disease. Best initial diagnostic test for anemia is complete blood count especially MCH, MCHC and peripheral blood smear examination.
- Additional initial tests include reticulocyte count, haptoglobin, lactic dehydrogenase, serum bilirubin, iron stores, serum vitamin/B_{12}/folic acid, serum TSH/T4 and complete urine examination.

Clinical History

Anemia in poorly nourished infants suggests a nutritional cause. Clinical history of jaundice suggests a possible hemolytic process. Ingestion of certain drugs and exposure to chemical agents may also predispose to anemia.

- History of chronic blood loss in females and males is most important cause of anemia.
- Family history, ethnic and geographical considerations provide valuable information in diagnosis anemias.

Physical Examination

Physical examination provides important information such as pallor, bruising, shock, palpable spleen, lymphadenopathy and jaundice. Leg ulcers may occur in sickle cell anemia and thalassemia. Residual neurological abnormalities are seen in sickle cell disease and vitamin B_{12} deficiency.

Evaluation of Basic Hematologic Studies

Basic blood studies include hemoglobin, PCV, reticulocyte count and examination of peripheral blood smears. MCV, MCH and MCHC are measured by blood cell count. Main hematologic parameters are given in Table 8.48.

Biochemical Parameter Studies

Biochemical parameter studies include analysis of serum ferritin, serum iron and serum total iron binding capacity.

Peripheral Blood Smear Examination

Peripheral blood smear examination provides valuable information in studying anemias, leukemias and platelets disorders and demonstration of parasites (e.g. microfilaria, Plasmodium and trypanosomes).

- Scanning for nucleated red blood cells and abnormal white blood cells can be done under high power (40X). In a normal well-prepared peripheral blood smear, one may see 1–3 leukocytes, 10–15 platelets and 200 red blood cells in each high power field.
- Finally, peripheral blood smear should be examined under oil immersion to study the morphology of red blood cells, white blood cells and platelets. Presence of malarial parasites and spirochetes should be carried out.

Table 8.48 Main hematologic parameters

Hematologic Parameter	Calculations	Normal Range	Interpretations
Hematocrit	▪ The ratio of RBC to serum expressed in percentage ▪ It is measured by centrifuging whole blood in a calibrated capillary tube	▪ Men (39–49%) ▪ Women (33–43%)	▪ Hematocrit value is low ▪ Hematocrit value is high in polycythemia
MCV of RBCs	The average calculated volume of a single RBC (hematocrit/erythrocyte count)	82–96 femtoliters	▪ MCV is decreased in iron deficiency anemia (<80 femtoliters) ▪ MCV is increased in megaloblastic anemia (>100 femtoliters)
MCH	The average content of hemoglobin in each RBC (hemoglobin/RBC count)	26–33 pg	MCH is decreased in iron deficiency anemia and increased in megaloblastic anemia
MCHC	The average concentration of hemoglobin in a given volume of packed RBCs (hemoglobin/hematocrit)	33–37 g/dl	▪ MCHC is decreased in iron deficiency anemia and increased in hereditary spherocytosis ▪ MCHC remains normal in megaloblastic anemia
Red blood cell distribution width (RDW)	RDW is the index to measure degree of anisocytosis (various in size of red blood cells)	11.5 to 14%	▪ RDW is increased in iron deficiency anemia, megaloblastic anemia and immune hemolytic anemia ▪ RWD remains within normal range in thalassemia

Diagnostic Approach of Anemia

Red Blood Cells: Morphologic Changes

- One should look for anisocytosis, poikilocytosis of RBCs, hypochromia, polychromasia, presence of abnormal cells like target cells, burr cells, acanthocytes, fragmented red blood cells (schistocytes), sickle cells, spherocytes.
- One should also look for red blood cell inclusions like nuclear fragments (Howell-Jolly bodies), aggregated ribosomes (stippling) or malarial parasites. Abnormal RBCs morphology and interpretations are given in Table 8.49 and Fig. 8.14.

Inclusions in Red Blood Cells

- **Howell-Jolly bodies:** Howell-Jolly bodies are nuclear remnants (aggregates of chromatin material) present in the RBCs and intermediate normoblasts. These are demonstrated by examination of Romanowsky stained smear in postsplenectomy/hyposplenism, megaloblastic anemia and acute hemolytic anemia.
- **Basophilic stippling:** Basophilic stippling is also known as punctate basophilia due to precipitated ribosomal RNA. Periphery of RBCs display small blue-black dots in Romanowsky stained blood films. These are demonstrated by examination of Romanowsky stained smear in lead poisoning, β-thalassemia, megaloblastic anemia, myelodysplastic syndrome and alcoholism.
- **Siderotic nodules/Pappenheimer bodies:** Siderotic nodules are aggregates of ferritin located close to the red blood cell membrane appear as pale blue; which can be demonstrated by examination of Perls Prussian blue stained smear in sideroblastic anemia, megaloblastic anemia, hemolytic anemia and postsplenectomy.
- **Hemoglobin H inclusions:** Hemoglobin H inclusions are β-chains appearing as golf ball-shaped inclusions in red blood cells. These are demonstrated by supravital stains on peripheral blood smear in α-thalassemia and some cases of myelodysplastic syndrome.
- **Heinz bodies:** Heinz bodies are denatured globin located close to the red blood cell membrane. These are demonstrated by examination of supravital stained.
- **Cabot's ring:** Cabot's ring is circular or figure of eight probably formed as a result of damage to RBCs stromal lipoproteins. These are demonstrated in megaloblastic anemia and lead poisoning.
- **Hemoglobin C inclusions:** Hemoglobin C inclusions are tetragonal in shape and are birefringent in a polarized light seen as a result of crystallization of hemoglobin C in 10% of RBCs following splenectomy in homozygous hemoglobin C disease. Red blood cell inclusions in hematologic disorders are given in Table 8.50.

Other Abnormalities of RBCs

- **Rouleaux formation:** RBCs are aggregated resembling a stack of coins known as rouleaux as a result of increased gamma globulins in cases of multiple myeloma, kala-azar, Waldenstörm macroglobulinemia and chronic inflammatory disease.
- **Polychromasia:** Reticulocytes appear as bluish red in Romanowsky stained blood film. These appear as fine reticular network in supravital staining in cases of hemorrhage, hemolysis and response to hematinic replacement.

Table 8.49 Abnormal RBCs morphology and interpretations

Abnormal Red Blood Cells	Interpretations
Macrocytes	Megaloblastic anemia, liver disease, myelodysplasia
Microcytes	Iron deficiency anemia, thalassemia, sideroblastic anemia
Microspherocytes	Hereditary spherocytosis, immune hemolytic anemia, HbC disease, splenectomy, stored blood, burns
Ovalocytes (elliptocytes)	Hereditary elliptocytes, thalassemia, pernicious anemia, iron deficiency anemia, myelofibrosis
Sickle cells (drepanocytes)	Hb sickle cell disease (HbSS), other sickle cell disorders
Target cells	Hemoglobinopathy, liver disease, iron deficiency anemia, splenectomy
Schistocytes (fragmented red blood cells)	Microangiopathic hemolytic anemia, thalassemia, drug-induced hemolytic anemia, mechanical hemolytic anemia, disseminated intravascular coagulation
Hypochromia	Iron deficiency anemia, thalassemia, sideroblastic anemia, chronic renal failure (sometimes)
Polychromasia	Hemolytic anemia, hypoxia, megaloblastic anemia, acute blood loss
Acanthocytes (spiny projections of the surface due to defect within the lipid bilayer of the red cell membrane may be associated with hemolysis)	Chronic liver disease (increased free cholesterol is deposited within the cell membrane), abetalipoproteinemia, splenectomy
Poikilocytes (tear-shaped red cells)	Megaloblastic anemia, thalassemia, myelosclerosis, microangiopathic hemolytic anemia, iron deficiency anemia

Contd...

Table 8.49 Abnormal RBCs morphology and interpretations (*Contd...*)

Abnormal Red Blood Cells	Interpretations
Burr cells (ethinocytes)	Uremia (chronic renal disease), liver disease, MDS, pyruvate kinase deficiency, disseminated malignancy
Stomatocytes (slit- or mouth-like area)	An artifact, alcoholism, stomatocytosis
Leptocytes (RBCs with thin ring of membrane due to grossly deficient hemoglobin)	Iron deficiency anemia, thalassemia
Helmet cells	Disseminated intravascular coagulation, thrombotic thrombocytopenic purpura
Bite cells	G6PD deficiency

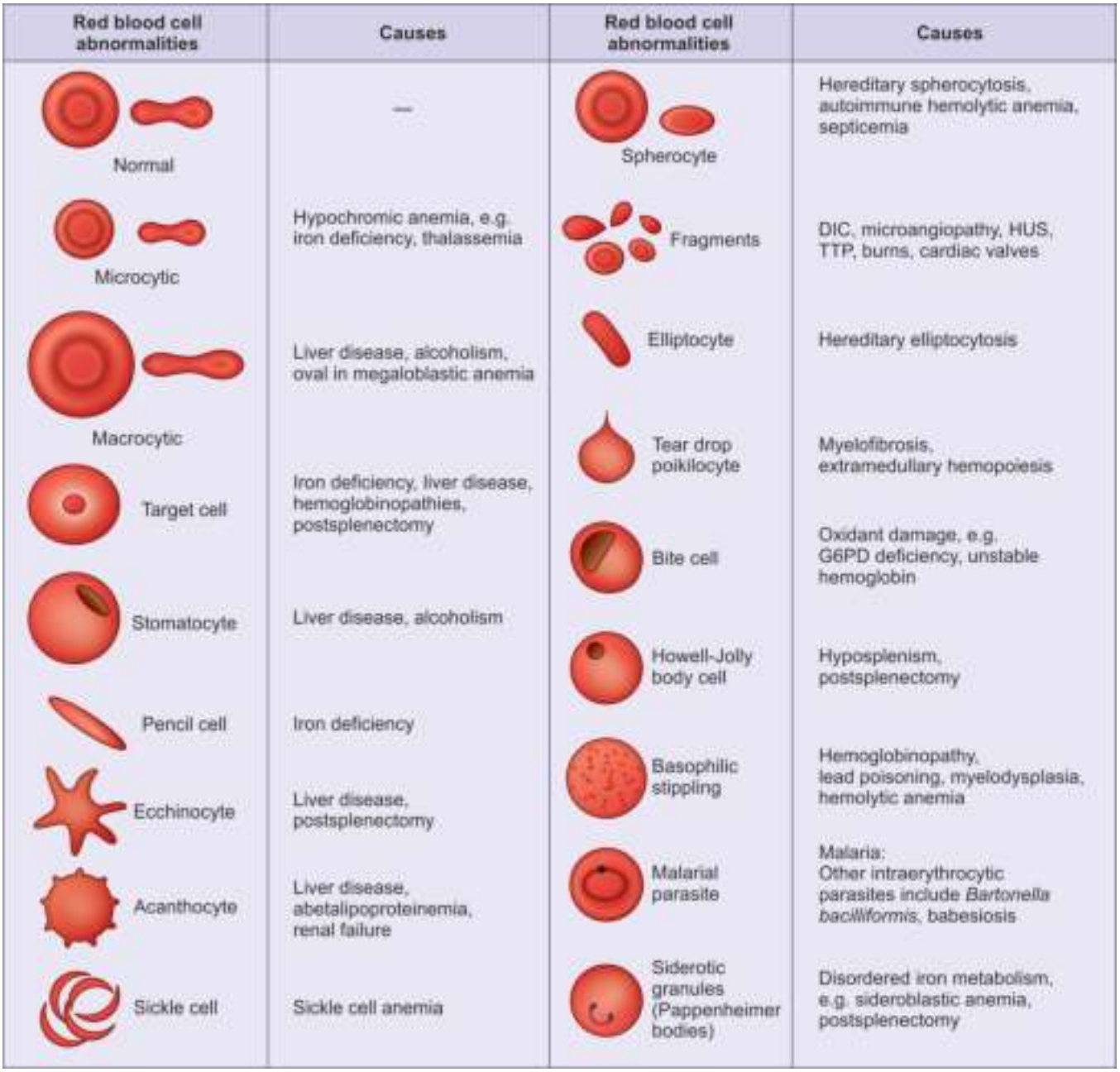


Fig. 8.14: Abnormal RBCs morphology and interpretations.

Table 8.50 Red blood cell inclusions in hematologic disorders

RBC Inclusion	Comment	Staining Technique	Hematologic Disorders	
Howell-Jolly bodies	DNA nuclear remnants	Romanowsky stains	▪ Postsplenectomy state ▪ Hypersplenism	▪ Megaloblastic anemia ▪ Acute hemolytic process
Basophilic staining	Ribosomal RNA	Romanowsky stains	▪ β-Thalassemia major and trait ▪ Megaloblastic anemia ▪ Myelodysplastic syndrome (MDS) ▪ Sideroblastic anemia	▪ Pyrimidine 5'-nucleosidase deficiency ▪ Heavy metal poisoning (coarse basophilic stippling in RBCs seen due to lead, zinc, arsenic, silver, mercury)
Pappenheimer body	Iron (siderotic) nodule	Perls Prussian stain	▪ Sideroblastic anemia ▪ Hemolytic anemia ▪ Megaloblastic anemia	▪ Postsplenectomy state ▪ Myelodysplastic syndrome
HbH inclusions	β-Globin tetramers (β4)	Supravital stain	▪ α-Thalassemia	▪ Myelodysplastic syndrome (some cases)
Heinz bodies	Denatured hemoglobin	Supravital stain	▪ G6PD deficiency ▪ Drugs and poisons with oxidative action	▪ Hypersplenism ▪ Unstable hemoglobin ▪ Postsplenectomy
Hemoglobin C crystals	Hemoglobin C homozygous state	Polarizing microscope	▪ Hemoglobin C homozygous state	▪ Postsplenectomy state
Cabot's rings (figure of 8)	Mitotic spindle remnants	Romanowsky stain	▪ Megaloblastic anemia	▪ Lead poisoning

Bone Marrow Aspirate Smear Examination

Bone marrow aspiration is usually performed on the back of hip bone or posterior iliac crest. Bone marrow aspirate smear examination gives more information and morphologic characteristics, better defined in Romanowsky stained bone marrow aspirate blood smears. Bone marrow examination is done to diagnose iron deficiency anemia, megaloblastic anemia, leukemias, ITP, multiple myeloma, Gaucher's disease, Niemann-Pick disease, metastatic deposits and parasites, e.g. LD bodies and malarial parasite. Iron content is demonstrated by Perls Prussian blue stain.

IRON METABOLISM AND IRON DEFICIENCY ANEMIA

Iron deficiency is the most common nutritional disorder across world, it is frequent cause of microcytic hypochromic anemia. Decreased dietary iron, increased demand, decreased iron absorption, or blood loss lead to iron deficiency anemia.

IRON METABOLISM

Iron participates in various chemical reactions and maintains homeostasis of iron at both the systemic and cellular levels. A well-balanced diet contains sufficient iron. Normal diet contains 10–20 mg of iron each day. Iron present in the ferrous form (Fe^{2++}) is mainly absorbed in the duodenum and upper jejunum. This is sufficient to balance the 1.0–2.0 mg daily loss from desquamation of epithelia of gastrointestinal tract and skin. Iron absorption is enhanced by citrate and ascorbate present in citrus fruits; and acidic pH, while decreased by tannates present in tea, phytates, tetracycline, milk and alkaline pH. Factors favoring and reducing iron absorption are given in Table 8.51.

REGULATION OF IRON METABOLISM

Heme iron derived from animal products such as liver and meat is more readily absorbed. Non-heme iron (inorganic iron) derived from raw vegetables and cereals is first reduced to ferrous (Fe^{2++}) form inside enterocytes of duodenum via cytochrome B. Regulation of iron metabolism is shown in Fig. 8.15.

Binding of Fe^{2++} with DMT1

Ferrous (Fe^{2++}) binds to apical transporter is called DMT1 (divalent metal transporter 1) encoded by **SLC11A2**

Table 8.51 Factors favoring and reducing iron absorption

Factors Favoring Iron Absorption	Factors Reducing Iron Absorption
Heme iron	Inorganic iron
Ferrous iron (Fe^{2+})	Ferrous iron (Fe^{3+})
Acids—HCl, vitamin C	Alkalis—antacids, pancreatic secretions
Stabilizing agents—sugars, amino acids	Iron precipitating agents—phytates, phosphates
Ineffective erythropoiesis	Tea consumption
Pregnancy	Infection
Hereditary hemochromatosis	Decreased erythropoiesis
Increased expression of DMT1 and ferroportin in duodenal enterocytes	Decreased expression of DMT1 and ferroportin in duodenal enterocytes
Decreased hepcidin concentration	Increased hepcidin concentration

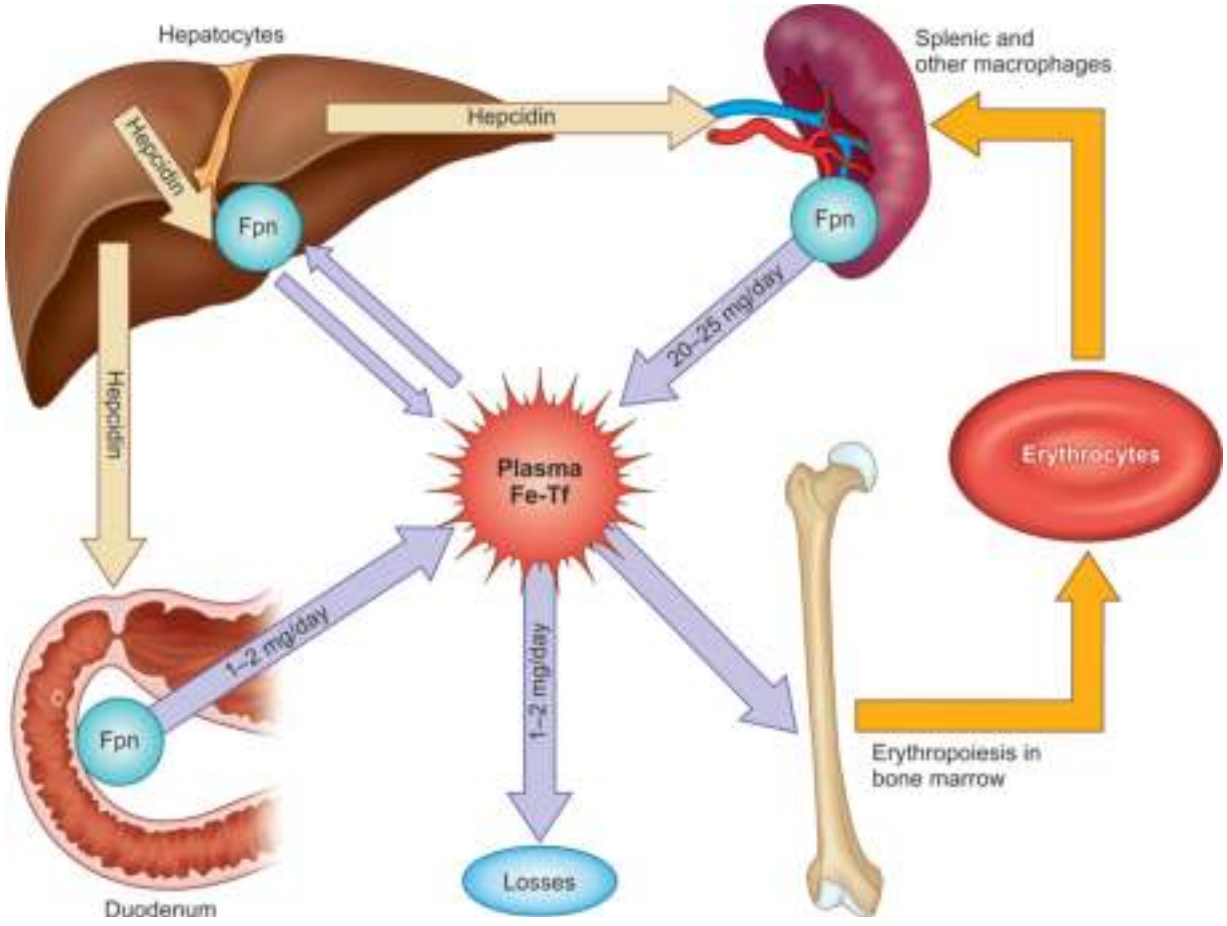


Fig. 8.15: Regulation of iron metabolism. Hepcidin (also known as LEAP1) encoded by HAMP gene is the main regulator of iron metabolism. It degrades ferroportin (protein involved in iron absorption). It blocks ferroportin carrying iron to spleen. It blocks iron release from macrophages required for heme synthesis during erythropoiesis.

gene. DMT1 participates in transfer of iron from endosomes into the cytosol of developing erythroid precursors. Mutation of SLC11A2 gene encoding DMT1 (divalent metal transporter 1) causes severe microcytic hypochromic anemia in newborns. Serum ferritin level in newborns is decreased due to defective release of iron into the blood. Patient's transferrin saturation percentage is increased.

Iron Transport Across Enterocytes

Absorbed iron is transported by transporter proteins such as ferroportin and hephaestin across basolateral membrane of enterocytes. These transporter proteins convert ferrous (Fe^{2++}) to ferric (Fe^{3+++}) form. Intracellular mucosal ferritin in the enterocytes is subsequently lost during sloughing of epithelia. Iron absorption in enterocytes in duodenum is shown in Fig. 8.16.

Hepcidin: Master Regulator of Iron Absorption

- Hepcidin is main regulator of iron absorption also known as liver expressed antimicrobial peptide or LEAP1, encoded by the HAMP gene.
- Hepcidin inactivates ferroportin 1 (iron-regulated transporter 1 protein encoded by SLC40A1 gene), prevents reabsorption of iron from duodenum.
- Decrease in hepcidin level increases expression of ferroportin 1 resulting in increased iron absorption by duodenum.
- Hepcidin also blocks the release of important source of iron from reticuloendothelial cells in bone marrow required for heme synthesis during erythropoiesis.
- Hepcidin also prevents excessive deposition of iron in liver, heart, pancreas, joints, skin and pituitary gland.
- Iron is demonstrated by Perls Prussian blue stain. Iron level >22 mg/g of liver dry weight carries increased risk of cirrhosis.

Transport of Iron in Plasma

Absorbed iron is carried in the bloodstream by the glycoprotein named transferrin. Normally, about 20–45% of transferrin binding sites are saturated with iron termed as the percent saturation. Familial atransferrinemia causes microcytic hypochromic anemia.

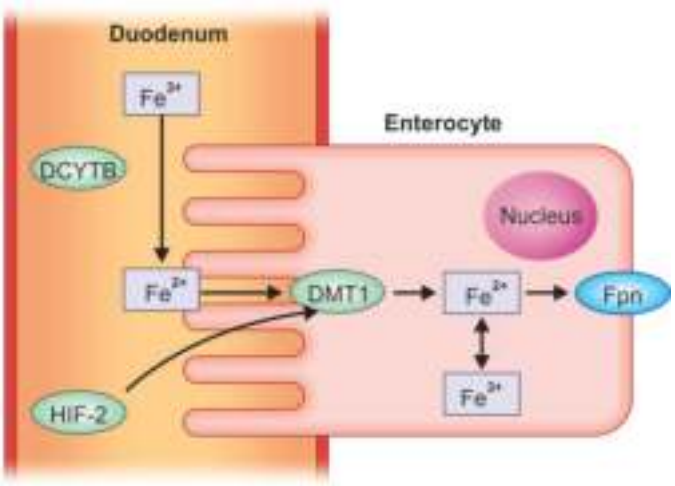


Fig. 8.16: Iron absorption in enterocytes in duodenum.

Storage of Iron

Serum iron (Fe^{3++}) is mainly stored in the liver (33%), spleen (33%), bone marrow (33%) and skeletal muscle. Iron is initially stored as ferritin. However, ferritin can be incorporated by phagolysosomes to form hemosiderin. Distribution of body iron in average adult male and female is given in Table 8.52. Differences between ferritin and hemosiderin are given in Table 8.53.

Utilization of Iron

Most absorbed iron (Fe^{3++}) is utilized in the bone marrow for erythropoiesis. About 10–20% of absorbed iron goes into a storage pool, which is also being recycled for erythropoiesis, so there is a balance of storage and use.

Table 8.52 The distribution of body iron in average adult male and female

Distribution of Iron Form	Distribution of Iron in Adult Male	Distribution of Iron in Adult Female	Percentage of Total Body Iron
Hemoglobin (functional iron)	2.4 gm	1.7 gm	65%
Heme enzymes for cellular respiration: functional iron (e.g. cytochromes, catalase, peroxidase, flavoproteins)	0.02 gm	0.015 gm	0.5%
Myoglobin (functional iron)	0.15 gm	0.12 gm	3.5%
Ferritin and hemosiderin (storage iron in bone marrow, liver and spleen)	1.0 gm (0.3–1.5 gm)	0.3 gm (0–1.0 gm)	30%
Transferrin-bound iron in plasma	0.004 gm	0.003 gm	0.1%

Table 8.53 Differences between ferritin and hemosiderin

Parameters	Ferritin	Hemosiderin
Color	Colorless (unstained smear)	Golden yellow (unstained smear)
Solubility in water	Water soluble	Water insoluble
Iron content	Contains less iron	Contains more iron
Storage sites	Bone marrow, liver and spleen	Storage: Bone marrow, liver and spleen

IRON DEFICIENCY ANEMIA

Iron deficiency anemia occurs due to dietary iron deficiency, increased iron demand during reproductive period in women, decreased absorption and chronic blood loss.

ETIOPATHOGENESIS

Dietary Iron Deficiency

Dietary iron deficiency is rare except in infants, because human milk is low in iron. Newborn iron stores are depleted within first 6 months of postnatal life unless it is replaced by dietary supplementation. Iron deficiency anemia is common in infants within the first 2 months of life, who are non-breastfed and supplemented with cow's milk, rather than iron-fortified formula. Other uncommon causes of iron deficiency anemia include poor economic status, anorexia in pregnancy, poor dentition and financial constraints.

Increased Iron Demand

During reproductive period, women require more iron due to menstrual blood loss. During pregnancy 0.5 g of extra iron is needed for growing fetus. Growth spurt during childhood requires more iron.

Decreased Iron Absorption

Diseases that could impair iron absorption include celiac disease, achlorhydria, partial or total gastrectomy. Absorption of iron is decreased by tannates present in tea, phytates, tetracycline, milk and alkaline pH.

Chronic Blood Loss

Chronic blood loss is most common cause of iron deficiency anemia in adults due to pathological disorders. Gastrointestinal tract bleeding may occur due to peptic ulcer disease, GIT carcinomas, hemorrhoids, chronic use of aspirin, hookworm disease.

- Bleeding from respiratory tract occurs due to hemoptysis and recurrent epistaxis. Renal pathology may lead to hematuria and hemoglobinuria.
- Chronic blood loss in women may occur due to dysfunctional uterine bleeding, early onset of menarche, postmenopausal bleeding and cervical carcinoma. Of course, hemorrhage will increase the iron need to replace lost RBCs. Hookworm infestation is a common cause of iron deficiency anemia.

CLINICAL FEATURES

Patient presents with pallor, fatigue, or dyspnea on exertion. Fatigue appears first than abnormal laboratory findings. Latent iron deficiency refers to deficient iron stores but hemoglobin remains within normal range. In severe iron deficiency anemia, associated features may include glossitis, gastritis, koilonychia (spooning of the nails) due to loss of essential iron containing enzymes (Fig. 8.17 and Table 8.54).

Plummer-Vinson Syndrome

Plummer-Vinson syndrome also known as Paterson-Kelly syndrome is a triad, i.e. chronic iron deficiency anemia, dysphagia due to partially obstructing postcricoid webs in esophagus and glossitis. Postcricoid esophageal web may undergo esophageal squamous cell carcinoma.

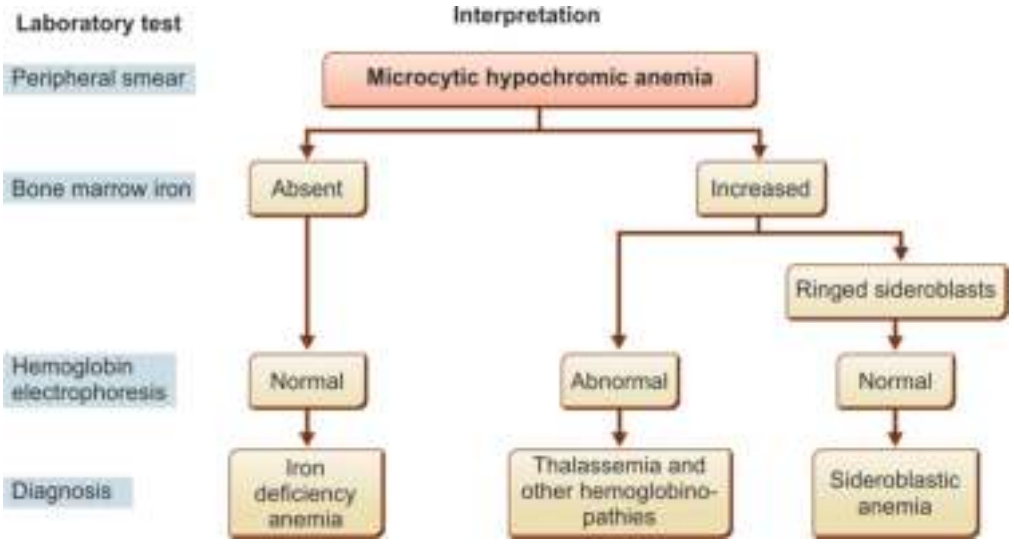


Fig. 8.17: Scheme for investigating patients with microcytic hypochromic anemia. This figure represents a convenient approach to the diagnosis of this form of anemia.

Table 8.54 Laboratory investigations of iron deficiency anemia

Laboratory Method	Reference Range	Iron Deficiency Anemia
Complete blood count and peripheral smear examination		
RBC count	▪ Male: 4.6–6 million/mm^3 ▪ Female: 4.2–5 million/mm^3	Decreased
Hemoglobin	▪ Male: 13–16 g/dl ▪ Female: 12–15 g/dl	▪ Male: <13 g/dl ▪ Female: <12 g/dl
MCV	80–98 femtoliter (fl)	<79 femtoliter (fl)
MCH	27–32 pg	<26 pg
MCHC	32–36 g/dl	<30 g/dl
RDW-CV = (standard deviation of MCV ÷ MCV) × 100	11.5–14.5%	Increased
Mentzer index in children: It is used as a rough guide to differentiate between iron deficiency anemia (>13) and thalassemia trait (<13)	<13	>13
RBC morphology	Normocytic normochromic anemia	Microcytic hypochromic cells, anisocytosis, poikilocytosis (ring cells, target cells, pencil cells, elliptical cells)
Serum ferritin	▪ Male: 12–300 ng/ml ▪ Female: 12–150 ng/ml	▪ <12 ng/ml ▪ <12 ng/ml
Serum iron	50–150 µg/dl	<30 µg/dl
Serum TIBC (total iron-binding capacity)	250–435 µg/dl	>400 µg/dl
Serum TIBC % saturation: It is calculated by serum iron 100/TIBC	20–45%	<16%
Bone marrow iron stores: Iron is graded by Galle's bone marrow aspirate smear iron grading (0–6+). Iron is demonstrated by Prussian blue reaction/Perls reaction	1–3 grade	0 grade in severe iron deficiency anemia
Bone marrow sideroblasts percentage	40–60%	<10%
RBC-free protoporphyrin level	30–50 µg/dl	>200 µg/dl (increased)
Serum soluble transferrin receptor (sTfR)	4–9 µg/L	Increased

Flow cytometric analysis of reticulocytes: Reticulocyte hemoglobin content (CHr) and percentage of hypochromic reticulocytes.

LABORATORY DIAGNOSIS

If during initial evaluation of peripheral blood smear, pathologist finds microcytic hypochromic picture, one must consider common causes of microcytic hypochromic anemia such as iron deficiency anemia, thalassemia and abnormalities of iron metabolism.

- **PCV analysis:** Extent of morphological abnormalities depend on the level of hemoglobin or PCV.
- **Laboratory diagnostic tools:** Laboratory diagnostic tools are hemoglobin estimation, complete blood count, peripheral smear examination, bone marrow aspiration, hematocrit values, packed cell volume, erythrocyte sedimentation rate, serum iron, serum iron-binding capacity, percentage saturation, serum ferritin, bone marrow biopsy and liver biopsy. Bone marrow iron is deficient in iron deficiency anemia.
- **Bone marrow iron:** Bone marrow iron is increased in β-thalassemia major and other hemoglobinopathies.

In sideroblastic anemia, bone marrow evaluation reveals sideroblasts and increased iron. Hemoglobin electrophoresis is normal in iron deficiency and sideroblastic anemia.

Laboratory Diagnosis of Iron Deficiency Anemia

Peripheral Blood Smear Examination

- RBCs show anisocytosis, poikilocytosis, microcytes, hypochromic red blood cells with central pallor area greater than half, ring cells, target cells, pencil cells, elliptical cells.
- Complete blood count gives an indirect measure of iron stores, because the mean corpuscular volume (MCV) is decreased in iron deficiency anemia.
- Microcytic hypochromic anemia is also observed in thalassemia major, thalassemia trait, thalassemia intermedia, HbE thalassemia and HbH disease. RDW is <15% in thalassemia trait and >15% in iron deficiency anemia (Fig. 8.18A to C).

Bone Marrow Smear Examination

Bone marrow smear examination is a diagnostic tool for differentiating iron deficiency from other causes of hypochromic anemia. By definition, iron deficiency anemia means that the bone marrow has no iron stores.

Iron store is most often determined by Perls Prussian blue iron stain. Iron granules appear as bright blue or blue-green aggregate contrasting sharply with pink stained background. Bone marrow smear shows following features (Fig. 8.19):

- **Cellularity:** Bone marrow smear examination shows hypercellular marrow as a result of erythroid hyperplasia.
- **Erythroid series:** Erythroid hyperplasia shows micronormoblastic erythropoiesis. Predominant cells are polychromatic (late) normoblasts with fraying of cell borders.
- **Myeloid series:** Myelopoiesis is normal.
- **Myeloid/erythroid ratio:** Myeloid/erythroid ratio is decreased due to erythroid hyperplasia.
- **Megakaryocytic series:** Megakaryopoiesis is normal.

Bone Marrow Iron

- Bone marrow store study is another reliable method for estimating iron stores, but drawback is invasive technique.
- The amount of storage iron for erythropoiesis can be quantified by performing an iron stain Perls Prussian blue stain) on a bone marrow aspiration. Potassium ferrocyanide combines with iron forming potassium ferrocyanide–ferricyanide complex giving bluish coloration.
- Iron stores are depleted in iron deficiency anemia. Excessive iron stores can be determined by bone marrow and by liver biopsies.

Biochemical Markers of Iron Deficiency Anemia

Biochemical markers of iron deficiency anemia comprise serum ferritin, serum iron, TIBC, % saturation of transferrin and serum transferrin receptors.

- **Serum iron:** Serum iron test gives indirect indication of iron stores. Normal serum iron concentration is the range of 60–150 μg/dl. Serum iron concentration is below normal in iron deficiency anemia. Serum iron concentration is increased in cases of β-thalassemia major, hemoglobinopathy, sideroblastic anemia and history of multiple blood transfusions.
- **Total iron-binding capacity:** Total iron-binding capacity (TIBC) measurement may provide useful in information about iron stores. Normal range of TIBC is 250–450 μg/dl and its saturation is 33%. Measurement of TIBC is based on saturating plasma with iron and removal of excess of unbound iron by adsorbing onto chemical agent and then estimation of iron in the iron-saturated serum.
- **Hemoglobin electrophoresis:** HbA_2 and HbF determinations are very useful for differentiating thalassemia from abnormal use of iron. HbA_2 and HbF are increased in patients with β-thalassemia. Hemoglobin electrophoresis is not useful in α-thalassemia. Therefore, demonstration of HbH in RBCs on peripheral blood smear stained by brilliant cersyl blue is useful in α-thalassemia.
- **Other hematological tests:** RBCs protoporphyrin level is increased due to nonavailability of iron for synthesis of heme (normal range 20–40 μg/dl). Osmotic fragility is increased.

Differential Diagnosis

Iron deficiency anemia must be distinguished from other causes of microcytic hypochromic anemia such as β-thalassemia major/minor and anemia of chronic disease.

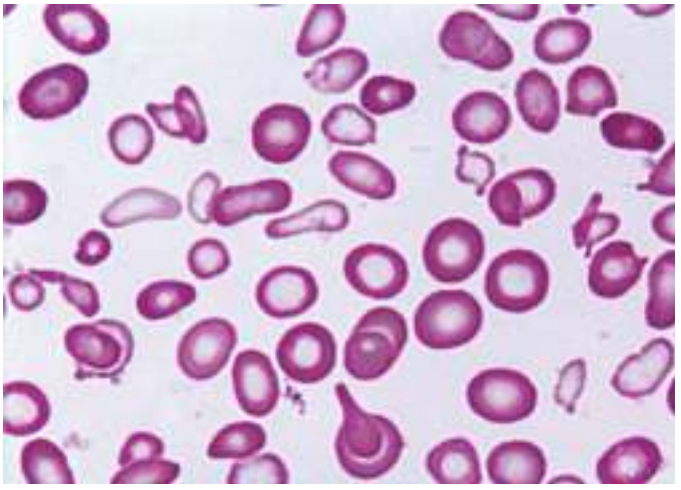


Fig. 8.18A: Peripheral blood smear examination shows microcytic hypochromic anemia. There is central pallor area of erythrocytes. Erythrocytes show anisocytosis and poikilocytosis. RBCs are elongated known as pencil cells.

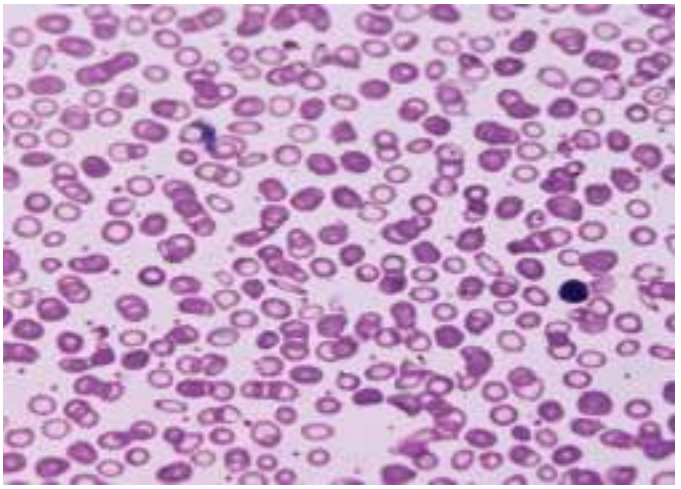


Fig. 8.18B: Peripheral blood smear examination in iron deficiency anemia. It shows marked anisocytosis, poikilocytosis of red blood cells, microcytic hypochromic picture with numerous target cells and a few pencil cells.

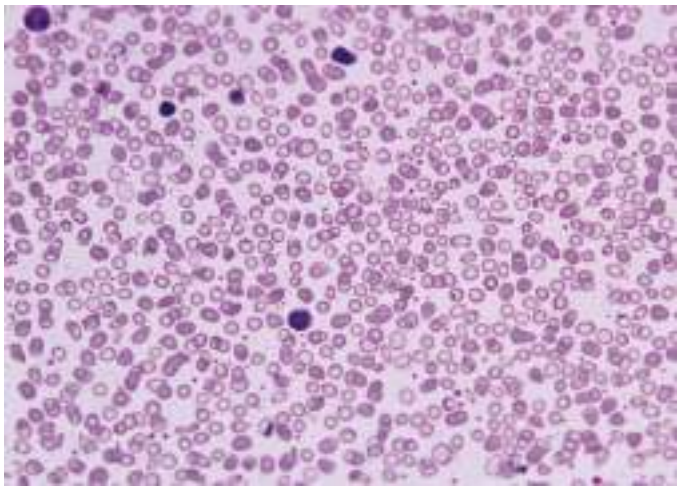


Fig. 8.18C: Peripheral blood smear examination in iron deficiency anemia. It shows microcytic hypochromic picture, target cells and nemerous target cells.

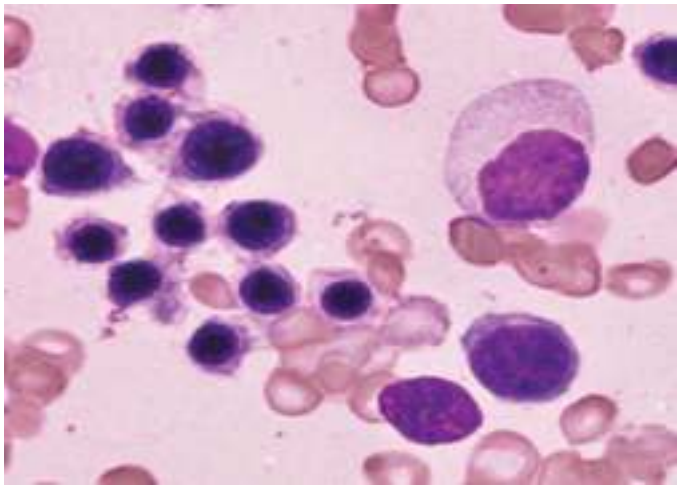


Fig. 8.19: Bone marrow aspiration shows micronormoblastic erythropoiesis in iron deficiency anemia.

- Free erythrocyte protoporphyrin level is increased in iron deficiency anemia and anemia of chronic disease; but normal in β-thalassemia major.
- In iron deficiency anemia, serum ferritin is markedly reduced (<15 µm/L). Bone marrow iron is very low or absent. In β-thalassemia major/minor, serum ferritin and bone marrow iron are increased along with increased HbF (20–90%). In β-thalassemia minor, the HbA_2 is increased.
- In anemia of chronic disease, the serum iron is low in iron deficiency anemia, but the TIBC is also low. Ratio of serum transferrin receptors (sTfR) to ferritin is important parameter to distinguish iron deficiency anemia and anemia of chronic disease. Ratio of serum transferrin receptors to ferritin >1.5 indicates iron deficiency anemia and ratio <1.5 indicates anemia of chronic inflammation.

RESPONSE TO IRON THERAPY

Oral iron preparations in the form of ferrous salts containing 100–200 mg of elemental iron is given per day for 3–6 months in severe iron deficiency anemia. Patients may be treated by administering injectable iron–dextran or iron–sorbitol citrate.

- **Subjective symptoms:** Iron supplement therapy leads to improvement in symptoms due to synthesis of iron containing enzymes and globin. Iron containing enzymes are cytochrome B, catalase, ferrochelatase countase and ribonucleotide reductase.
- **Reticulocyte count:** Response to iron therapy is evaluated by reticulocyte count on day 7 of therapy. There is increase in the reticulocyte count within 7–10 days after iron therapy.
- **Hemoglobin content:** Hemoglobin estimation is the most accurate measure of degree of iron deficiency anemia. Hemoglobin level achieves normal range within 2 months after iron therapy. Atrophic gastritis disappears. Koilonychia (spooning of the nails) due to loss of essential iron containing enzymes disappears by 3–6 months after iron therapy.

IRON OVERLOAD

Excessive iron can accumulate and induce either acute iron poisoning or chronic iron overload.

Acute Iron Poisoning

Acute iron poisoning is mainly seen in children following ingestion of 20 mg of elemental iron per kg of body weight, which occurs when free iron not bound to transferrin appears in the blood. Free iron can damage blood vessels resulting in vasodilation with increased vascular permeability, hypotension and metabolic acidosis.

- In addition, excessive iron damages mitochondria and causes lipid peroxidation, and manifest mainly as renal damage and hepatocellular damage.
- Early signs of acute iron poisoning include vomiting and diarrhea, fever, hyperglycemia, and leukocytosis.
- Later signs of acute iron poisoning include hypotension, metabolic acidosis, lethargy, seizures, and coma. Hyperbilirubinemia and elevated liver enzymes suggest hepatocellular injury, while proteinuria and appearance of tubular cells in urine suggest renal tubular injury.

Chronic Iron Overload

Chronic iron overload can occur in patients who receive multiple blood transfusions for anemias caused by anything other than blood loss. Patients with congenital anemias may require numerous blood transfusions for many years. Each unit of blood bag has 250 mg of iron.

MEGALOBLASTIC ANEMIA, FOLIC ACID AND VITAMIN B_{12} DEFICIENCY

MEGALOBLASTIC ANEMIA

The megaloblastic anemias are a group of disorders characterized by macrocytic anemia picture in peripheral blood and megaloblastic erythropoiesis in bone marrow. Megaloblastic anemia occurs due to deficiency of vitamin B_{12} or folate resulting in defective DNA synthesis. Vitamin B_{12} is not required directly for DNA synthesis; however, it is required as methylcobalamin to convert 5-methyltetrahydrofolate (methyl-THF), which enters cells from plasma into other folate coenzyme forms through its involvement in the methionine synthase reaction in which homocysteine gets methylated to methionine. Vitamin B_{12} participates in utilization of folate in synthesis of nucleic acid; and essential for maintenance of nervous system. Folate acts as coenzyme involving one carbon unit transfer resulting in the synthesis of nucleic acid. Major functions of folate and vitamin B_{12} deficiency syndrome are given in Table 8.55. Differences between vitamin B_{12} and folate nutritional aspects are given in Table 8.56.

MEGALOBLASTIC ANEMIA: CLASSIFICATION

MACROCYTIC ANEMIA WITH MEGALOBLASTIC ERYTHROPOIESIS

Megaloblastic macrocytic anemia occurs due to deficiency of vitamin B_{12} and/or folic acid, which is characterized by decreased DNA synthesis, with a consequent delay in DNA replication and nuclear division.

Table 8.55 Major functions of folate and vitamin B_{12} deficiency syndrome

Nutrient	Functions	Deficiency Syndrome
Folate	Acts as a coenzyme in transfer and utilization of 1-carbon unit, an essential step in nucleic acid synthesis	Megaloblastic anemia
Cyanocobalamin (vitamin B_{12})	▪ Participates in utilization of folate in nucleic acid synthesis ▪ Essential for maintenance of nervous system	▪ Megaloblastic anemia ▪ Subacute combined degeneration of spinal cord in midthoracic region

Table 8.56 Differences between vitamin B_{12} and folate nutritional aspects

Parameters	Vitamin B_{12} (Cyanocobalamin)	Folate
Normal daily dietary intake	7–30 µg	200–250 µg
Minimal adult daily requirement	1–2 µg	100–150 µg
Main dietary foods	Animal (dairy) products	Liver, green raw leafy vegetables, yeast, fresh fruits, whole grain cereals
Cooking effect	Little effect	Easily destroyed
Absorption site	Ileum	Duodenum and jejunum
Mechanism of absorption	Vitamin B_{12} combines with intrinsic factor synthesized by gastric parietal cells	Folate converted to methyltetrahydrofolate
Limit of absorption	2–3 µg	50–80% of dietary content
Enterohepatic circulation	5–10 µg	80 µg
Transport in plasma	Most bound to haptocorrin (transcobalamin 1) essential for cell uptake	Folate weakly bound to albumin
Major intracellular physiologic forms	▪ Methylcarbylamine ▪ Deoxyadenosylcobalamin	Reduced polyglutamate derivatives
Storage sites	Liver (main site), kidney, heart and brain	Liver acts as a coenzyme in transfer and utilization of 1-carbon unit, an essential step in synthesis of nucleic acid
Functions	Participates in utilization of folate in synthesis of nucleic acid; and essential for maintenance of nervous system	Folate acts as a coenzyme involving one carbon unit transfer resulting in the synthesis of nucleic acid
Usual therapeutic form	Hydroxocobalamin	Folic acid (pteroylglutamic acid)
Serum assays	Serum vitamin B_{12} (160–925 ng/L)	Serum folate (3–15 µg/L)

- Cytoplasmic maturation is relatively unimpeded. Bone marrow examination shows megaloblastic erythropoiesis and manifests as nuclear-cytoplasmic asynchrony of large erythroid precursor cells with an open, loose sieve-like appearing chromatin pattern.
- Peripheral blood smear examination shows oval macrocytes, leukopenia, hypersegmented neutrophils, and thrombocytopenia.

MACROCYTIC ANEMIA WITH NORMOBLASTIC ERYTHROPOIESIS

Normoblastic macrocytic anemia occurs due to liver disease, alcoholism, hypothyroidism, postsplenectomy, pregnancy, hemolytic anemia, posthemorrhagic anemia, aplastic anemia, hypothyroidism, cytotoxic therapy, chronic myeloproliferative neoplasm (erythroleukemia), myelodysplastic syndrome and chronic obstructive pulmonary disease. Causes of macrocytic anemia (MCV >100 fl) with megaloblastic erythropoiesis are given in Table 8.57. Causes of macrocytosis other than megaloblastic anemia are given in Table 8.58. Vitamin B_{12} and folate nutritional aspects are given in Table 8.59. Comparison between vitamin B_{12} and folate deficiency inducing megaloblastic anemia is given in Table 8.60. Causes of macrocytic anemia due to congenital disorders are given in Table 8.61.

Table 8.57 Causes of macrocytic anemia (MCV >100 fl) with megaloblastic erythropoiesis

Vitamin B_{12} (Cobalamin) Deficiency	
Decreased dietary intake of vitamin B_{12}	Vegetarians
Impaired absorption of vitamin B_{12}	▪ Gastric causes (pernicious anemia, partial or total gastrectomy, Zollinger-Ellison syndrome) ▪ Intestinal causes (ileal resection, celiac disease, malabsorption syndrome, intestinal lymphoma, competitive consumption of vitamin B_{12} by *Diphyllobothrium latum*—fish tapeworm) ▪ Pancreatic insufficiency
Increased requirement of vitamin B_{12}	Pregnancy, neoplasms, hyperthyroidism, chronic pancreatic disease
Impaired utilization of vitamin B_{12}	Impaired utilization of vitamin B_{12} (transcobalamin II deficiency, abnormal serum cobalamin-binding protein, enzyme deficiencies, nitrous oxide administration)
Folate Deficiency	
Decreased dietary intake of folate	Malnutrition, premature infants, old persons, alcoholic persons, goat's milk anemia, hemodialysis
Impaired absorption of folate	Steatorrhea, celiac disease, anticonvulsant drugs, tropical sprue, intrinsic intestinal disease, Whipple disease, scleroderma
Increased requirement of folate	Pregnancy, infancy, neoplasms, hypothyroidism, hyperactive hematopoiesis, exfoliative dermatitis
Increased utilization of folate	▪ Physiologic state, e.g. pregnancy, lactation, premature infants ▪ Pathologic state: (a) chronic hemolytic anemia, e.g. sickle cell disease, β-thalassemia major and myelofibrosis; (b) malignant disorders, e.g. leukemias, lymphomas, multiple myeloma and carcinoma; (c) inflammatory disorders, e.g. tuberculosis, Crohn's disease, psoriasis, malaria and exfoliative dermatitis
Antifolate drugs	Anticonvulsant drugs, e.g. phenytoin, barbiturates and pyrimidine; methotrexate potent inhibitor of dihydrofolate, sulfasalazine, trimethoprim, omeprazole and oral contraceptives
Inborn errors	▪ Lesch-Nyhan syndrome ▪ Hereditary otitic aciduria ▪ Deficiency of formiminotransferase ▪ Deficiency of methyltransferase
Unresponsive to Vitamin B_{12} and Folate in Cases of Metabolic Inhibitors	
Purine synthesis metabolic inhibitors	6-Mercaptopurine, 6-thioguanine, azathioprine
Pyrimidine synthesis metabolic inhibitors	6-Azauridine
Thymidylate synthesis metabolic inhibitors	Methotrexate, 5-fluorouracil
Deoxyribonucleotide synthesis metabolic inhibitors	Hydroxyurea, cytarabine, severe iron deficiency anemia

Contd...

Table 8.57 Causes of macrocytic anemia (MCV >100 fl) with megaloblastic erythropoiesis (*Contd...*)

Inborn errors of cobalamin and folate	▪ Cobalamin deficiency, e.g. Imeslund-Gräsbeck disease, congenital deficiency of intrinsic factor, transcobalamin deficiency, cobalamin mutant syndromes with homocysteinemia and/or methylmalonic acidemia ▪ Folate deficiency, e.g. congenital folate malabsorption, dihydrofolate reductase deficiency, N5-methyltetrahydrofolate homocysteine methyltransferase deficiency
Other inborn errors	▪ Hereditary otitic aciduria ▪ Lesch-Nyhan syndrome ▪ Deficiency of formiminotransferase ▪ Deficiency of methyltransferase
Unexplained mechanism	▪ Congenital dyserythropoietic anemia ▪ Refractory megaloblastic anemia ▪ Erythroleukemia

Table 8.58 Causes of macrocytosis other than megaloblastic anemia

Macrocytosis in Other Disorders
Liver disease
Alcohol
Pregnancy
Aplastic anemia
Chronic myeloproliferative neoplasm (e.g. erythroleukemia)
Myelodysplastic syndrome (MDS)
Cytotoxic drugs
Hemolytic anemia
Reticulocytosis
Multiple myeloma
Paraproteinemias due to other disorders
Chronic obstructive pulmonary disease
Neonates

Table 8.59 Major functions of folate and vitamin B_{12} deficiency syndrome

Vitamin B_{12} (Cyanocobalamin)
Vitamin B_{12} participates in utilization of folate in synthesis of nucleic acid and maintenance of nervous system. Its deficiency causes megaloblastic anemia and subacute combined demyelination of posterolateral columns of spinal cord in midthoracic region.
Metformin blocks vitamin B_{12}. Hence, reticulocyte count is low in vitamin B_{12} deficiency.
Vitamin B_{12} deficiency raises LDH and indirect bilirubin by destroying red blood cells early, as they come out of the bone marrow. This phenomenon is called 'ineffective erythropoiesis. That is why the reticulocyte count is low.
After vitamin B_{12}, reticulocyte count improves first and neurological abnormalities improve last.
Folate (Pteroylglutamic Acid)
Folate acts as a coenzyme in transfer and utilization of 1-carbon unit, an essential step in the synthesis of nucleic acid.
Folic acid deficiency causes megaloblastic anemia without central nervous system involvement.

Table 8.60 Comparison between vitamin B_{12} and folate deficiency inducing megaloblastic anemia

Parameters	Vitamin B_{12} Deficiency	Folate Deficiency
Etiology	▪ Decreased dietary intake in vegetarians, impaired absorption, increased requirement and impaired utilization of vitamin B_{12} ▪ Failure to synthesize intrinsic factor by gastric parietal cells results in pernicious anemia	▪ Decreased dietary intake, impaired absorption, increased requirement and impaired utilization of folate ▪ Synthesis of intrinsic factor by gastric parietal cells is normal
Pathologic findings	▪ Demyelination of the posterior and lateral columns of spinal cord in the midthoracic region ▪ Peripheral blood smear examination shows pancytopenia, macrocytes, hypersegmented neutrophils ▪ Bone marrow shows megaloblastic erythropoiesis ▪ Schilling test abnormal in pernicious anemia	▪ Spinal cord demyelination absent ▪ Peripheral blood smear examination shows pancytopenia, macrocytes, hypersegmented neutrophils ▪ Bone marrow shows megaloblastic erythropoiesis ▪ Schilling test normal
Clinical features	Neurologic manifestations present, e.g. ataxia, impaired proprioception and vibrating sensations; anemia and glossitis	Neurologic manifestations absent; but anemia and glossitis present
Serum assays	▪ Serum vitamin B_{12} assay decreased ▪ Anti-intrinsic factor autoantibodies demonstrated in pernicious anemia	▪ Red blood folate decreased ▪ Anti-intrinsic factor autoantibodies absent
Treatment	Vitamin B_{12} supplement and intrinsic factor administration in pernicious anemia caused by autoimmune gastritis	Folate supplement

Table 8.61 Causes of macrocytic anemia due to congenital disorders

Congenital Disorders	Bone Marrow Findings	Comments
Diamond-Blackfan syndrome	Isolated profound decrease in erythroid elements, defective erythroid cells maturation and increased hematogone (multiparameter analysis of bone marrow precursor cells)	Autosomal dominant disorder (40%) associated with short stature, abnormalities of head, upper limbs and mild to moderate macrocytic anemia
Congenital dyserythropoietic anemia type 1 (CDA I), HEMPAS (hereditary erythroblastic multinuclearity with positive acidified serum)	Megaloblastic erythropoiesis, 1–3% of erythroid cells show intranuclear chromatin bridges or nuclear budding	▪ Autosomal recessive disorder ▪ CDN1 gene mutation (15q15.1–q15.3) ▪ Mild to moderate macrocytic anemia
Congenital dyserythropoietic anemia type 2 (CDA II)	Normoblastic to megaloblastic erythropoiesis, 10–40% erythroid precursor cells show binucleation or multinucleation, and karyorrhexis	▪ Autosomal recessive disorder ▪ CDN2 gene mutation (20q11.2) ▪ Mild to moderate macrocytic anemia
Congenital dyserythropoietic anemia type 3 (CDA III)	Megaloblastic erythropoiesis, 10–40% erythroid precursor cells show multinucleation, including giant erythroblasts (up to 12 nuclei), and karyorrhexis	▪ Autosomal dominant disorder ▪ CDN3 gene mutation (15q21–25) ▪ Mild to moderate anemia

MEGALOBLASTIC ANEMIA DUE TO FOLIC ACID (PTEROYLGLUTAMIC ACID) DEFICIENCY

Megaloblastic anemia due to folate deficiency is similar to vitamin B_{12} deficiency. But no neurological abnormalities and gastric atrophy occur due to folate deficiency in contrast to vitamin B_{12} deficiency.

- Folate deficiency results in megaloblastic anemia in approximately 20 weeks.
- Deficiency of vitamin B_{12} and folic acid occurs due to less intake, intestinal malabsorption and alcoholism, increased demand during pregnancy and hemolytic anemia; and chemotherapeutic agents containing folic acid antagonists.
- Causes of megaloblastic anemia due to folic acid deficiency are given in Table 8.62.

FOLIC ACID METABOLISM

Folates are derived from leafy vegetables, fresh fruits, whole grain cereals, and dairy products. Heating and boiling of foodstuffs destroy 90–95% of folic acid.

- Goat's milk is lower in folate than cow's milk. Daily requirement is 50–100 µg.
- Daily intake is 500–800 µg. During cooking, 70–100% of folates are lost. Folate is present in diet in the form of polyglutamate. It is broken down to monoglutamate. It is absorbed in duodenum and jejunum and stored in the liver (capacity of 5–10 mg).

FOLIC ACID FUNCTIONS

Under physiologic state, folate acts as a coenzyme in transfer and utilization of 1-carbon unit, an essential step in nucleic acid synthesis. Folate is taken into cells in the form of N5-methyltetrahydrofolate (N5-methyl-THF). Reduction to tetrahydrofolate derivative is essential for folate to participate in metabolic reactions. Tetrahydrofolate must be demethylated before conjugation can occur. Demethylation is mediated by cobalamin.

PATHOGENESIS

If cobalamin supply is inadequate, demethylation does not occur. If N5-methyl-THF is not conjugated, it will leak out of the cell again. Normally, deoxyuridine monophosphate (dUMP) is entirely converted to deoxythymidine monophosphate (dTMP). Deoxyuridine gets converted to deoxyuridylate and then thymidylate. Thymine is necessary for DNA synthesis. Due to folate deficiency, dUMP is not converted to dTMP. As a consequence, dUTP concentration rises and begins to replace dTTP in DNA synthesis, which leads to impairment of DNA synthesis.

CLINICAL FEATURES

Folic acid deficiency does not cause neurological changes in contrast to vitamin B_{12} deficiency, which causes megaloblastic anemia and neural tube defects in fetus *in utero*. Folic acid is used for treatment of chronic hemolytic anemia.

LABORATORY DIAGNOSIS

Peripheral blood smear examination shows oval macrocytes, leukopenia, hypersegmented neutrophils, and decreased platelets. Normal Schilling test (vitamin B_{12} absorption) indicates folic acid deficiency. Serum folate assay may be variable and should be carried out with a RBC folate assay. Bone marrow examination reveals megaloblastic erythropoiesis.

Table 8.62 Causes of megaloblastic anemia due to folic acid deficiency

Mechanism	Etiology
Decreased intake	Malnutrition, elderly persons
Increased demand	Pregnancy, infancy, cancers, hemolytic anemia (hyperplasia of bone marrow)
Impaired absorption	Malabsorption syndrome, oral contraceptives, diphenylhydantoin (antiepileptic drug), alcoholism and cirrhosis
Impaired utilization	Folic acid antagonists such as anticancer drug (methotrexate, 6-mercaptopurine, 5-fluorouracil, cytosine arabinoside and vinca alkaloids)
Increased loss	Steatorrhea or tropical sprue, hemodialysis

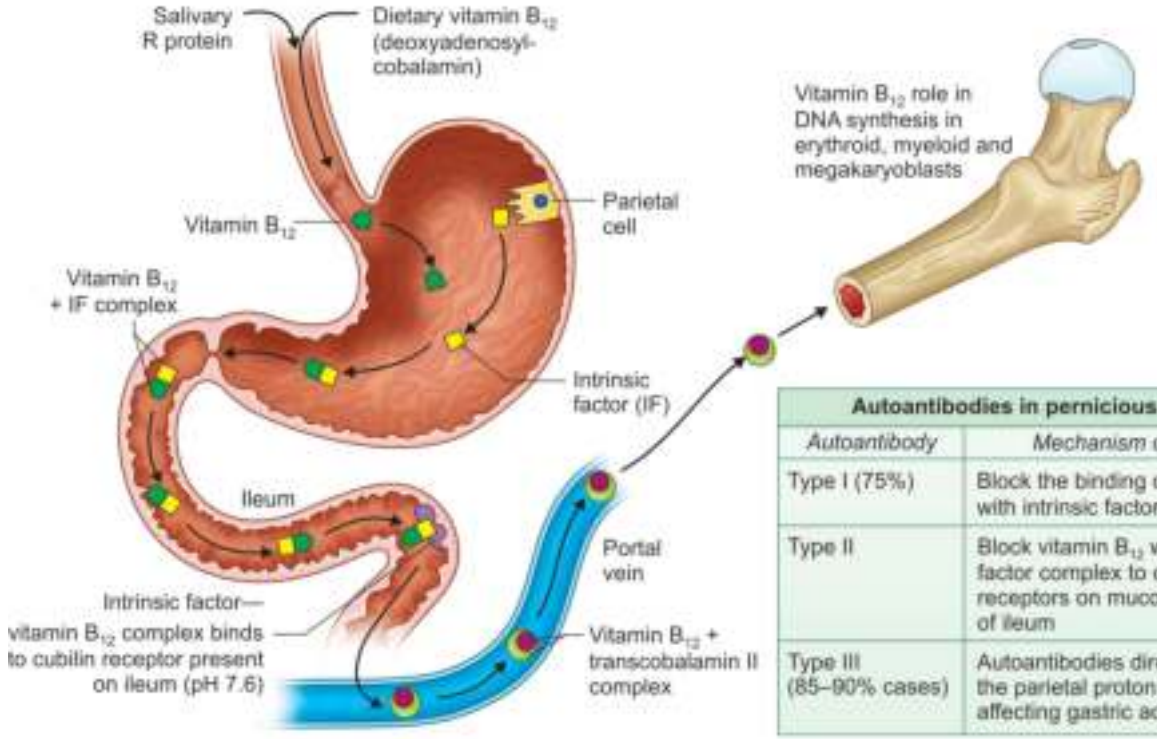
VITAMIN B_{12} DEFICIENCY

Vitamin B_{12} deficiency is reflected by impaired DNA synthesis in developing precursors in the bone marrow and defective fatty acid degradation leading to excessive demyelination. Vitamin B_{12} therapeutic administration gives excellent response. Vitamin B_{12} occurs in three forms: dehydroxyadenosylcobalamin forms in diet, methylcobalamin forms in plasma and hydroxocobalamin used for therapeutic purpose (Fig. 8.20).

VITAMIN B_{12} METABOLISM

Dietary dehydroxyadenosylcobalamin is mostly derived from animal products. Peptic digestion releases cobalamins from the dietary vitamin B_{12}.

- **Binding of vitamin B_{12} to salivary R protein:** Deoxyadenosylcobalamin binds with salivary R protein is known as haptocorrin temporarily.
- **Release of vitamin B_{12} by pancreatic enzymes:** Cobalamin is liberated in duodenum by pancreatic enzymes.
- **Binding of vitamin B_{12} with intrinsic factor (IF):** Cobalamin combines with intrinsic factor synthesized by parietal cells of stomach, which becomes resistant to proteolysis.
- **Absorption of vitamin B_{12}:** Intrinsic factor (IF) synthesized by parietal cells of stomach is required for absorption of vitamin B_{12}. IF vitamin B_{12} complex binds to IF receptors (cubilin) on mucosal epithelium of ileum. Vitamin B_{12} is absorbed in ileum, which is facilitated by calcium at pH >6.
- **Transport of vitamin B_{12}:** Vitamin B_{12} is present in the form of methylcobalamin in plasma. It is transported by transcobalamin II (β-globulin) synthesized by liver. Congenital deficiency of transcobalamin II results in severe megaloblastic anemia.
- **Storage sites of vitamin B_{12}:** Vitamin B_{12} is mainly stored in liver. Other storage sites are kidneys, heart and brain.



Autoantibodies in pernicious anemia

Autoantibody	*Mechanism of action*
Type I (75%)	Block the binding of vitamin B_{12} with intrinsic factor
Type II	Block vitamin B_{12} with intrinsic factor complex to cubilin (IF) receptors on mucosal surface of ileum
Type III (85–90% cases)	Autoantibodies directed against the parietal proton pump proteins affecting gastric acid synthesis

Fig. 8.20: Vitamin B_{12} absorption. It also shows autoantibodies demonstrated in pernicious anemia.

CAUSES OF VITAMIN B_{12} DEFICIENCY

Deficiency of vitamin B_{12} most often occurs in strict vegetarian and chronic alcoholism and impaired absorption. Most common cause of vitamin B_{12} deficiency is impaired absorption. Causes of megaloblastic anemia due to vitamin B_{12} deficiency are given in Table 8.63.

- **Increased demand of vitamin B_{12} deficiency:** Vitamin B_{12} deficiency may also occur due to increased demand during pregnancy or disseminated malignant tumor; and decreased absorption due to gastrectomy or malabsorption syndromes affecting terminal ileum.
- **Gastric causes of vitamin B_{12} deficiency:** In achlorhydria, vitamin B_{12} is not released from salivary R binding proteins.
  - Gastrectomy causes loss of synthesis of intrinsic factor by parietal cells in the gastric fundus, the clinical picture is the same as in pernicious anemia.
  - Pernicious anemia is the most common form of vitamin B_{12} deficiency megaloblastic anemia. It is immune-mediated disorder associated with formation of autoantibodies against intrinsic factor essential for vitamin B_{12} absorption.
- **Intestinal causes of vitamin B_{12} deficiency:** Vitamin B_{12} is not absorbed in patient, who has undergone resection of ileum. Bacterial overgrowth in a surgically induced intestinal blind loop results in the depletion of vitamin B_{12}.
  - Broad-spectrum antibiotic therapy can result in intestinal bacterial overgrowth with vitamin B_{12} depletion.
  - The giant fish tapeworm (*Diphyllobothrium latum*) infestation in man is acquired by ingestion of freshwater fish. The parasite inhabits the intestine and causes vitamin B_{12} depletion.

ROLE OF VITAMIN B_{12} AND FOLIC ACID IN DNA SYNTHESIS

Vitamin B_{12} and folic acid are essential for maturation of nuclei of developing precursors in bone marrow. Deficiency of vitamin B_{12}/folic acid causes megaloblastic erythropoiesis due to decreased DNA synthesis. The nuclear maturation of developing precursors lags behind than maturation of cytoplasm. All the developing precursors in the bone marrow show abnormalities.

- Peripheral blood smear examination shows macrocytosis, hypersegmented neutrophil and thrombocytopenia.
- Patient with vitamin B_{12} deficiency develops subacute combined demyelination of posterolateral columns of spinal cord in midthoracic region. Role of vitamin B_{12} and folic acid in DNA synthesis is shown in Fig. 8.21.

Role of Vitamin B_{12} in DNA Synthesis

Vitamin B_{12} (methylcobalamin) is indirectly required for DNA synthesis, which converts homocysteine to methionine and forms tetrahydrofolate, which is then converted to polyglutamate used for DNA synthesis. Vitamin B_{12} is also required for conversion of methylmalonyl-CoA to succinyl-CoA. Due to deficiency of vitamin B_{12}, these chemical reactions do not take place.

Role of Folic Acid in DNA Synthesis

5,10-methylene TH_4 is required for conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). Thus, deficiency of folic acid hinders synthesis of DNA.

- **Physiologic state:** Histidine is metabolized to form formiminoglutamic acid (FIGLU), which combines with tetrahydrofolate.

Table 8.63 Causes of megaloblastic anemia due to vitamin B_{12} deficiency

Mechanism	Etiology
Decreased intake	Dietary deficiency, strict vegetarians
Increased demand	During pregnancy, disseminated malignancies
Impaired absorption	▪ Achlorhydria (vitamin B_{12} not released from salivary R binding proteins) ▪ Pernicious anemia (autoantibodies against intrinsic factor essential for vitamin B_{12} absorption) ▪ Partial gastrectomy (loss of parietal cells synthesizing intrinsic factor) ▪ *Diphyllobothrium latum* parasite (inhibiting vitamin B_{12} absorption) ▪ Blind loop syndrome (depletion of vitamin B_{12} due to overgrowth of bacteria) ▪ Surgical resection of ileum ▪ Broad-spectrum antibiotic therapy (depletion of vitamin B_{12} due to bacterial overgrowth)

Neurologic symptoms develop in vitamin B_{12} deficiency, secondary to degeneration of the posterior and lateral columns of the spinal cord.

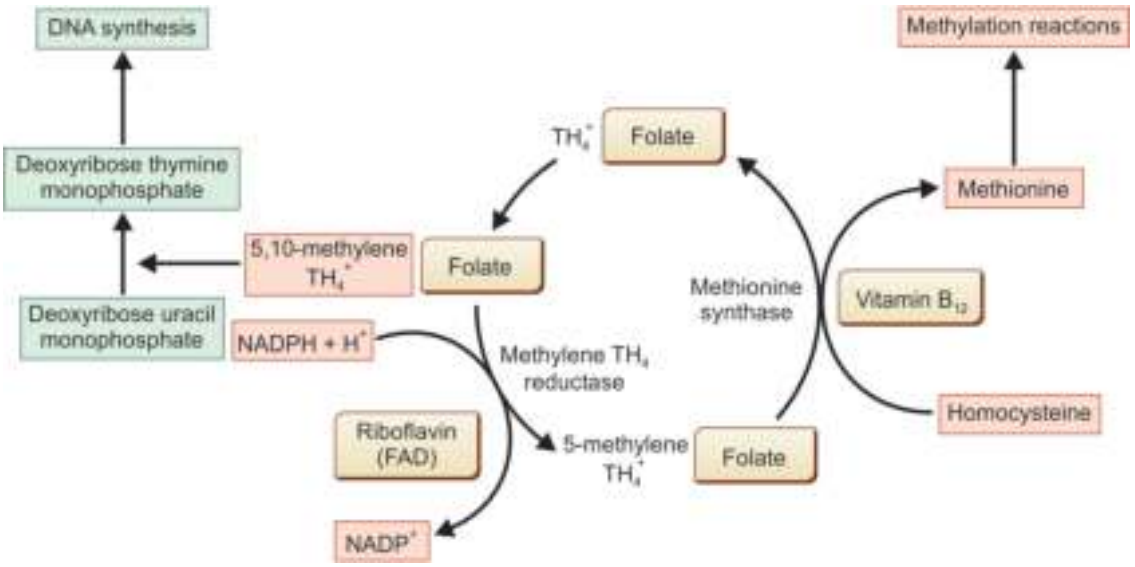


Fig. 8.21: Role of vitamin B_{12} and folic acid in DNA synthesis. 5,10-methylenetetrahydrofolate (TH_4) is required for the formation of methionine from homocysteine. Methionine, in the form of S-adenosylmethionine is required for many biological reactions including DNA methylation. Methylene TH_4 reductase is a flavin-dependent enzyme required to catalyze the reduction of 5,10-methylene TH_4 to 5-methylene TH_4.

- **Pathologic state:** Deficiency of folic acid leads to accumulation of formiminoglutamic acid and excreted in urine. FIGLU test is performed to diagnose folic acid deficiency.

PATHOPHYSIOLOGY OF MEGALOBLASTIC ANEMIA DUE TO VITAMIN B_{12} DEFICIENCY

Megaloblastic anemia occurs due to decreased DNA synthesis, ineffective erythropoiesis and mild hemolysis as a result of intramedullary death of bone marrow erythroid precursors. Patient may have mild jaundice due to increased serum bilirubin.

Clinical Features

Patient presents with pallor, glossitis, central nervous system manifestations and peripheral neuropathy. Clinical manifestations of peripheral neuropathy include spastic paraparesis, sensory ataxia and marked paresthesia leading to total paralysis of trunk and lower limbs. CNS results in ataxic gait, hyperreflexia with extensor plantar reflexes, and impaired position and vibration sensation. There may also be associated with optic atrophy and axonal peripheral neuropathy. Mechanism of neuropathy is described as under.

Peripheral Neuropathy in Vitamin B_{12} Deficiency

- **Physiologic state:** Methylcobalamin converts homocysteine to methionine, which acts as a donor in the synthesis of choline containing phospholipids, an important component of myelin. Normally deoxyadenosylcobalamin converts methylmalonyl-CoA into succinyl-CoA.
- **Pathologic state:** Deficiency of methylcobalamin results in decreased synthesis of choline required for myelin sheath synthesis. Vitamin B_{12} deficiency leads to accumulation of methylmalonyl-CoA, which is converted into methylmalonate and propionate. It leads to increased production of abnormal fatty acids like methylmalonic acid and propionic fatty acids. These fatty acids incorporate into neuronal lipids resulting in breakdown of myelin sheath.

Subacute Combined Demyelination of Spinal Cord in Midthoracic Region due to Vitamin B_{12} Deficiency

Subacute combined demyelination of spinal cord occurs in midthoracic and cervical regions. Spinal cord shows pale areas of demyelination in the posterior columns and the lateral corticospinal tracts. On histopathologic examination, spinal cord shows swollen myelin sheath with disintegration and axonal degeneration.

Laboratory Diagnosis of Megaloblastic Anemia

Main laboratory tests performed in diagnosing megaloblastic anemia include serum vitamin B_{12}, serum and RBCs folate level, autoantibodies to intrinsic factor, lactate dehydrogenase (LDH), homocysteine and methylmalonic acid in urine. Patient with macrocytic anemia should be investigated by examination of peripheral blood smear, bone marrow, reticulocyte count and therapeutic response (Fig. 8.22). Laboratory investigations needed in patient with macrocytic anemia are given in Table 8.64.

Peripheral Blood Smear Examination

Peripheral blood smear examination reveals macrocytic anemia, leukopenia and mild to moderate thrombocytopenia. Hypersegmented neutrophils may be demonstrated (Fig. 8.23A to D).

- **Red blood cells:** Large number of oval macrocytes with MCV >100 femtoliters are present. RBCs are normochromic and show polychromasia, anisocytosis and poikilocytosis. Macrocytes are oval in megaloblastic anemia, while round macrocytes in normoblastic macrocytic anemia. Presence of tear drop cells, Howell-Jolly bodies, basophilic stippling and Cabot's rings indicate dyserythropoiesis.
- **White blood cells:** Total leukocyte count is decreased. Hypersegmented neutrophils constitute >5% of all neutrophils, which should have >5 lobes and even >6 lobes. This finding indicates shift-to-the-right in megaloblastic anemia.
- **Platelets:** Moderate thrombocytopenia with giant platelets is seen.

Reticulocyte Count

- Reticulocyte count is decreased as a result of dyserythropoiesis in megaloblastic anemia.
- Post-therapeutic response should be assessed by demonstration of increased reticulocyte count on the sixth day.
- Reticulocytes are round with polychromasia in peripheral blood smear.

Bone Marrow Smear Examination

Vitamin B_{12} and folic acid are essential for maturation of nuclei developing precursors in bone marrow. Deficiency of either of the two results in megaloblastic erythropoiesis due to decreased DNA synthesis. Abnormalities will be observed in all the developing precusors in the bone marrow. Bone marrow smears show changes at all stages of RBCs development. Maturation of the nuclei of erythroid, myeloid and megakaryocytes lags behind and thus chromatin of these cells remains open sieve-like. Megaloblastic erythropoiesis in vitamin B_{12} deficiency is shown in Fig. 8.24.

- **Cellularity and M:E ratio:** Bone marrow becomes hypercellular with reversal of myeloid to erythroid ratio (M:E ratio 1:1) due to erythroid hyperplasia. Normal myeloid to erythroid ratio is 4:1. Ineffective erythropoiesis leads to intramedullary hemolysis of erythroid precursors. Bone marrow iron store is increased due to dyserythropoiesis.
- **Erythropoiesis:** Bone marrow shows dyserythropoiesis. Erythroid precursors show hyperplasia. Erythroid precursors are extremely large called megaloblastic cells. Early megaloblasts do not mature to late normoblasts. Intermediate and late erythroid precursors (megaloblasts) undergo ineffective apoptosis in the bone marrow. It is known as dyserythropoiesis.
  - Promegaloblasts and basophilic megaloblasts constitute 50% of population indicate maturation arrest. Polychromatic megaloblasts are well marked, while orthochromatic megaloblasts are less common.
  - Megaloblasts show nuclear budding, irregular dumbbell-shaped nuclei, irregular mitosis and Howell-Jolly bodies.
  - Megaloblasts are extremely large erythroid precursors. Chromatin remains open and arranged in a fine reticular fashion to give stippled appearance.
  - Although some of the megaloblasts are well hemoglobinized, the nucleus is still present suggesting nuclear/cytoplasmic developmental asynchrony. Oval macrocytes may also be demonstrated.
- **Leukopoiesis:** Presence of giant band form of neutrophil and metamyelocytes in bone marrow give a clue of megaloblastic anemia. Many myeloid precursor cells die within bone marrow and rest develop into giant stab forms and hypersegmented neutrophils.
- **Megakaryopoiesis:** Megakaryocytes demonstrate nuclear hypersegmentation with open sieve-like nuclear chromatin. Maturation of the cytoplasm of these cells is normal. The size of these cells remains large. Most of megakaryocytes die within bone marrow.

Other Laboratory Tests

- **Serum bilirubin:** Serum bilirubin is increased due to short life span of macrocytes. Imbalanced nuclear and cytoplasmic maturation of erythroid precursors result in hemolysis.
- **Serum vitamin B_{12} assay:** Normal serum vitamin B_{12} is 160–925 ng/L. Serum vitamin B_{12} is decreased to <100 ng/L in vitamin B_{12} deficiency.
- **Serum folate assay:** Serum folate level is decreased in folic acid deficiency. It is determined by isotope dilution and microbial chemiluminescence methods.
- **Striking reticulocyte response to vitamin B_{12} therapy:** Reticulocyte count is increased in megaloblastic anemia due to vitamin B_{12} deficiency, when the patient is given vitamin B_{12}.
- **Striking neurological improvement to vitamin B_{12} therapy:** Patient shows neurological improvement in response to vitamin B_{12} therapy.
- **Urinary analysis:** Methylmalonic acid level is raised in vitamin B_{12} deficiency.
- **FIGLU excretion in urine:** FIGLU test is performed to diagnose folic acid deficiency. Normally, histidine is metabolized to form FIGLU, which combines with tetrahydrofolate. Deficiency of folic acid leads to accumulation of FIGLU and excreted in urine.

TREATMENT

Shotgun therapy may be used where the etiology is unclear. Folate, then vitamin B_{12} must be attempted to make the patient well. Intramuscular hydroxycobalamin is administered daily for a week. Regime will depend on the cause of megaloblastic anemia.

Vitamin B_{12} and Folic Acid Deficiency: Therapeutic Response

Therapeutic response to vitamin B_{12} and folic acid in patients with megaloblastic anemia is dramatic described as under. Patient starts recovering from sore throat and glossitis in a day or two after therapy. Tongue becomes normal within 2 weeks.

- **Hemoglobin:** Hemoglobin starts increasing within one week after the therapy.

- **Peripheral blood smear:** Macrocytic anemia picture returns to normocytic morphology within 4–8 weeks after therapy. Platelet count returns to normal within a week.
- **Reticulocyte count:** Decreased platelet count in megaloblastic anemia returns to normal within 2–3 weeks after therapy.
- **Bone marrow:** Megaloblastic erythropoiesis returns to normal within 24 hours of therapy.

PERNICIOUS ANEMIA

Pernicious anemia, an autoimmune disorder causes megaloblastic anemia. Immunoreactive T cells cause autoimmune gastritis. Autoantibodies are formed against intrinsic factor that is essential for vitamin B_{12} absorption. Demonstration of autoantibodies against intrinsic factor is highly specific for pernicious anemia. Atrophic glossitis is common finding in pernicious anemia characterized by shiny red and glazed tongue.

EPIDEMIOLOGY

Pernicious anemia is more prevalent in European persons and rare in India and South Asia. Females are more affected than males.

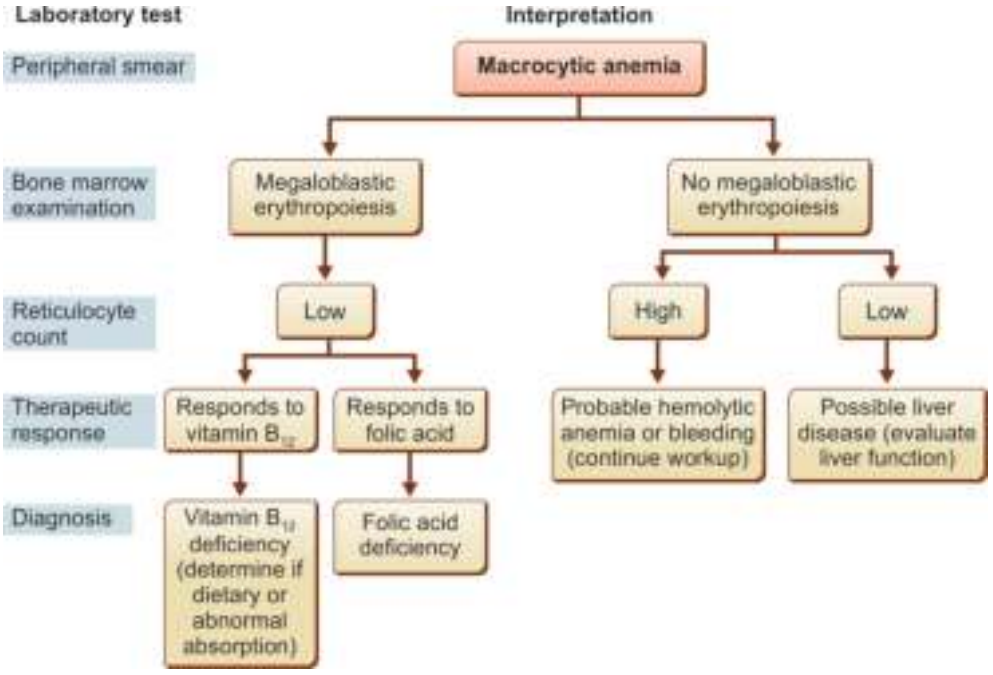


Fig. 8.22: Scheme for investigating patients with macrocytic anemia. It represents a convenient approach to this form of anemia by peripheral blood smear and bone marrow examination, reticulocyte count, therapeutic response to administration of vitamin B_{12} or folic acid.

Table 8.64 Laboratory investigations needed in patient with macrocytic anemia

Laboratory Investigations in Macrocytic Anemia
Liver function tests
Thyroid function tests
Endoscopy for gastric biopsy for vitamin B_{12} deficiency; or duodenal biopsy for folate deficiency
Peripheral smear examination
Reticulocyte count
Bone marrow examination shows megaloblastic erythropoiesis suggestive of vitamin B_{12} or folate deficiency or alternative diagnoses, e.g. myelodysplastic syndrome, aplastic anemia and myeloma
Serum protein electrophoresis
For vitamin B_{12} deficiency such as serum parietal cell antibodies and intrinsic factor antibodies, radioactive vitamin B_{12} absorption with or without intrinsic factor (Schilling test), possibly serum gastrin concentration
For folate deficiency such as FIGLU test, serum and red blood cell folate assay antigliadin antibody, anti-endomysial antibody and anti-reticulum antibody

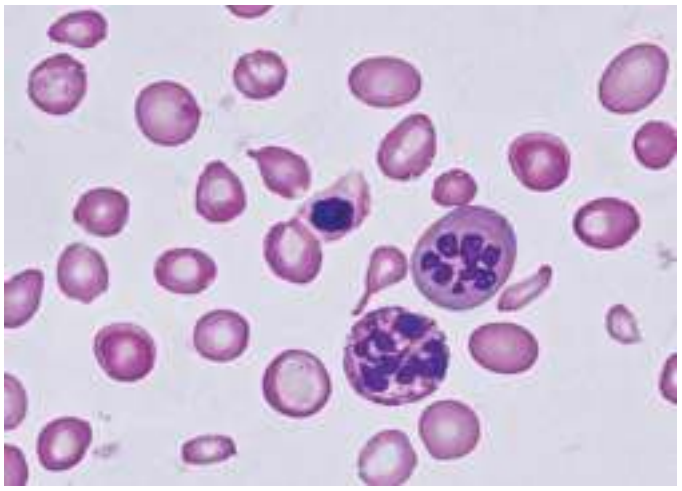


Fig. 8.23A: Peripheral blood smear examination in megaloblastic anemia. It shows anisocytosis, poikilocytosis with macrocytosis and a tear drop cell. Hypersegmented neutrophils with >5 lobes are also present.

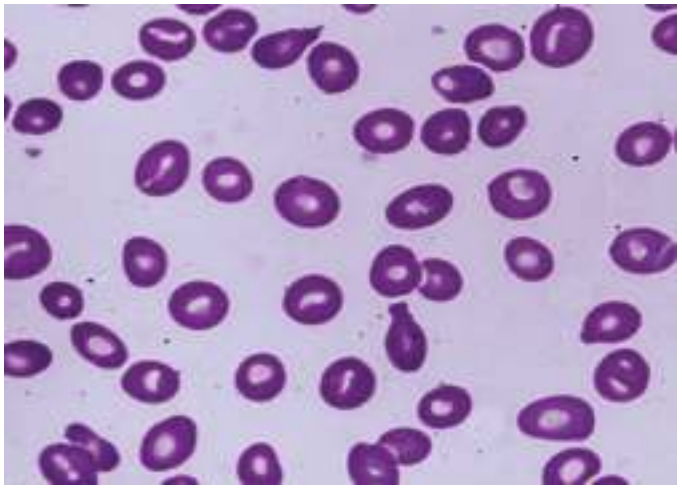


Fig. 8.23B: Peripheral blood smear examination in megaloblastic anemia It shows anisocytosis with numerous macrocytosis and scattered tear drop cells.

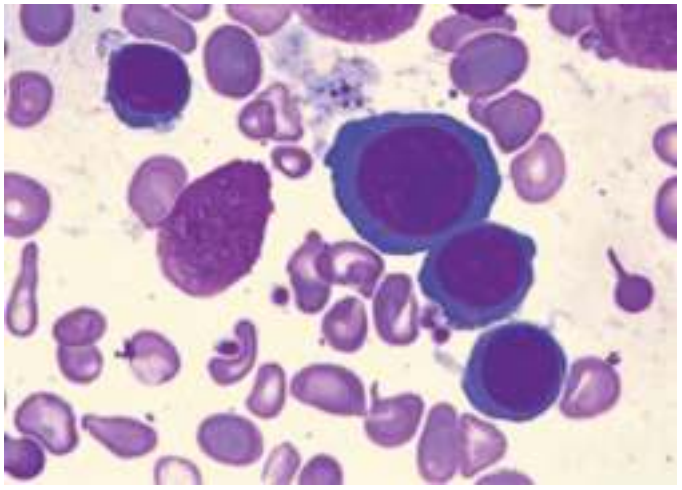


Fig. 8.24: Megaloblastic erythropoiesis in vitamin B_{12} deficiency. There is preponderance of early and intermediate normoblasts showing stippled nuclear chromatin (open sieve-like) on bone marrow smear examination.

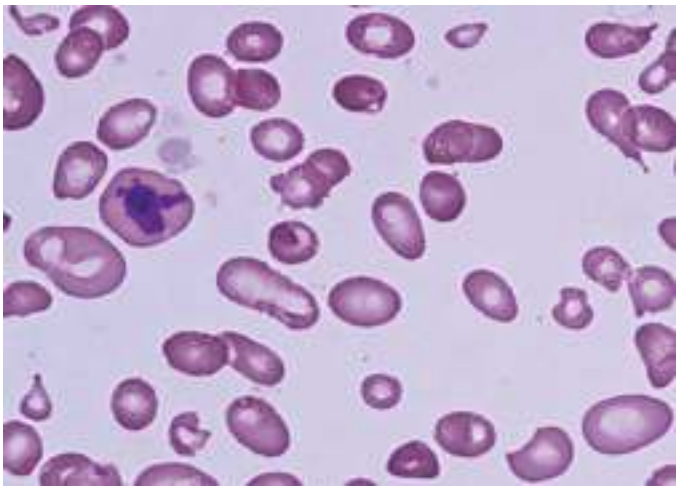


Fig. 8.23C: Peripheral blood smear examination in megaloblastic anemia It shows anisocytosis with numerous macrocytosis and scattered tear drop cells. Howell-Jolly body in red blood cell is seen.

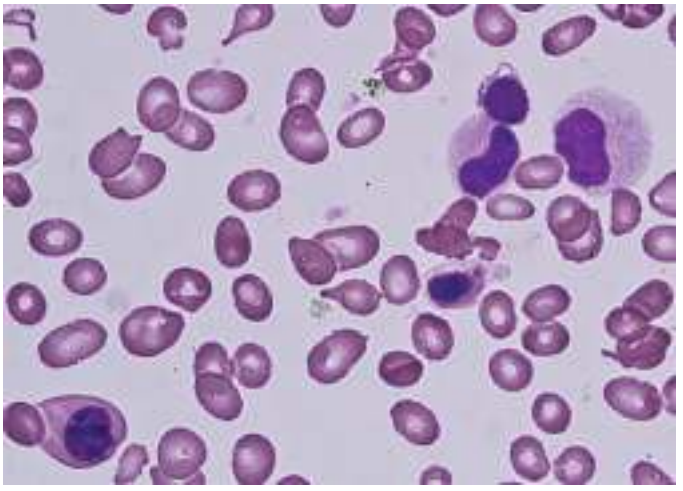


Fig. 8.23D: Peripheral blood smear examination in megaloblastic anemia. It shows anisocytosis with numerous macrocytes and scattered tear drop cells. Giant band form of neutrophil is seen.

PATHOGENESIS

Pernicious anemia is considered autoimmune disorder, which may be associated with Hashimoto's thyroiditis and type 1 diabetes mellitus and Addison's disease. Three types of autoantibodies in pernicious anemia are given in Table 8.65.

CLINICAL FEATURES

Patient presents with anemia, paresthesia, glossitis, recurrent diarrhea, anorexia, weight loss, abdominal pain, mental disturbance and visual disturbance.

- **Atrophic glossitis:** Tongue becomes shiny red and glazed in pernicious anemia.
- **Chronic atrophic gastritis:** Sections from gastric biopsy of fundus region show features of chronic atrophic gastritis due to antiparietal autoantibodies.

Table 8.65 Autoantibodies in pernicious anemia

Autoantibody	%	Mechanism of Action
Type 1 blocking autoantibodies	75–80	Block binding of vitamin B_{12} with intrinsic factor
Type 2 autoantibodies	50	Block vitamin B_{12} with intrinsic factor complex to cubilin receptors on mucosal surface of ileum
Type 3 autoantibodies	85–90	Autoantibodies directed against the parietal proton pump proteins affecting gastric acid synthesis

Replacement of parietal cells by mucin-secreting goblet cells is known as gastric intestinalization.

- **Central nervous system:** Central nervous system is most commonly involved in >75% cases of pernicious anemia. Due to incorporation of methylmalonic acid and propionic fatty acids into neuronal lipids, there is breakdown of myelin sheath of posterior and lateral white columns of mid-thoracic spinal cord involved. Patient develops spastic paraparesis, sensory ataxia and marked paresthesia leading to total paralysis of trunk and lower limbs.

Laboratory Diagnosis of Pernicious Anemia

Peripheral Blood Smear Examination

Peripheral blood smear examination shows oval macrocytes, leukopenia, hypersegmented neutrophils and moderate thrombocytopenia.

- **Red blood cells:** Large number of oval macrocytes with MCV >100 femtoliters are present. RBCs are normochromic picture with polychromasia, anisocytosis and poikilocytosis. Macrocytes are oval in megaloblastic anemia, while round macrocytes in normoblastic macrocytic anemia. Presence of tear drop cells, Howell-Jolly bodies, basophilic stippling and Cabot's rings indicate dyserythropoiesis.
- **White blood cells:** Total leukocyte count is decreased. Hypersegmented neutrophils constitute >5% of all neutrophils, which should have >5 lobes and even has >6 lobes. This finding indicates shift-to-the-right in megaloblastic anemia.
- **Platelets:** Moderate thrombocytopenia with giant platelets is seen.

Reticulocyte Count

Reticulocyte count is decreased as a result of dyserythropoiesis in megaloblastic anemia.

Bone Marrow Smear Examination

- Vitamin B_{12} and folic acid are essential for maturation of nuclei developing precursors in bone marrow. Deficiency of either of the two results in megaloblastic erythropoiesis due to decreased DNA synthesis.
- Pernicious anemia is one of the causes of vitamin B_{12} deficiency of megaloblastic anemia. Autoantibodies are formed against intrinsic factor synthesized by gastric parietal cells, which is essential for vitamin B_{12} absorption.
- Abnormalities will be observed in all the developing cells in the bone marrow. Bone marrow smears show changes at all stages of RBCs development. Maturation of the nuclei of erythroid, myeloid and megakaryocytes lags behind and thus chromatin of these cells remains open sieve-like.
  - **Cellularity and M:E Ratio:** Bone marrow becomes hypercellular with reversal of myeloid to erythroid ratio (M:E ratio 1:1) due to erythroid hyperplasia. Normal myeloid to erythroid ratio is 4:1. Ineffective erythropoiesis leads to intramedullary hemolysis of erythroid precursors. Bone marrow iron store increased due to dyserythropoiesis.
  - **Erythropoiesis:** Bone marrow shows megaloblastic erythropoiesis.
  - **Leukopoiesis:** Presence of giant band form of neutrophil and metamyelocytes in bone marrow give a clue of megaloblastic anemia. Many myeloid precursor cells die within marrow and rest develop into giant stab forms and hypersegmented neutrophils.
  - **Megakaryopoiesis:** Megakaryocytes demonstrate nuclear hypersegmentation with open sieve-like nuclear chromatin. Maturation of the cytoplasm of these cells is normal. The size of these cells remains large. Most of these cells die within bone marrow.

Serum Assays

Serum B_{12} levels are low. Due to diminished thymidine and methionine synthesis, serum concentration of homocysteine and methylmalonic acid is increased.

Therapeutic Response

Diagnosis of pernicious anemia is confirmed by increased reticulocyte count, following parenteral administration of vitamin B_{12}.

Schilling Test

- Schilling test is done to establish the diagnosis of pernicious anemia. Injectable dose of vitamin B_{12} is given to saturate body vitamin B_{12} stores.
- Oral dose of radioactive vitamin B_{12} is given. If intrinsic factor is intact, then vitamin B_{12} is absorbed otherwise not. Its excretion is estimated in urine.
- Normally, >8% of oral radioactive vitamin B_{12} is demonstrated in urine.
- But in pernicious anemia and malabsorption syndrome, its excretion will be decreased depending upon the severity of the disease.
- Now oral dose of radioactive vitamin B_{12} combined with intrinsic factor is given to the patient with pernicious anemia, its excretion is within normal range.
- In patient with chronic renal failure not suffering from pernicious anemia, Schilling test is false positive due to impaired renal functions.

RED BLOOD CELLS DESTRUCTION AND CLASSIFICATION OF HEMOLYTIC ANEMIA

HEMOLYTIC ANEMIA: OVERVIEW

Hemolytic anemia is produced by an increased rate of destruction of red blood cells that cannot be compensated by hematopoiesis in bone marrow. RBC destruction may be due to intrinsic defects within red blood cells or extrinsic etiology.

- Intravascular hemolytic anemia is marked by intrinsic defects, most often genetically determined, in the red blood cell itself.
- Extracorpuscular hemolytic anemia is marked by extrinsic defects, most often acquired such as circulating antibodies against RBCs or splenomegaly.
- Normal life span of RBCs is 100–120 days. But in hemolytic anemia, life span of red blood cells may be reduced to as low as 15 days. Classification of inherited hemolytic anemia is given in Table 8.66. Acquired causes of hemolytic anemia are given in Table 8.67. Differences between extravascular hemolysis and intravascular hemolysis are given in Table 8.68.

Table 8.66 Classification of inherited hemolytic anemia

Disorder	Gene Mutation and Inheritance	RBC Morphology	Laboratory Diagnosis
Red blood cell membrane defects			
Hereditary spherocytosis	▪ ANK1 (autosomal dominant/ autosomal recessive disorder) ▪ SPTB (autosomal dominant disorder) ▪ SPTA1 (autosomal recessive disorder) ▪ SLC4A1 (autosomal dominant disorder) ▪ EPB42 (autosomal recessive disorder)	▪ Microspherocytosis ▪ Microspherocytosis ▪ Microspherocytosis ▪ Microspherocytosis ▪ Microspherocytosis	▪ RBC morphology ▪ RBC morphology ▪ RBC morphology ▪ RBC morphology ▪ RBC morphology
Hereditary ovalocytosis (elliptocytosis)	▪ SPTA1 (autosomal dominant disorder) ▪ SPTB (autosomal dominant disorder) ▪ EPB41 (autosomal dominant disorder)	▪ Elliptocytosis >25% ▪ Elliptocytosis >25% ▪ Elliptocytosis >25%	▪ Coombs' test negative ▪ Coombs' test negative ▪ Coombs' test negative
Southeast Asian ovalocytosis	RHAG (autosomal dominant disorder)	↑Ovalocytosis with transverse bars or single longitudinal slit	RBC morphology
Dehydrated hereditary stomatocytosis	▪ PIEZOI (autosomal dominant disorder) ▪ KCN4 (autosomal dominant disorder)	▪ Macrocytosis, stomatocytosis, target cells, schistocytes, spiculated cells ▪ Macrocytosis, stomatocytosis, target cells, schistocytes, spiculated cells	▪ RBC morphology ▪ RBC morphology
Dehydrated hereditary stomatocytosis	PIEZOI (autosomal dominant disorder)	Macrocytosis, stomatocytosis, target cells, schistocytes, spiculated cells	RBC morphology
Overhydrated hereditary stomatocytosis	RHAG (autosomal dominant disorder)	Spiculated cells	RBC morphology
Hereditary pyropoikilocytosis	▪ EPB4 (autosomal recessive disorder) ▪ SPTA1 (autosomal recessive disorder) ▪ SPTB (autosomal recessive disorder)	▪ Bizarre RBC fragments ▪ Bizarre RBC fragments ▪ Bizarre RBC fragments	▪ RBC morphology ▪ RBC morphology ▪ RBC morphology
Red blood cell enzyme disorders			
G6PD deficiency	G6PD (X-linked recessive disorder)	Usually normal, occasional bite cells	G6PD enzyme measurement
Pyruvate kinase deficiency	Pyruvate kinase (autosomal recessive trait)	Usually normal	Pyruvate kinase measurement
Sickle cell disease, HbC, HbD, HbE and HbG			
Sickle cell disease	▪ Change in 6th amino acid of the β-globin chain from glutamate to valine (β_6 glutamine → valine)	▪ Sickle cells in peripheral blood smear	▪ Hemoglobin electrophoresis

Contd...

Table 8.66 Classification of inherited hemolytic anemia (*Contd...*)

Disorder	Gene Mutation and Inheritance	RBC Morphology	Laboratory Diagnosis
	▪ Sickle cell trait (A/S) ▪ Sickle cell disease (S/S)	▪ Sickle cells in peripheral blood smear ▪ Sickle cells in peripheral blood smear	▪ Hemoglobin electrophoresis ▪ Hemoglobin electrophoresis
Hemoglobin C (HbC) disease	▪ Change in 6th amino acid of the β-globin chain from glutamate to valine (β_6 glutamine → lysine) ▪ Hemoglobin C trait (A/C) ▪ Hemoglobin disease (C/C)	▪ Target cells in peripheral blood smear ▪ Target cells in peripheral blood smear ▪ Target cells in peripheral blood smear	▪ Hemoglobin electrophoresis ▪ Hemoglobin electrophoresis ▪ Hemoglobin electrophoresis
Hemoglobin D (HbD) disease (affects East Indian population)	Change in 121st amino acid of the β-globin chain from glutamate to glutamine (β_{121} glutamine → glutamine)	Mild hemolytic anemia	Hemoglobin electrophoresis (HbD migrates with HbS at pH 8.6)
Hemoglobin E (HbE) disease (affects South East Asian population)	▪ Change in 26th amino acid of the β-globin chain from glutamate to lysine (β_{26} glutamine → lysine) ▪ Hemoglobin E trait (A/E) ▪ Hemoglobin E disease (E/E)	▪ Microcytic hypochromic picture ▪ Microcytic hypochromic picture ▪ Microcytic hypochromic picture	▪ Hemoglobin electrophoresis (HbE migrates with HbC and HbA_2 at pH 8.6) ▪ Hemoglobin electrophoresis (HbE migrates with HbC and HbA_2 at pH 8.6) ▪ Hemoglobin electrophoresis (HbE migrates with HbC and HbA_2 at pH 8.6)
Hemoglobin G (HbG) disease	Change in 68th amino acid of the α-globin chain from asparagine to lysine (α_{68} asparagine → lysine)	Mild hemolytic anemia	Hemoglobin electrophoresis
α-Thalassemia (in a normal person, α-gene synthesizes α-globin chains)			
Silent carrier	–α/αα due to HBA gene mutation	Normal hematologic findings	Genetic testing
α-thalassemia minor/trait	–α/–α or –/αα due to HBA gene mutation	▪ Hemoglobin normal ▪ Mild microcytic hypochromic picture	▪ Hemoglobin electrophoresis ▪ Genetic testing
Hemoglobin H disease	–α/– due to HBA gene mutation	▪ Hemolytic anemia picture ▪ HbA (70–90%) ▪ HbH (5–30%)	Genetic testing
Hydrops fetalis	–/– (loss of all four α-globin alleles due to HBA gene mutation)	▪ Hb Bart (>80%) ▪ HbH (10–20%) ▪ HbA (nil) ▪ Severe disease not compatible with life	Genetic testing
β-Thalassemia (in a normal person, β-gene synthesizes β-globin chains)			
Thalassemia major	β^0/β^0 due to HBB gene mutation	▪ Microcytic hypochromic picture, numerous target cells, basophilic stippling ▪ HbA (nil) ▪ HbA_2 (3.8%) ▪ HbF (>90%)	▪ Hemoglobin electrophoresis ▪ Genetic testing ▪ High performance liquid chromatography (HPLC) ▪ Genetic testing

Contd...

Table 8.66 Classification of inherited hemolytic anemia (*Contd...*)

Disorder	Gene Mutation and Inheritance	RBC Morphology	Laboratory Diagnosis
Thalassemia minor/trait	β^0/β due to HBB gene mutation	▪ Microcytic hypochromic picture, target cells, basophilic stippling ▪ HbA (90%) ▪ HbA_2 (3.8%) ▪ HbF (+/–)	▪ High performance liquid chromatography (HPLC) ▪ Genetic testing
Thalassemia intermedia	β^+/β^+ due to HBB gene mutation	▪ Mild microcytic hypochromic picture ▪ HbA (50–70%) ▪ HbA_2 (3.8%) ▪ HbF (20–40%)	No definite investigations
δβ-Thalassemia			
δβ-Thalassemia major	$\delta\beta^0/\delta\beta^0$	▪ HBA (absent) ▪ HbA_2 (absent) ▪ HbF (100%)	High performance liquid chromatography (HPLC)
δβ-Thalassemia minor	$\delta\beta^0/\delta\beta$	HBA (80–90%)	High performance liquid chromatography (HPLC)

Table 8.67 Acquired causes of hemolytic anemia

Immune-mediated Hemolytic Anemia

- Autoimmune hemolytic anemia
  - Warm autoantibody mediated hemolytic anemia (at 37°C)
  - Cold autoantibody mediated hemolytic anemia (at <37°C)
- Alloimmune hemolytic anemia
  - Hemolytic disease of newborn
  - Hemolytic blood transfusion reactions
  - Allografts especially stem cell transplantation
- Paroxysmal nocturnal hemoglobinuria (PNH)
- Drug-induced hemolytic anemia
  - α-methyldopa
  - Penicillin
  - Oxidant drugs
  - Primaquine
  - Dapsone

Red Blood Cell Fragmentation Syndromes

- Cardiac hemolysis
  - Prosthetic cardiac valves
  - Severe aortic valvular diseases
  - Grafts used in vessels
  - Perivascular leaks
- March hemoglobinuria
- Arteriovenous malformations
- Microangiopathic hemolytic anemia
  - Thrombotic thrombocytopenic purpura
  - Hemolytic uremic syndrome
  - Disseminated intravascular coagulation (DIC)
  - Malignant hypertension
  - Polyarteritis nodosa (PAN)
  - Pre-eclampsia/eclampsia of pregnancy
  - HELLA syndrome (life-threatening pregnancy associated complication during later half of pregnancy or sometimes after childbirth)
  - Renovascular disorders
  - Cyclosporine immunosuppressive drug
  - Homograft rejection

Severe Infections induced Hemolytic Anemia

- *Clostridium welchii*
- *Vibrio cholerae*
- Malarial parasite
- Bartonella gram-negative bacteria

Chemicals and Physical Agents induced Hemolytic Anemia

- Industrial chemical: Naphthalene
- Domestic substances: Nitrites and nitrates
- Thermal injury: Severe burns

Secondary Causes induced Hemolytic Anemia

- Liver disease
- Renal disease

Miscellaneous Causes induced Hemolytic Anemia

- Hypersplenism
- Snake venom
- Celiac disease
- Vitamin E deficiency

Contd...

Table 8.68 Differences between extravascular hemolysis and intravascular hemolysis

Features	Extravascular Hemolysis	Intravascular Hemolysis
Site of hemolysis	Reticuloendothelial cells (spleen, bone marrow)	Inside blood circulation
Examples	Red blood cell membrane defect, HbS, thalassemia	Fragmentation syndrome, mismatched blood transfusion, *Clostridium welchii* sepsis, paroxysmal nocturnal hemoglobinuria
Clinical features	Anemia, jaundice	Anemia, jaundice
Splenomegaly	Present	Absent
Serum bilirubin (unconjugated)	Increased (++)	Increased (+)
Serum haptoglobin	Normal or reduced	Decreased
Serum methemalbumin	Absent	Present
Plasma hemoglobin	Absent	Present
Serum LDH	Increased (+)	Increased (++)
Urine hemoglobinuria	Absent	Present
Urine hemosiderin	Absent	Present
Body iron stores in spleen, bone marrow, liver	Increased	Decreased

Morphologically changes in both types of hemolysis are identical, except for the fact that erythrophagocytosis in extravascular hemolysis causes hypertrophy of the mononuclear phagocytic system, and this may lead to splenomegaly.

- Red blood cell destruction may occur in the extravascular or intravascular compartments. Clinical data of the patient gives clue to the diagnosis, which includes a family history of hemoglobinopathies or hereditary spherocytosis, a recent blood transfusion, malaria or splenectomy or malignant tumor.
- In addition to the basic symptoms of anemia, splenomegaly and jaundice may be present. There is presence of reticulocytes higher than normal in the blood, which cannot carry as much as oxygen as the fully developed red blood cells.

CLINICAL FEATURES

Clinical features of hemolytic anemia depend on duration and severity of red blood cell destruction. Patient presents with anemia, jaundice, cholelithiasis, leg ulcers, dark or red-colored urine, cortical bone thinning, extramedullary hematopoiesis and splenomegaly. Main features of hemolytic anemia are given in Table 8.69.

- **Pallor:** Depending on the severity of hemolysis, patient presents with anemia. Pallor is demonstrated in conjunctiva and tongue.
- **Jaundice:** Hemolytic jaundice occurs due to rapid destruction of red blood cells resulting in production of excess of bilirubin. Liver is not able to conjugate excess of bilirubin. Most hemoglobinopathies are characterized by mild jaundice. Patient with hereditary spherocytosis or G6PD deficiency may develop moderate to severe jaundice.
- **Splenomegaly:** In chronic hemolytic anemia, patient presents with vague abdominal discomfort. Splenomegaly is appreciated on clinical examination.
- **Gallbladder pigment stones:** Chronic intravascular hemolysis is the most common cause of gallbladder pigment stones, which are commonest and associated with hemolysis and cirrhosis. Pigment stones are confined to the gallbladder. Bile juice is sterile.
  - Gallbladder pigment stones consist of large amounts of polymerized degradation product of oxidized bilirubin.
  - Patient presents with sudden pain in right upper quadrant that may radiate to scapular region, lasting for 2–4 hours following ingestion of fatty meals, alcohol, and caffeine. Pain is associated with nausea and vomiting.
- **Skeleton abnormalities:** Skeleton abnormalities occur in congenital hemolytic anemia such as β-thalassemia major. Erythroid hyperplasia leads to widening of diploë of skull bones (parietal and frontal bones) and maxilla. Physical examination reveals frontal bossing, eminent cheek bones and widening of teeth. Radiograph of skull shows crewcut appearance as a result of widening of diploë and formation of new bone especially in young patients.
- **Chronic leg ulcers:** Patient suffering from hereditary spherocytosis or β-thalassemia major may develop leg ulcers as a result of thrombosis of small blood vessels and necrosis of skin. Leg ulcers are demonstrated on medial and lateral malleoli.

Table 8.69 Main features of hemolytic anemia

Features	Comments
Evidence of increased extravascular and intravascular red blood cell destruction	
Hyperbilirubinemia	▪ Mild, moderate or severe jaundice (yellow discoloration of sclera) depending on extent of hemolysis ▪ Increased risk of gallbladder stones
Physical examination	▪ Anemia depends on extent of hemolysis ▪ Jaundice (yellow discoloration of sclera) ▪ Frontal bossing, eminent cheek and widening of teeth as a result of widening of skull bones ▪ Splenomegaly due to extravascular hemolysis
Laboratory findings	▪ Decreased serum haptoglobin level ▪ Decreased serum hemopexin level ▪ Increased urinary urobilinogen ▪ Increased fecal stercobilinogen ▪ Increased body iron stores ▪ Extravascular hemolytic anemia (increased body iron store) ▪ Intravascular hemolytic anemia (hemoglobinemia, hemoglobinuria, hemosiderinuria, methemoglobinemia and decreased iron stores)
Compensatory mechanisms of red blood cell hemolysis	
Bone marrow findings	Evidence of erythroid hyperplasia as a result of expansion of bone marrow and decreased myeloid to erythroid ratio
Peripheral blood smear findings	▪ Polychromasia and reticulocytosis ▪ Macrocytosis due to increased requirement of folate

COMPENSATORY MECHANISMS OF HEMOLYTIC ANEMIA

Hematopoiesis is increased as a consequence of hemolytic process to compensate RBCs destruction. Compensatory mechanisms include erythroid hyperplasia and reticulocytosis.

Evidence of Erythroid Hyperplasia

Increased erythropoiesis compensates in part for the shortened red blood cell survival in hemolytic anemia. Bone marrow examination shows normoblastic erythroid hyperplasia.

- There is increased number of circulating reticulocytes (newly formed red blood cells identified by residual stainable RNA). Because reticulocytes are larger than mature red blood cells, the MCV may be moderately increased (up to about 105 fl).
- Peripheral blood smear examination shows nucleated red blood cells, polychromasia and leukocytosis.

Reticulocytosis

Reticulocyte count is increased following massive bleeding, acute hemolysis, and response to specific therapy in nutritional anemia and even after voluntary blood donation.

- Reticulocyte count is used to assess the capacity of the bone marrow to increase RBC production in response to increased demand.
- Reticulocytes are demonstrated by supravital stains such as new methylene blue (best stain) and brilliant cresyl blue.

Peripheral Blood Smear Examination

Peripheral blood smear examination provides valuable information about hemolytic anemia.

- Red blood cells exhibit polychromasia and increased reticulocyte count.
- Normoblasts are demonstrated in peripheral blood smear following moderate to severe hemolysis.
- One must look for spherocytes, sickle cells, target cells, fragmented red blood cell and acanthocytes.
- Neutrophilia with shift to left with myelocytes and metamyelocytes is seen. Thrombocytosis is demonstrated in acute hemolysis.

EXTRAVASCULAR HEMOLYSIS INVOLVING RBCs DESTRUCTION

After 100–120 days, RBCs are destroyed by reticuloendothelial system and liberate hemoglobin, which dissociates to form heme (porphyrin, iron and globin).

- Iron goes to iron storage sites for further utilization. Globin goes to amino acid pool. Iron goes to iron storage sites (e.g. bone marrow, spleen and liver) for reutilization.
- Metabolic products of porphyrin such as urobilinogen and stercobilinogen are excreted via urine and stool

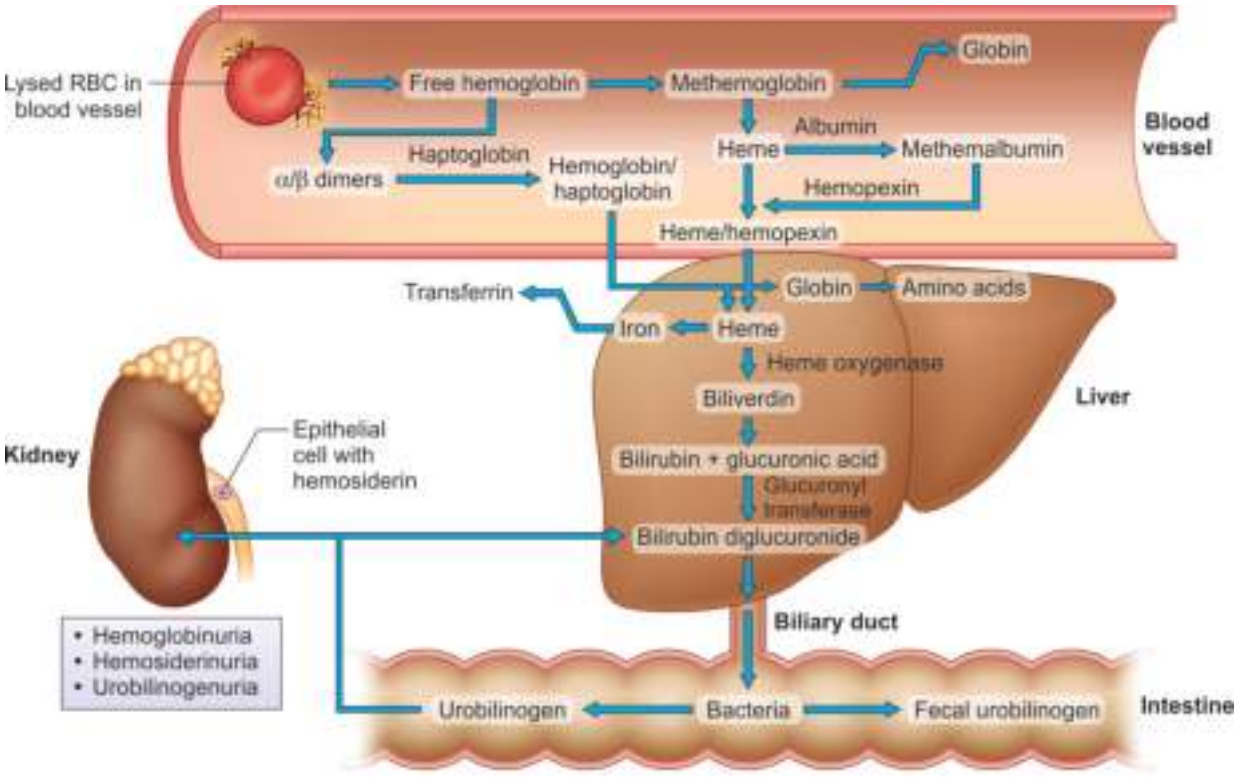


Fig. 8.25: Simplified pathway of bilirubin metabolism. Hemolytic anemia leads to increased biliary excretion of bilirubin.

respectively. Total normal bilirubin level is 0.3–1.0 mg/dl. Simplified pathway of bilirubin metabolism is shown in Fig. 8.25.

BIOCHEMICAL ALTERATIONS

Increased Serum Bilirubin

Absence of jaundice does not exclude hemolytic anemia. Increased red blood cell destruction results in liberation of hemoglobin or its degradation products. Hemolytic anemia is not accompanied with pruritus. Hyperbilirubinemia may lead to pigment-containing gallbladder stones as a late complication.

- Clinical jaundice is not apparent until the serum bilirubin exceeds 40 µmol per liter. Urine contains increased level of urobilinogen. Aqueous diazotized sulphanilic acid reacts only with conjugated bilirubin, giving red color.
- Addition of alcohol (methanol) frees the albumin, hence, rest of the unconjugated bilirubin reacts with the reagent. Bilirubin is destroyed by direct sunlight or ultraviolet light including fluorescent light.

Increased Excretion of Urobilinogen

Extravascular hemolysis involving red blood cell destruction leads to increased excretion of urobilinogen in urine giving high-colored urine.

Increased Excretion of Stercobilinogen

Extravascular hemolysis involving red blood cell destruction leads to increased excretion of stercobilinogen exhibiting dark-colored stool.

Increased Iron Stores in Tissues

Hemolytic anemia due to extravascular etiology leads to excess release and deposition of iron in tissues.

INTRAVASCULAR HEMOLYSIS INVOLVING RBCs DESTRUCTION

Estimation of serum haptoglobin, hemopexin and methemalbumin provides good indicators of severity of intravascular hemolytic process. Indicators of intravascular hemolytic anemia are shown in Fig. 8.26. Causes of intravascular hemolysis are given in Table 8.70. Biochemical alterations in intravascular hemolysis destruction are discussed below.

BIOCHEMICAL ALTERATIONS

Hemoglobinemia

Normal free hemoglobin in plasma is 0.6 mg/dl. When free hemoglobin is markedly raised, the color of the plasma becomes pink or red. When rise of free hemoglobin is moderate, plasma color is lacking due to

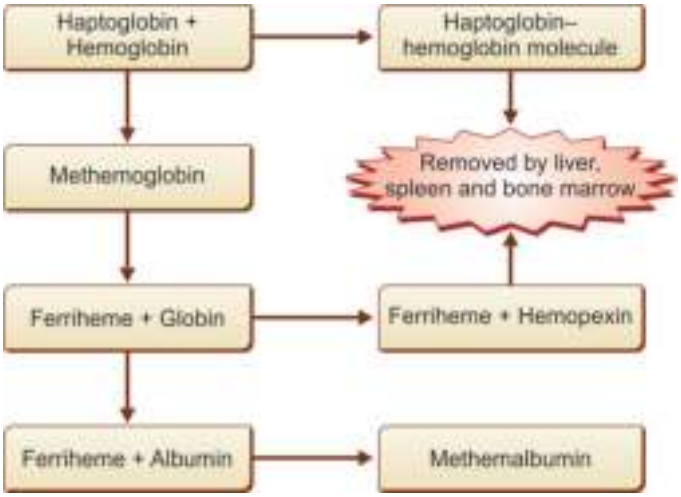


Fig. 8.26: Indicators of intravascular hemolytic anemia.

Table 8.70 Causes of intravascular hemolysis

Mismatched blood transfusion
G6PD deficiency with oxidative stress
Paroxysmal nocturnal hemoglobinuria (PNH)
Unstable hemoglobin
Drug-induced hemolytic anemia (e.g. α-methyldopa, penicillin, oxidant drugs, primaquine, dapsone)
Infection-induced hemolytic anemia (*Clostridium welchii*, *Vibrio cholerae*, malarial parasite, Bartonella gram-negative bacteria)
Red cell fragmentation syndromes (march hemoglobinuria, prosthetic cardiac valves, thrombotic thrombocytopenic purpura, hemolytic–uremic syndrome, malignant hypertension, pre-eclampsia/eclampsia of pregnancy, HELLP syndrome associated with pregnancy, disseminated intravascular coagulation (DIC), homograft rejection and ciclosporin immunosuppressive drug)

low hemoglobin concentration. Hemoglobin in plasma is detected by **benzidine test**.

- Presence of other pigments such as bilirubin gives yellow color.
- **Methemoglobin** is dark brown in acidic urine, while **oxyhemoglobin** is bright red in alkaline urine.

Hemoglobinuria

Hemoglobinuria occurs in severe intravascular hemolysis. Hemoglobin in urine is demonstrated by spectroscopy in bright day light. Oxyhemoglobin appears in the yellow-green, while methemoglobin in the red by spectroscopy, which must be distinguished from hematuria. In hematuria, intact RBCs are seen in freshly voided specimens. *In vitro* lysis of the red blood cells in hematuria results in artificial hemoglobinuria.

Hemosiderinuria

Hemosiderinuria is a valuable sign for chronic hemolytic process, which persists for several weeks after a hemolytic episode. Examination of urinary sediment stained by **Perls Prussian blue** stain demonstrates iron-containing renal tubular cells.

Plasma Haptoglobin

Haptoglobin is α_2 glycoprotein synthesized by liver, which combines with any hemoglobin in plasma and prevents its excretion in urine. Haptoglobin–hemoglobin molecule is removed by liver, spleen and bone marrow. Hence, plasma haptoglobin levels are decreased with each episode of hemolysis, which come to normal within 3 days. Decreased level of plasma haptoglobin is very sensitive indicator of mild hemolytic process (normal range 0.5–1.5 g/L).

Plasma Hemopexin

Hemopexin is a glycoprotein synthesized by liver, which does not bind to hemoglobin rather binds only to ferriheme. When hemolysis occurs, hemoglobin liberated is reduced to methemoglobin, which splits into ferriheme and globin.

- Globin goes to amino acid pool for further utilization, while ferriheme combines with hemopexin and cleared by liver. Hemopexin value is decreased only in severe degree of hemolysis.
- Plasma hemopexin level is a good index of sensitivity of the hemolytic process as it is not reduced by minor degrees of hemolysis. Decreased plasma hemopexin levels indicate severe hemolytic process (normal range 0.5–1.0 g/dl).

Plasma Methemalbumin

Methemalbumin occurs in hemolytic anemia, especially when hemolysis is intravascular. Methemoglobin consists heme and albumin, which gives brown color to plasma in intravascular RBCs hemolysis.

- Schumm's test is performed to differentiate intravascular hemolysis from extravascular hemolysis in a case of hemolytic anemia.
- Methemalbumin has a characteristic absorption in the red band (at 624 nm), which may be seen by means of spectroscope. Now cover the sample with a layer of ether, and add 1/10th of saturated yellow ammonium sulfide and mix, which leads to formation of an ammonium hemochromatogen, a more intense band can be seen in the green part of the spectrum (588 nm). This is called Schumm's test.

HEREDITARY SPHEROCYTOSIS, OVALOCYTOSIS AND STOMATOCYTOSIS: RBC MEMBRANE DEFECTS

HEREDITARY SPHEROCYTOSIS

Hereditary spherocytosis is an autosomal disorder caused by deficiency of cytoskeleton isolated or combined proteins resulting in reduced RBC membrane stability and loss of RBCs membrane lipid bilayer.

- Red blood cells assume shape, i.e. microspherocytes having small surface area for the given volume.
- Life span of red blood cells is reduced to 10–20 days against a normal 100–120 days.
- Spectrin cytoskeleton protein deficiency linked to hereditary spherocytosis is more common.
- Combined deficiency of spectrin and ankyrin cytoskeleton proteins is demonstrated in majority of hereditary spherocytosis cases.
- Band 3 cytoskeleton protein deficiency occurs in 25% of cases.
- Band 4.2 cytoskeleton protein deficiency linked to hereditary spherocytosis is more common in Japan.

Pathology Pearls: Hereditary Spherocytosis

Physiologic State

Normal biconcave shape and flexibility of red blood cells are maintained by the presence of cytoskeleton proteins composed of spectrin, ankyrin, actin, protein 4.1 and protein 3 (Fig. 8.27).

Pathologic State

- Hereditary deficiency of cytoskeleton proteins in red blood cells leads to reduced membrane stability, loss of lipid bilayer membrane and microspherocytes formation.
- The microspherocytes fail to pass through interendothelial fenestrations of sinusoids and get trapped in the spleen.
- Due to accumulation of lactic acid in microspherocytes disrupts sodium pump resulting in sequestration in the spleen and chronic extravascular hemolysis. Life span of red blood cells is decreased to 10–20 days.
- Hereditary spherocytosis is autosomal dominant (more common and less severe) or recessive disorder (less common but more severe). Pathophysiology of hereditary spherocytosis is shown in Fig. 8.28.

GENE MUTATIONS

Isolated or combined mutation in spectrin and ankyrin genes account for most cases. Isolated mutation occurs in band 3 or band 4.2. Glycophorin is not involved in the pathogenesis of hereditary spherocytosis. Hereditary spherocytosis (HS) and inherited defects of cytoskeleton proteins are given in Table 8.71.

Spectrin Proteins

Approximately, 65% of hereditary spherocytosis/autosomal dominant cases are due to SPTAI gene mutation

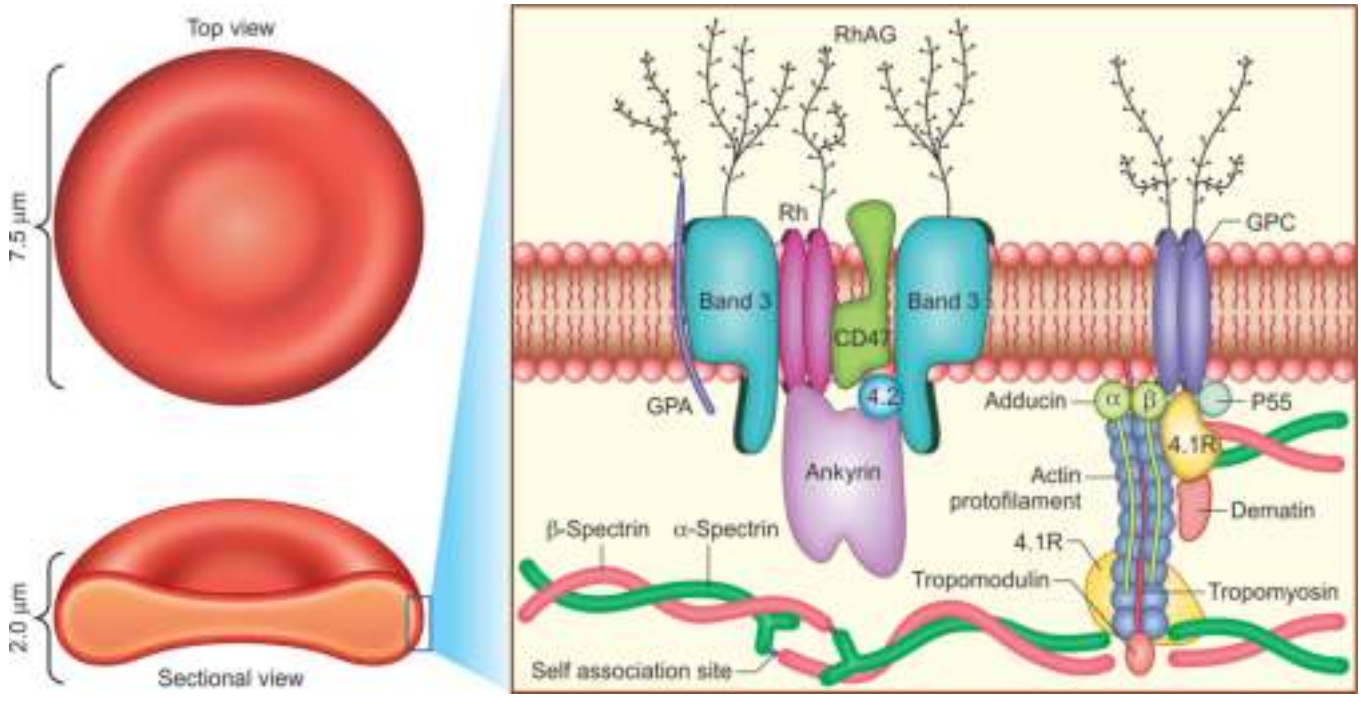


Fig. 8.27: Cross-sectional side view of the biconcave structure of red blood cell. Diagram shows cytoskeleton and flexible spectrin, ankyrin, actin, protein 4.1 and protein 3. Vertical interaction proteins include spectrin, ankyrin, protein 4.2 and band 3. Horizontal interaction proteins include actin, protein 4.1 and adducin.

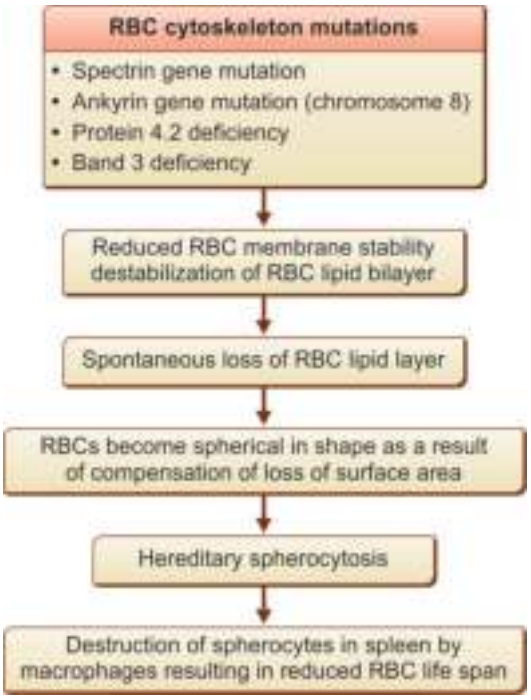


Fig. 8.28: Pathophysiology of hereditary spherocytosis.

located on chromosome 1q22–q23 encoding spectrin protein, and 30% cases due to SPTB gene mutation located on chromosome 14q23–q24.1 encoding spectrin protein. α-Spectrin mutation correlates with severity of hemolysis and osmotic fragility.

Ankyrin Protein

ANK1 gene mutation located on chromosome 8p11.2 encoding ankyrin protein is associated with majority of hereditary spherocytosis (as autosomal dominant disorder). It is most common mutation in hereditary spherocytosis.

Band 3 (Anion Channel) Protein

Band 3 is anion transport protein. SLC4A1 gene mutation located on chromosome 17q21 encoding band 3 (anion channel) protein accounts for 25% of hereditary spherocytosis is an autosomal dominant disorder.

Band 4.2 Protein (Pallidin)

EPB42 gene mutation located on chromosome 15q15–q21 encoding mutant band 4.2 protein accounts for 3% of hereditary spherocytosis an autosomal recessive disorder. Protein 4.2 deficiency is common in Japan.

CLINICAL FEATURES

Due to chronic hemolysis of microspherocytes in the spleen, patient presents with intermittent jaundice, splenomegaly, pigmented gallstones in 50% of cases, mild to moderate anemia and chronic leg ulcers. The severity of the disease is quite variable among individuals.

- Patient may develop aplastic crisis due to bone marrow depression by Parvovirus B19 infection. Splenectomy is the treatment of choice.
- Splenectomy cures the anemia caused by hereditary spherocytosis by removing the site of extravascular hemolysis.
- Genetic defect is incurable. Microspherocytes persist in blood circulation in postsplenectomy patients.

Age and Sex

Hereditary spherocytosis manifests in neonates, children and adults affecting both sex groups.

Anemia

Patient presents with mild to moderate anemia. Severe anemia rarely occurs. Aplastic crisis occurs due to bone marrow depression caused by Parvovirus B19 infection. Majority of patients present with moderate anemia showing 15–30 microspherocytes/HPF and splenomegaly.

Jaundice

Intermittent jaundice is the most common presentation in children. Pregnancy, fatigue and infection may aggravate jaundice.

Splenomegaly

Mild to moderate splenomegaly is most common clinical finding in hereditary spherocytosis as a result

Table 8.71 Hereditary spherocytosis (HS) and inherited defects of cytoskeleton proteins

Encoding Proteins	Gene	Location on Chromosome	Autosomal Dominant/Recessive Hereditary Spherocytosis	Percentage
Spectrin	▪ SPTAI gene ▪ SPTB gene	▪ 1q22–q23 ▪ 14q23–q24.1	▪ Autosomal dominant ▪ Autosomal dominant	▪ 65% of cases ▪ 30% of cases
Ankyrin	ANK1 gene	8p11.2	Autosomal dominant	Majority of cases
Band 3 (anion channel)	SLC4A1	17q21	Autosomal dominant	25% of cases
Band 4.2 (pallidin)	EPB42	15q15–q21	Autosomal recessive	3% of cases (common in Japan)

of trapping of microspherocytes in the red pulp of the spleen.

Gallbladder Pigment Stones

Gallbladder pigment stones are most often demonstrated during first to second decade in 50–75% cases of hereditary spherocytosis.

Leg Ulcers

Patient with hereditary spherocytosis may rarely develop leg ulcers.

Laboratory Diagnosis of Hereditary Spherocytosis

- **Hemoglobin:** Hemoglobin is low depending on degree of hemolysis.
- **Reticulocyte count:** Reticulocyte count is increased (>8%).
- **Hematocrit values:** MCHC is often increased. MCH is within normal range. MCV is decreased.

Peripheral Blood Smear Examination

- Peripheral blood smear examination shows round, slightly smaller than normal red blood cells. The microspherocytes lack central pallor area. Nucleated and polychromatic red blood cells are demonstrated.
- To demonstrate microspherocytes properly, it is important to visualize in the area of the peripheral blood smear, where red blood cells are nearly touching each other but not stretched out.
- Normal red blood cells when stretched, as at the tail or thin end of the peripheral blood smear have an artificial spherical appearance.
- Platelet count is within normal range. Peripheral blood smear showing hereditary spherocytosis is shown in Fig. 8.29A to C.
- Hereditary spherocytosis should be differentiated from other disorders showing spherocytes, which include immune hemolytic anemia (Coombs' test positive), paroxysmal nocturnal hemoglobinuria, G6PD deficiency, blood transfusion reactions, *Clostridium perfringens* sepsis, snakebites, microangiopathic hemolytic anemia and malarial parasitic infection.

Bone Marrow Smear Examination

Bone marrow is hypercellular due to normoblastic erythroid hyperplasia. Myelopoiesis and megakaryopoiesis are within normal range.

Serum Bilirubin

- There is increase in indirect unconjugated serum bilirubin, but not direct (conjugated).
- The jaundice is acholuric (no bilirubin in the urine, so bilirubinuria would not be expected).

Incubation Osmotic Fragility

Osmotic fragility is **most reliable diagnostic test** for hereditary spherocytosis. Incubation increases sensitivity of osmotic fragility test by enhancing loss of surface area of red blood cells. It is performed to measure the erythrocyte's resistance to hemolysis by osmotic stress. It mainly depends on volume of the cell, surface area and membrane function. Osmotic fragility test on human red blood cells in hereditary spherocytosis is shown in Fig. 8.30.

- **Physiologic state:** Normal red blood cells begin to hemolyze at 0.5% NaCl concentration. Hemolysis is complete at about 0.3%. There is increased RBCs osmotic fragility in hypotonic saline.
- **Pathologic state:** Red blood cells are incubated in varying concentrations of hypotonic NaCl solution. The erythrocytes take in water in an effort to achieve osmotic equilibrium. Further uptake of water by red blood cells results in rupture of membrane. Osmotic fragility curve demonstrates a shift-to-the-right in hereditary spherocytosis, and autoimmune hemolytic anemia.

Coombs' Test

- Presence of microspherocytes in the peripheral blood smear and along with family history, strongly suggest a diagnosis of hereditary spherocytosis.
- Spherocytes are also observed in warm antibody autoimmune hemolytic anemia and G6PD deficiency. Coombs' test is negative in hereditary spherocytosis, but positive in autoimmune hemolytic anemia.

Flow Cytometry Test

Flow cytometric analysis is done by binding eosin-5-maleimide to red blood cells for identifying deficient band 3 protein in hereditary spherocytosis.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is performed to detect abnormalities of cytoskeleton proteins, e.g. spectrin, ankyrin, band 4.2 (pallidin) and band 3 (anion channel).

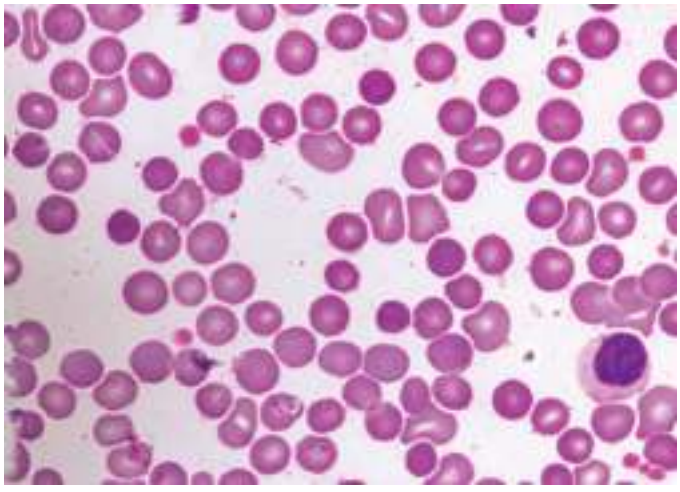


Fig. 8.29A: Peripheral blood smear examination in hereditary spherocytosis shows darkly stained microspherocytes lacking central pallor.

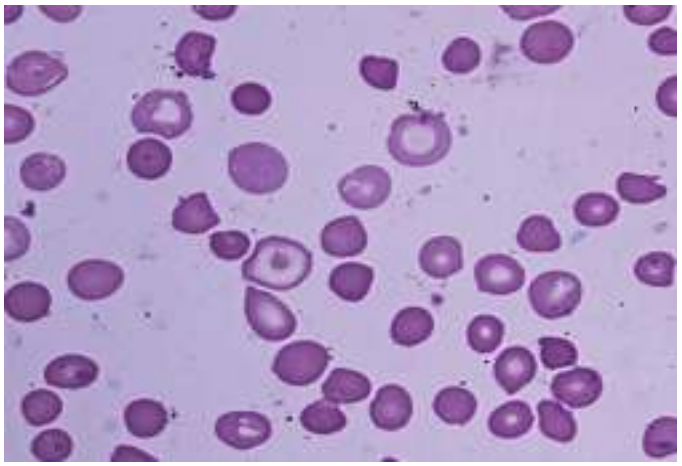


Fig. 8.29B: Peripheral blood smear in hereditary spherocytosis. It shows darkly stained microspherocytes.

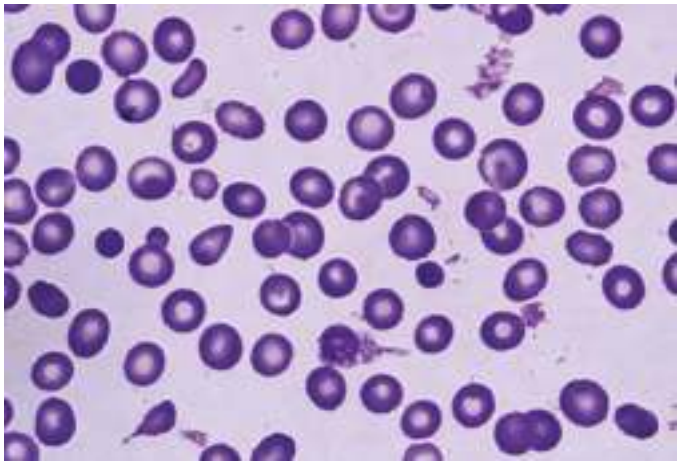


Fig. 8.29C: Peripheral blood smear in hereditary spherocytosis shows many spherocytes.

HEREDITARY OVALOCYTOSIS

Hereditary ovalocytosis (elliptocytosis) is an autosomal dominant disorder affecting assembly of membrane associated cytoskeleton horizontal proteins band 3 (anion channel) and band 4.1.

- Hereditary ovalocytosis is characterized by elongated, oval red blood cells in peripheral blood smears in some populations residing in Melanesia.

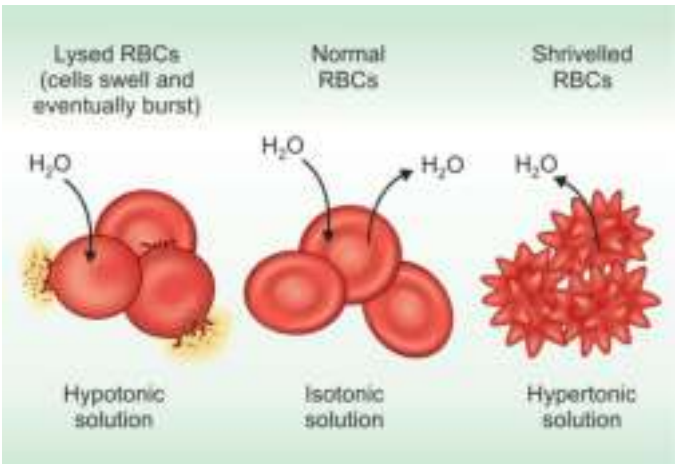


Fig. 8.30: Osmotic fragility test on human red blood cells in hereditary spherocytosis.

- There may be mild hemolysis during infancy. Homozygous persons may rarely develop chronic hemolytic anemia, which does not cause anemia in most of the cases.

GENE MUTATIONS

Hereditary ovalocytosis occurs due to gene mutations encoding cytoskeleton horizontal proteins band 3 or band 4.1. Hereditary ovalocytosis and inherited defects of cytoskeleton proteins are given in Table 8.72.

- **Band 3 (anion channel) protein:** SLC4A1 gene mutation (polymorphic mutation-deletion of 9 amino acids) located on chromosome 17q21 encoding band 3 (anion channel) is associated with autosomal dominant ovalocytosis in South-East Asia. The patients are clinically symptomatic and resistant to *Plasmodium falciparum.*
- **Band 4.1 protein:** EPB41 gene mutation located on 1p33–p34.2 encoding band 4.1 accounts for 5% of cases.

CLINICAL FEATURES

Most patients are occasionally symptomatic. Patient may present with pallor, jaundice, anemia and gallbladder stones.

Table 8.72 Hereditary ovalocytosis and inherited defects of cytoskeleton proteins

Encoding Proteins	Gene	Location on Chromosome	Disorder	Percentage and Comments
Band 3 (anion channel)	SLC4A1	17q21	Autosomal dominant	▪ Polymorphic deletion of 9 amino acids ▪ South-East Asian persons are asymptomatic and resistant to *Plasmodium falciparum*
Band 4.1	EPB41	1p33–p34.2	Autosomal dominant	▪ 5% of cases ▪ Severe hemolysis in homozygotes ▪ No hemolysis in heterozygotes

Laboratory Diagnosis of Hereditary Ovalocytosis

- **Hematocrit values:** MCH, MCHC and MCV are within normal range.
- **Hemoglobin value:** Hemoglobin value is most often within normal range.
- **Peripheral blood smear:** Peripheral blood smear examination shows ovalocytes constituting 20–90% of red blood cells (Fig. 8.31).

RBC Autohemolysis Test

Autohemolysis is increased in hereditary ovalocytosis. It can be corrected by addition of glucose.

Molecular Genetic Testing

The confirmatory test to diagnose hereditary ovalocytosis is based on molecular genetic testing for the presence of gene mutations in the specific protein molecules of the RBCs.

HEREDITARY STOMATOCYTOSIS

Hereditary stomatocytosis is an autosomal dominant disorder, which affects the red blood cells, in which the membrane of RBCs leaks sodium and potassium ions. It is caused by SLCA4A-I gene mutation located on chromosome 17q21 encoding RBCs membrane cytoskeleton band 3 protein. Hereditary stomatocytosis and inherited defects of cytoskeleton proteins are given in Table 8.73.

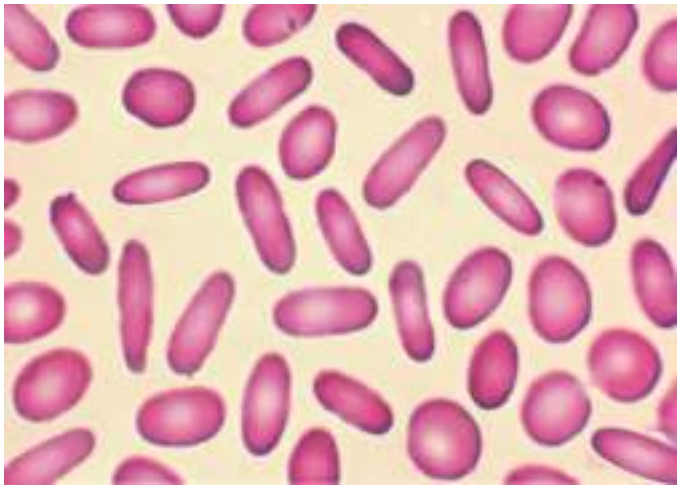


Fig. 8.31: Hereditary ovalocytosis shows large number of ovalocytes.

PATHOPHYSIOLOGY

Normally, ion exchanger pump on RBCs membrane pumps out sodium out cell and potassium inside cell. This action is balanced by 'the passive leak'. Due to SLCA4A-1 gene mutation encoding band 3 protein, the passive leak is increased resulting in swelling of red blood cells as a result of accumulation of salt and water. RBCs lyse and hemolytic anemia results. Stomatocytes are trapped and consequently hemolyzed in the

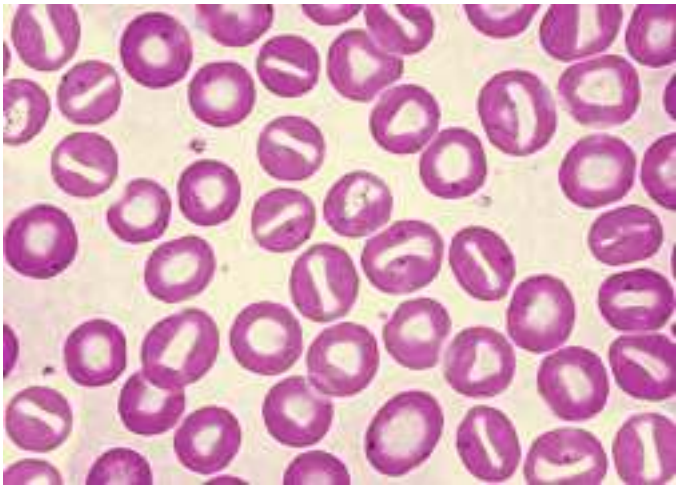


Fig. 8.32A: Peripheral blood smear in hereditary stomatocytosis. It shows stomatocytes with central slit-shaped area. These stomatocytes are also demonstrated in liver disease.

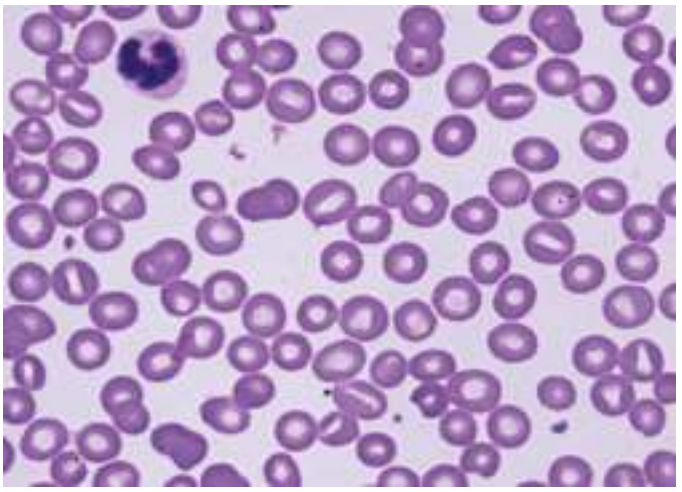


Fig. 8.32B: Peripheral blood smear in hereditary stomatocytosis.

Table 8.73 Hereditary stomatocytosis and inherited defects of cytoskeleton proteins

Encoding Proteins	Gene	Location on Chromosome	Disorder	Percentage and Comments
Band 3 (anion channel)	SLC4A1	17q21	Hereditary stomatocytosis	Certain specific missense mutations shift protein function from anion exchanger to cation conductance

microvasculature of spleen and other organs. Patient with hereditary stomatocytosis is protected against malarial parasite infection.

CLINICAL COURSE

Many patients present with hereditary stomatocytosis with hemolytic anemia in the neonatal period. Other patients remain asymptomatic throughout their lifetime.

Laboratory Diagnosis of Hereditary Stomatocytosis

Peripheral Blood Smear Examination

Peripheral blood smear examination shows many stomatocytes with a slit-like central pallor giving them the appearance of 'coffee beans'. In three dimensions, stomatocyte has lost its biconcave morphology due to membrane defect (Fig. 8.32A and B).

THALASSEMIAS

THALASSEMIAS: OVERVIEW

Thalassemia is a heterogenous group of inherited blood disorders affecting the hemoglobin genes coding for α- and β-chains of hemoglobin resulting in ineffective erythropoiesis. Hemoglobin serves as the oxygen-carrying component of red blood cells. Patients present with anemia in early childhood, who require frequent blood transfusions to maintain hemoglobin levels throughout life.

- **Physiologic state:** A normal hemoglobin molecule contains four globin chains, consisting of two α- and two β-chains. Three normal variants of hemoglobin are encountered, based on the nature of the non-α-chains. Hemoglobin A ($\alpha_2\beta_2$) accounts for 95–98% of the total hemoglobin in adults; only minor amounts of HbF ($\alpha_2\gamma_2$) and HbA_2 ($\alpha_2\delta_2$) are present.
- **Pathologic state:** Thalassemia is group of genetic disorders characterized by complete or deficient synthesis of either α- or β-globin chains of structurally normal hemoglobin chains. It is worth mentioning that heme synthesis is not affected. Thalassemias occur across world with high frequency in Africa, India, South-East Asia, and the Mediterranean area.

CLASSIFICATION

Thalassemias are classified according to the type of chains that are missing.

- If the α-chains are not synthesized, it is called α-thalassemia; if the β-chains are missing, it is called β-thalassemia.
- Homozygous β-thalassemia is a more serious disease, and silent carrier of α-thalassemia is asymptomatic.
- Thalassemia intermedia is double heterozygotes and less severe. Classification of α-, β- and δβ-thalassemias is shown in Fig. 8.33 and Table 8.74.

β-THALASSEMIAS

β-THALASSEMIA MAJOR

β-Thalassemia major is also known as Mediterranean anemia or Cooley's anemia. In transfusion-dependent anemia, morbidity and mortality occur due to cardiac failure as a result of expansion of plasma volume and iron overload. It is a serious disease which often results in death during childhood unless frequent blood transfusions are given. Life expectancy is 15–25 years with or without treatment death occurs by 5 years of age.

Pathophysiology

The lack of β-chains leads to accumulation of unpaired α-chains (which are free and uncombined) within developing red blood cells.

- These α-chains aggregate and result in premature destruction of maturing erythroblasts within bone marrow (ineffective erythropoiesis) and premature destruction of abnormal mature red blood cells in the spleen (hemolysis).
- Serum ferritin and serum iron levels are increased. Excess of iron is deposited in liver, heart, spleen, hypothalamus and endocrine glands (pituitary, islets of Langerhans' and parathyroid glands). Pathophysiology of β-thalassemia major is shown in Fig. 8.34.

Point Mutations of Globin Gene Clusters

β-Thalassemia major occurs due to various point mutations of the globin gene clusters. Suppression of β-globin occurs as a consequence of single nucleotide base pair substitution in the promoter region or messenger RNA processing region.

- Gene mutation at terminator region causes β-thalassemia characterized by absence of synthesis of β-chains.

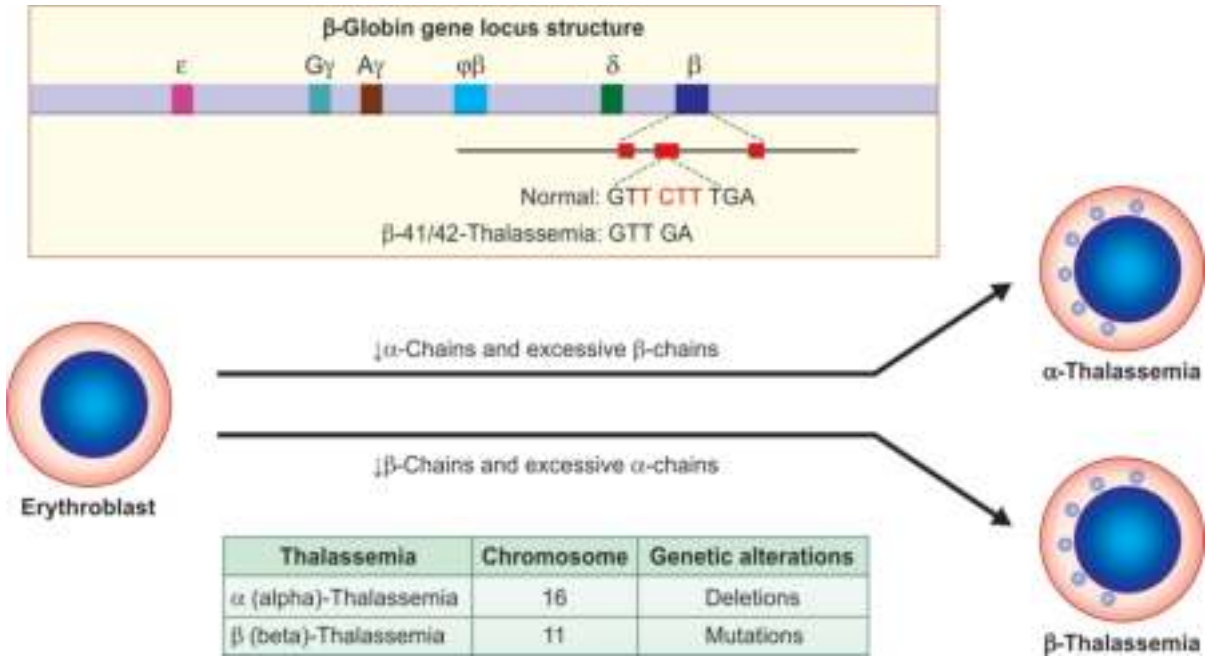


Fig. 8.33: Classification of thalassemias.

Table 8.74 Classification of α-, β- and δβ-thalassemias

Phenotype of Thalassemia	Globin Chain Synthesis Affected	Hemoglobin Synthesis in Patient	Peripheral Blood Smear, Bone Marrow, Hematocrit Values and Serum Assays	Clinical Features
β-Thalassemias				
Thalassemia major	β^0/β^0 due to total HBB gene mutation and absence of β-globin chain synthesis (homozygous state)	▪ HbA (nil) ▪ HbA_2 (3.8%) ▪ HbF (>90%)	▪ Peripheral blood smear examination shows microcytic hypochromic picture, numerous target cells, basophilic stippling ▪ Bone marrow shows erythroid hyperplasia and increased bone marrow iron due to hemolysis and repeated blood transfusion (generalized hemosiderosis) ▪ Serum iron increased ▪ Serum ferritin increased ▪ MCH, MCHC and MCV decreased ▪ Hemoglobin electrophoresis diagnostic significance ▪ High-performance liquid chromatography (HPLC) diagnostic significance	▪ Pallor due to severe anemia since childhood and failure to thrive ▪ Mongoloid face and other skeletal abnormalities ▪ Marked splenomegaly ▪ Hepatomegaly moderate ▪ Septicemia ▪ Transfusion-dependent disease
Thalassemia minor/trait	β^0/β due to HBB gene mutation (heterozygous state)	▪ HbA (90%) ▪ HbA_2 (3.8%) ▪ HbF (+/–)	▪ Peripheral blood smear examination shows mild microcytic and hypochromic picture, few target cells and basophilic stippling ▪ Hemoglobin electrophoresis diagnostic ▪ High-performance liquid chromatography diagnostic significance	Usually asymptomatic and mild pallor

Contd...

Table 8.74 Classification of α-,β- and δβ-thalassemias (*Contd...*)

Phenotype of Thalassemia	Globin Chain Synthesis Affected	Hemoglobin Synthesis in Patient	Peripheral Blood Smear, Bone Marrow, Hematocrit Values and Serum Assays	Clinical Features
Thalassemia intermedia	β^0/β^+ due to HBB gene mutation (double heterozygous state)	▪ HbA (50–70%) ▪ HbA_2 (3.8%) ▪ HbF (20–40%)	▪ Peripheral blood smear shows microcytic hypochromic picture ▪ Bone marrow shows erythroid hyperplasia and increased iron stores ▪ MCH, MCHC and MCV decreased	Anemia manifests during third to fourth decade of life
α-Thalassemias				
Silent carrier	α/αα due to HBA gene mutation (deletion of single α-globin chain located on chromosome 16)	Normal findings	Minority of patients show reduced mean cell volume and mean corpuscular hemoglobin	Asymptomatic
α-Thalassemia minor/trait	α/–α or –/αα due to HBA gene mutation (deletion of two α-globin chains located on chromosome 16)	Normal findings	▪ Hemoglobin is normal or slightly reduced decreased mean corpuscular volume and corpuscular hemoglobin ▪ Peripheral blood smear shows mild microcytic and hypochromic picture	Usually asymptomatic
Hemoglobin H disease	–α/– due to HBA gene mutation (deletion of three α-globin chains located on chromosome 16)	▪ HbA (70–90%) ▪ HbH (5–30%)	▪ Formation of unstable nonfunctional hemoglobin, i.e. failure to transport oxygen ▪ Moderate microcytic hypochromic hemolytic anemia	Anemia proportional to concentration of hemoglobin
Hydrops fetalis	–/– (loss of all four α-globin alleles due to HBA gene mutation)	▪ Hb Bart (>80%) ▪ HbH (10–20%) ▪ HbA (nil)	Severe disease not compatible with life. Intrauterine death or stillborn at 25–40 weeks or dies after birth	Intrauterine death of fetus
δβ-Thalassemias				
δβ-Thalassemia major	$\delta\beta^0/\delta\beta^0$	▪ HBA (absent) ▪ HbA_2 (absent) ▪ HbF (100%)	▪ Mild anemia (microcytic hypochromic picture and target cells) ▪ Recent onset of jaundice	▪ Pallor ▪ Palpable spleen
δβ-Thalassemia minor	$\delta\beta^+/\delta\beta^0$	▪ HBA (80–90%) ▪ HbA_2 (3%) ▪ HbF (100%)	▪ Mild anemia (microcytic hypochromic picture and target cells) ▪ Recent onset of jaundice	▪ Pallor ▪ Palpable spleen

β: *Normal β-globin gene and normal synthesis of β-globin chain.*
β^0: *Total depletion of β-globin gene and absence of synthesis of β-globin chain.*
β^+: *Partial depletion of β-globin gene and reduced synthesis of β-globin chain.*

- On the other hand, gene mutation in the splicing region causes β_1-thalassemia characterized by some synthesis of β-chains. Possible effects of point mutation of globin gene point and functional consequences (β-thalassemia) (Table 8.75).

Bone Marrow Changes

Development of hemolytic anemia stimulates erythropoietin synthesis resulting in erythroid hyperplasia in bone marrow. Expansion of bone marrow leads to widening of diploë of the skull and other bones. Skull radiograph shows hair-end-on appearance.

Extramedullary Hematopoiesis

Extramedullary hematopoiesis occurs in liver and spleen.

Fetal Hemoglobin (HbF) Synthesis

Synthesis of γ-chains continues even after 6 months of age. These γ-chains combine with α-chains resulting in synthesis of HbF ($\alpha_2\gamma_2$) varying from 20 to 90%.

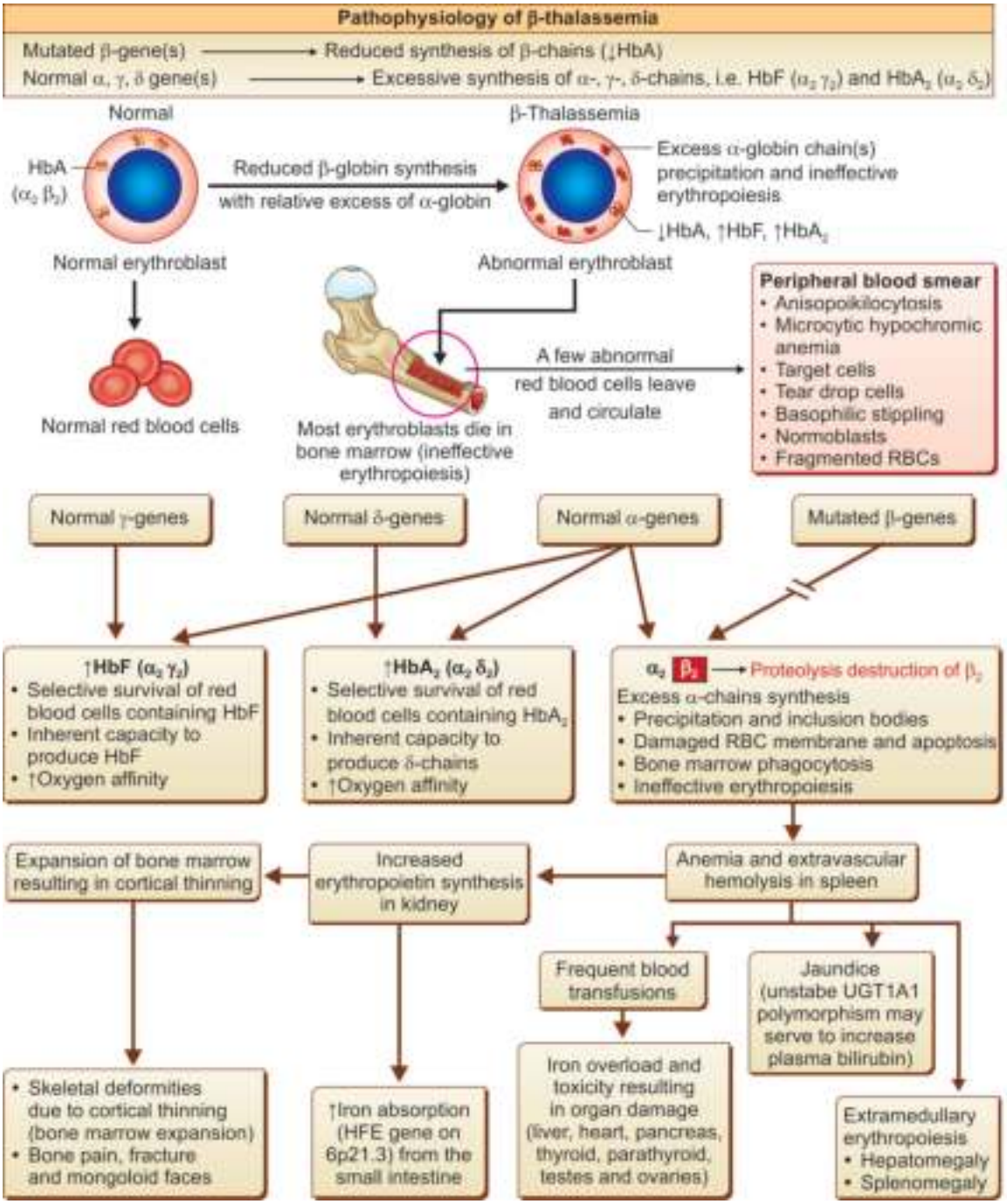


Fig. 8.34: Pathophysiology of β-thalassemia major. In β-thalassemia major, the decreased synthesis of β-globin chains reduces the production of HbA, and increases the synthesis of non-β-globin chain containing HbA_2 and HbF. Excess α-globin chains form insoluble precipitates inside red blood cells, damaging the red blood cell membrane and reducing their life span through splenic sequestration and ineffective erythropoiesis. All these factors contribute to a reduced oxygen delivery to the body tissues resulting in anemia and hypoxia. The compensatory erythroid hyperplasia in the bone marrow expands the bone marrow cavity by increased synthesis of erythropoietin, resulting in pathological fracture and mongoloid facial features.

Clinical Features

Clinical features begin during childhood after attaining 6–9 months of age as hemoglobin synthesis switch from HbF to HbA. Bone marrow transplantation is the only curative therapy. Clinical features of β-thalassemia major are shown in Fig. 8.35.

Severe Anemia

Anemia becomes within first year of life due to decrease in hemoglobin synthesis, marked shortening of RBC life span due to aggregation of insoluble excess α-chains, ineffective erythropoiesis and relative folate deficiency. Severe anemia impairs cardiac functions.

Table 8.75 Possible effects of point mutation of globin gene point and functional consequences (β-thalassemia)

Site of Point Mutation of Globin Chain	Possible Effects of Globin Gene Mutation	Example of Functional Consequences
Promoter site	Reduced transcription	β^+-Thalassemia
Initiation codon site	Methionine not encoded, hence absence of transcription	β^0-Thalassemia
Splice site	Absence of normal transcription	β^0-Thalassemia
Consensus site	Reduced transcription	β^+-Thalassemia
Intron site	Creation of false spice	β^+-Thalassemia
STOP codon site	STOP codon is converted to a coding sequence	α-Thalassemia due to elongation of globin chain and decreased synthesis at a reduced rate

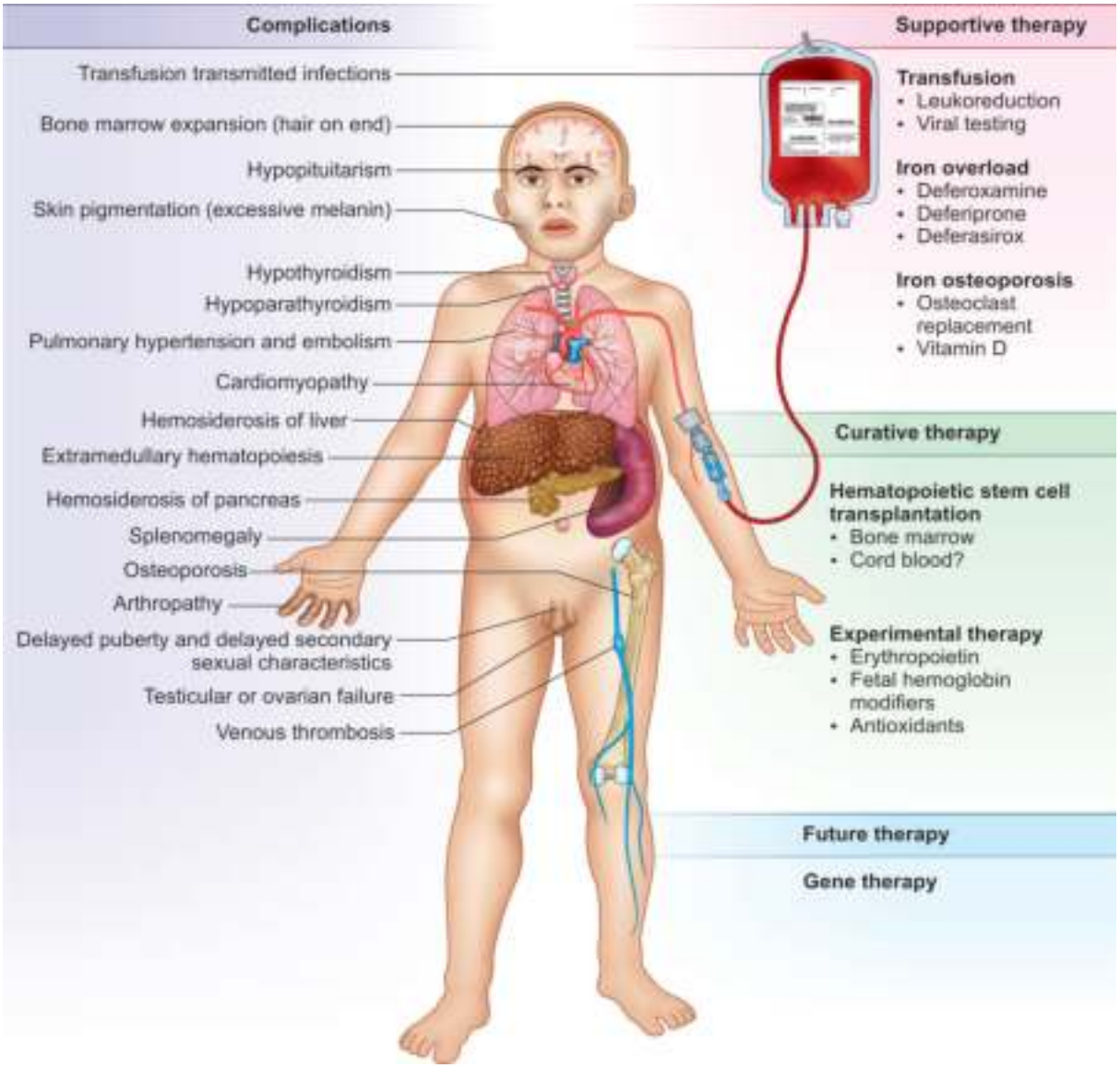


Fig. 8.35: Clinical features of β-thalassemia major.

Failure to Thrive

If untreated, the β-thalassemia major often results in failure to thrive. Impairment of growth results in short stature, diarrhea and recurrent infections.

Bone Marrow Expansion

Increased oxygen affinity of hemoglobin F (HbF) and the underlying anemia impair oxygen delivery and resulting in marked bone marrow erythroid hyperplasia.

Expansion of bone marrow causes facial and cranial bone deformities resulting in mongoloid face. These bone changes are demonstrated by X-rays in skull, long bones, small bones of hands and feet. Pathological fractures and bone pain may occur.

Marked Splenomegaly

Child develops marked splenomegaly by attaining age of 3 years due to sequestration, intramedullary destruction of red blood cells and extramedullary hematopoiesis.

Hepatomegaly

Child develops moderate to marked hepatomegaly as a result of iron overload and extramedullary hematopoiesis. Excessive absorption of iron due to ineffective erythropoiesis and repeated blood transfusions cause iron overload.

Spinal Cord Compression

Extramedullary hematopoiesis can occur in patients with β-thalassemia major, which can lead to spinal cord compression.

Generalized Hemosiderosis

Excess iron deposition in tissues is a major cause of morbidity and mortality in these patients. Extravascular hemolysis together with repeated blood transfusions creates iron overload. Excessive iron is deposited in bone marrow, liver (Kupffer's cells), myocardium, pituitary gland, hypothalamus, pancreas (islets of Langerhans) and parathyroid glands. Hemochromatosis is rare, if it occurs, patient may develop bronze diabetes comprising of cirrhosis, diabetes mellitus and skin pigmentation.

Septicemia

Septicemia is one of the leading causes of mortality in patients with β-thalassemia major.

Laboratory Diagnosis of β-Thalassemia Major

Hemoglobin

Patient suffers from moderate to severe anemia. Hemoglobin level ranges between 3 and 8 g/dl.

Routine Investigations

Hemoglobin, MCH, MCHC and MCV are decreased. Serum bilirubin is slightly increased. Reticulocyte count is increased. Osmotic fragility is decreased.

Peripheral Blood Smear Examination

Peripheral blood smear findings are shown in Fig. 8.36A and B.

- **RBCs morphology:** RBCs show marked anisocytosis, poikilocytosis with microcytic hypochromic picture.
  - Presence of target cells is characteristic finding.
  - **Basophilic stippling** is constant findings. Normoblasts range between 5 and 40/100 WBCs.
  - There is presence of tear drop cells, inclusion bodies in red blood cells due to aggregation of α-chains, and fragmented red blood cells. Target cells in various disorders are given in Table 8.76.
- **WBCs morphology:** TLC normal or increased. DLC shows slight shift-to-the-left with presence of some myelocytes and metamyelocytes.
- **Platelets:** Platelet count is normal but may be decreased due to sequestration of platelets in cases showing splenomegaly.

Reticulocyte Count

Reticulocyte count is increased 3–10%. Brilliant cresyl blue stain is used for reticulocyte count.

Bone Marrow Smear Examination

Bone marrow of β-thalassemia major is shown in Fig. 8.37A and B.

- **Cellularity:** Bone marrow is hypercellular as a result of erythroid hyperplasia.
- **Erythroid series:** Erythroid hyperplasia with micronormoblastic erythropoiesis (e.g. basophilic and polychromatic normoblasts) is seen.
  - Siderotic nodules are commonly seen in developing normoblasts.
  - Some normoblasts demonstrate dyserythropoiesis with irregular nuclear borders.
- **Myeloid series:** Myeloid precursors are within normal range. Myeloid hyperplasia occurs due to infection.
- **Myeloid–erythroid ratio:** M:E ratio is decreased.
- **Megakaryocytes:** Megakaryocytes are normal.

Bone Marrow Iron

Iron stores are increased in bone marrow due to hemolysis and multiple blood transfusions, demonstrated by Perls reaction/ Prussian blue reaction. Iron study in iron deficiency and β-thalassemia major is given in Table 8.77.

Naked Eye Single Tube Red Cell Osmotic Fragility Test (NESTROFT)

NESTROFT is most common screening test for thalassemia in India and across world. Normal saline (0.35%) is added to the test tube containing blood sample. Keep it for 30 minutes. A plain white paper with a black line is placed behind the test tube. Black line is visible in normal person as a result of hemolysis. On the other hand, black line is not visible in blood sample of thalassemia patient, because red blood cell membranes are relatively stable and resistant to hemolysis.

Fetal Hemoglobin (HbF) Estimation

Fetal hemoglobin is high ranging between 30 and 90% especially in β-thalassemia major estimated by alkali denaturation test. HbF may also be demonstrated by acid elution test. RBCs containing HbF are well stained. Red blood cells containing HbA appear as ghost cells.

Hemoglobin Electrophoresis

Diagnosis of β-thalassemia major is confirmed by hemoglobin electrophoresis with increased level of HbF in range of 30–90%. HbA_2 is also increased in these patients. Synthesis of HbF ($\alpha_2\gamma_2$) continues throughout life even after 6 months of age. The γ-chains combine with α-chains result in formation of fetal hemoglobin. In β-thalassemia, HbF and HbA_2 are present but no HbA.

High Performance Liquid Chromatography (HPLC)

HPLC is one of the commonest laboratory investigations performed for the identification of abnormal hemoglobin.

Molecular Genetic Alterations

DNA analysis is carried out to demonstrate gene mutations.

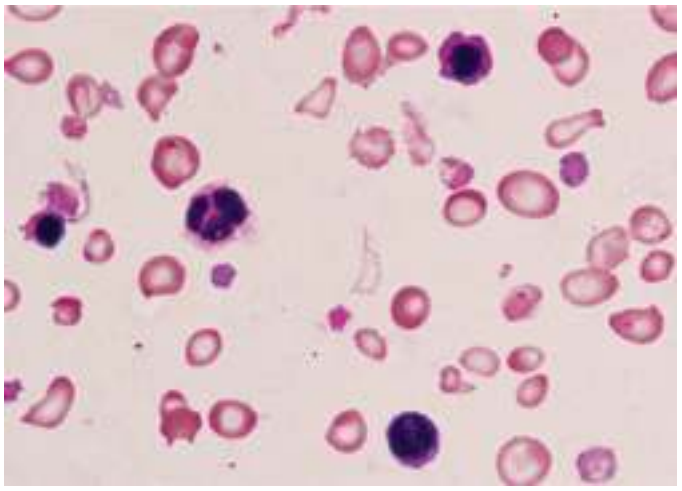


Fig. 8.36A: β-Thalassemia major of peripheral blood smear shows anisopoikilocytosis, many target cells and a few normoblasts.

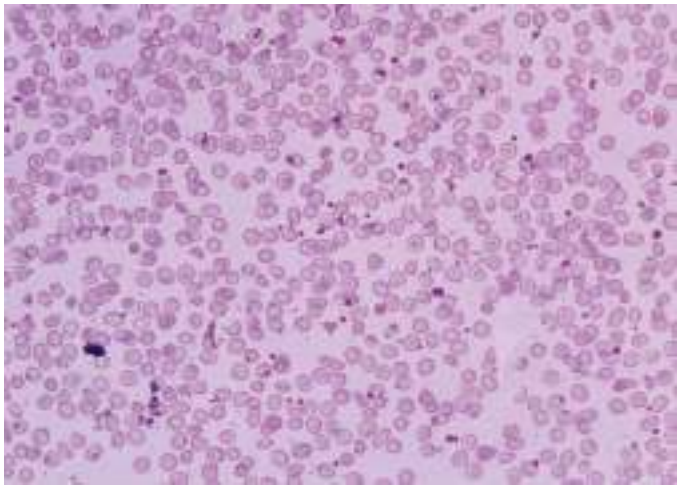


Fig. 8.36B: Peripheral blood smear examination in β-thalassemia major shows anisocytosis and poikilocytosis of red blood cell, microcytic hypochromic picture and numerous target cells.

β-THALASSEMIA MINOR (TRAIT)

In β-thalassemia minor (trait), a single aberrant β-globin gene is present on chromosome 11. The disorder may result in mild anemia, or no anemia at all. Heterozygotes are usually asymptomatic due to sufficient synthesis of β-globin. These patients have protection against malaria. Differences between β-thalassemia minor and iron deficiency anemia are given in Table 8.78.

Table 8.76 Target cells in various disorders

Microcytic Picture
▪ β-Thalassemia major (homozygous)
▪ β-Thalassemia trait (heterozygous)
▪ HbE homozygous and heterozygous
▪ HbH disease
▪ HbAC + HbC homozygous
▪ Hb Lepore homozygous and heterozygous
▪ HbO disease
▪ Iron deficiency
▪ Hb Lepore trait
Normocytic or Macrocytic Picture
▪ Obstructive jaundice
▪ LCAT deficiency (lecithin-cholesterol acyltransferase deficiency)
▪ Liver disease
▪ HbSC disease
▪ Hyposplenic state
▪ HbO-Arab disease

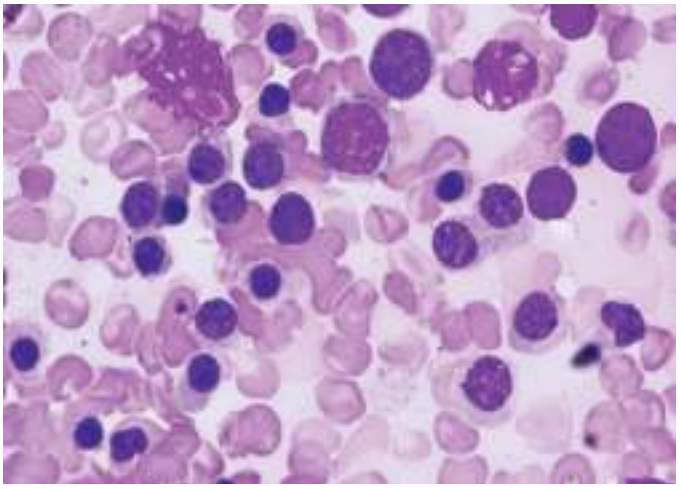


Fig. 8.37A: β-Thalassemia major. Bone marrow shows erythroid hyperplasia. Some normoblasts demonstrate dyserythropoiesis with irregular nuclear borders.

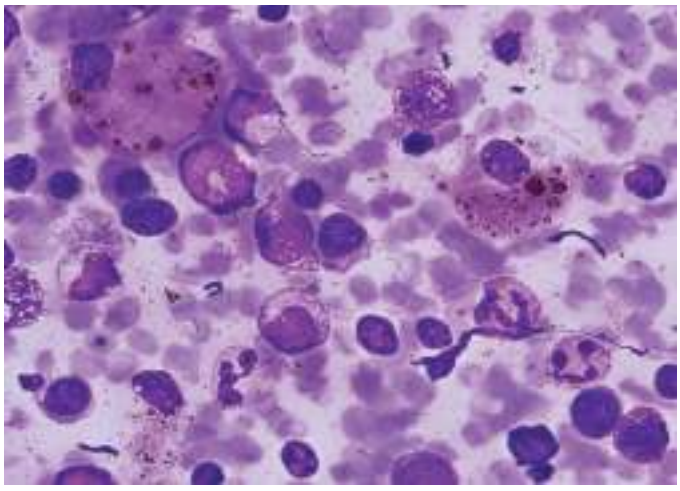


Fig. 8.37B: Bone marrow in β-thalassemia major shows erythroid hyperplasia with decreased myeloid to erythroid ratio. Bone marrow iron is increased. Iron can demonstrated by Perls Prussian blue stain.

Table 8.77 Iron study in iron deficiency and β-thalassemia major

Iron Staudy	Iron Deficiency	β-Thalassemia Major
Serum iron	Decreased	Normal/increased
Serum ferritin	Decreased	Normal/increased
TIBC	Increased	Normal/increased
Percent saturation	Decreased	Increased
Storage iron	Absent	Increased
FEP (free erythrocyte protoporphyrin)	Increased	Normal

Laboratory Diagnosis of β-Thalassemia Minor

- **Peripheral blood smear:** Peripheral blood smear examination shows minimal hypochromic microcytic anemia, target cells, and basophilic stippling.
- **Hematocrit values:** RBCs have reduced MCV and MCH.
- **Hemoglobin electrophoresis:** Increased HbA_2 ($\alpha_2\delta_2$) level between 3 and 6% is diagnostic for β-thalassemia minor. It demonstrates double the normal amount of hemoglobin A_2 ($\alpha_2\delta_2$), about 3–6%, and up to 5% fetal hemoglobin ($\alpha_2\gamma_2$). This finding is useful in distinguishing β-thalassemia minor from iron deficiency anemia and the anemia of chronic disease.

β-THALASSEMIA INTERMEDIA

β-Thalassemia intermedia is of less severity due to sufficient quantity of HbA. Insoluble α-globin chain aggregates are absent. These patients are anemic, who become blood transfusion-dependent later during adulthood.

- Complications of β-thalassemia intermedia are not as severe as for β-thalassemia major. These patients are diagnosed between 15 and 30 years of age. Hemolysis and increased iron absorption result in increased body iron stores.
- β-Thalassemia intermedia is double heterozygotes due to interaction of α, β with HbD, HbE, HbS and HbE.
- The clinical presentation of β-thalassemia intermedia is intermediate between β-thalassemia major and β-thalassemia minor.
- Hemolysis and increased iron absorption result in increased body iron stores.

Clinical Features

Patient presents with pallor, mild to moderate splenomegaly, skeleton changes (many cases), leg ulcers and infections.

Laboratory Diagnosis of β-Thalassemia Intermedia

- **Peripheral blood smear:** Peripheral blood smear shows microcytic hypochromic anemia, moderate degree of anisocytosis, poikilocytosis, target cells and tear drop cells.
- **Hemoglobin and hematocrit values:** Hemoglobin varies between 7 and 10 g/dl. MVC, MCH, and MCHC are decreased.
- **Bone marrow:** Bone marrow examination shows erythroid hyperplasia with increased iron stores.
- **Fetal hemoglobin:** HbF ranges between 10 and 30% in comparison to thalassemia major with HbF 40–90% (usually >90%).

α-THALASSEMIAS

α-Thalassemia is caused by α-globin gene deletion, which results in reduced or absent synthesis of α-globulin chains. Alpha-globin gene consists of 4 alleles and disease severity ranges from mild to severe disorders depending on the number of deletions of the alleles. Four alleles deletion is most severe disorder in which no α-globins are synthesized and the excess γ-chains form tetramers during intrauterine.

Table 8.78 Differences between β-thalassemia minor and iron deficiency anemia

Parameters	β-Thalassemia Minor	Iron Deficiency Anemia
Peripheral blood smear examination	Microcytic hypochromic picture	Microcytic hypochromic picture
RDW	Normal	Increased
Mentzer's index calculated by dividing MCV by RBCs	<13	>13

It is incompatible with life and results in hydrops fetalis. One allele deletion is the mildest form and is mostly clinically silent.

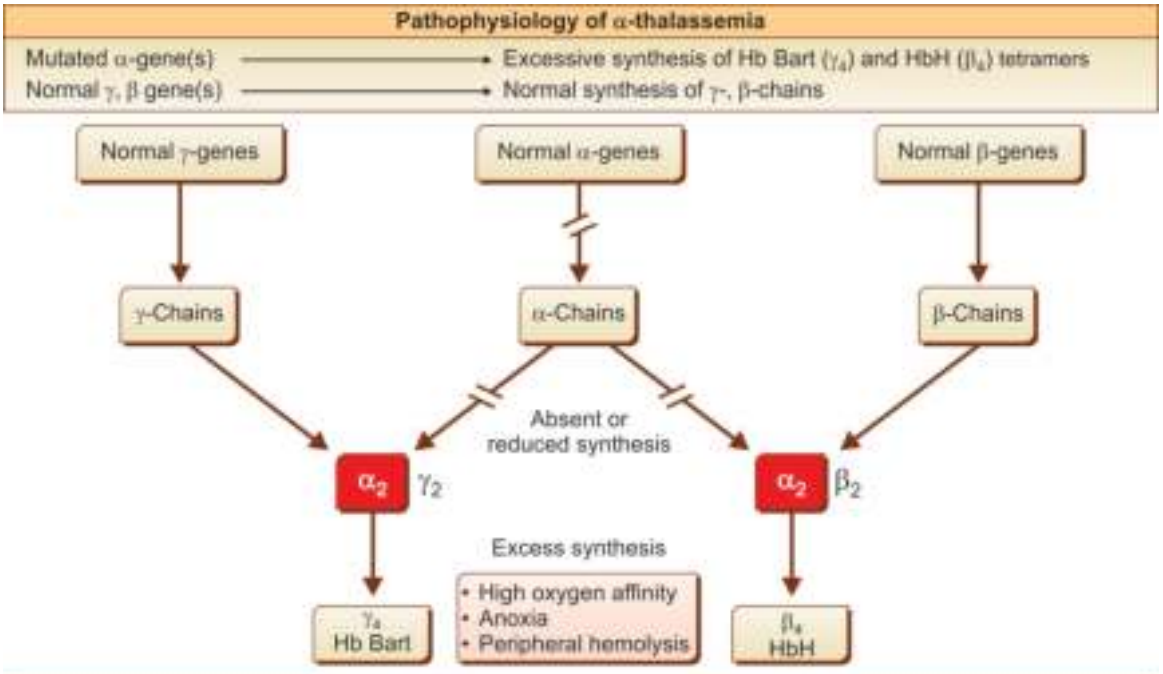
- α-Thalassemias are most common disorders in South-East Asia. Deletions of single or more α-globin genes on chromosome 16 result in reduced or deficient synthesis of α-globin.
- Clinical manifestations occur due to imbalanced synthesis of α- and non-α-chains such as γ-chain during infancy and β- and δ-chains at 6 months of age. Pathogenesis of α-thalassemia is shown in Fig. 8.38.
- α-Thalassemias are classified on the basis of the number and position of the α-globin genes deletion: silent carrier, α-thalassemia trait, hemoglobin H disease (β_4) and hydrops fetalis.

SILENT CARRIER OF α-THALASSEMIA

Silent carrier of α-thalassemia occurs due to deletion of single α-gene on chromosome 16. These silent carrier of α-thalassemia patients are asymptomatic without development of anemia.

α-THALASSEMIA TRAIT

α-Thalassemia trait occurs due to deletion of two α-genes on chromosome 16 (Asians) or two α-genes (one each on chromosome) in Africans. Peripheral blood smear examination shows absence or minimal anemia, and some microcytic red blood cells. These patients are asymptomatic without physical signs, and clinical findings remain identical to β-thalassemia minor showing microcytic hypochromic picture.



α-Thalassemia syndrome	Hematologic findings	Electrophoresis findings
Silent carrier (–α/αα) one gene deletion	No anemia or microcytosis	1–2% Hb Bart (γ_4) tetramers
Thalassemia trait (–α/–α) two genes deletion	Mild anemia and microcytosis	3–10% Hb Bart
HbH disease (–/–α) three genes deletion	Moderate microcytic, hypochromic hemolytic anemia and increased reticulocytes	20–40% Hb Bart
Hb Bart's hydrops syndrome (–/–) four genes deletion	Severe microcytic hypochromic anemia (lethal)	80% Hb Bart, 20% HbH (β_4) tetramers

Where αα/αα is normal (that is, 4 α-genes) and –α represents deletion of one α-gene on a chromosome
Only three genes deletion α-thalassemia has high reticulocytes

Fig. 8.38: Pathogenesis of α-thalassemia

HEMOGLOBIN H DISEASE (β_4)

Hemoglobin H disease occurs due to deletion of three α-genes on chromosome 16, which leads to formation of unstable tetramers of β-globin (β_4) known as HbH. These unstable tetramers are nonfunctional in oxygen transport, so anemia is disproportionate to hemoglobin level. Patient presents with moderate hemolytic anemia, which resembles β-thalassemia intermedia.

Laboratory Diagnosis of Hemoglobin H Disease

- Hemoglobin level is decreased.
- Peripheral blood smear examination shows microcytic hypochromic picture, target cells and Heinz bodies with anisocytosis and poikilocytosis.
- Hematocrit values (MCV, MCH, and MCHC) are decreased. Reticulocyte count is increased.

HYDROPS FETALIS

Hydrops fetalis disorder occurs due to deletion of all four α-globin chains. Due to absence of α-chains, fetus synthesizes excess of γ-globin chains forming tetramers known as hemoglobin Bart (γ_4). During intrauterine life, hemoglobin Bart (γ_4) is unable to deliver the oxygen to tissues. Without intrauterine transfusions, the fetus invariably dies. Newborn is either stillborn or death occurs immediately after birth due to pulmonary hypoplasia or cardiac failure. Hydrops fetalis, the most common, occurs in persons of South-East Asian ancestry.

Clinical Features

In hydrops fetalis, fetus shows pallor, generalized edema and massive hepatosplenomegaly.

Laboratory Diagnosis of Hydrops Fetalis

- **Peripheral blood smear:** Peripheral blood smear examination shows macrocytic hypochromic anemia, marked anisocytosis, poikilocytosis, with numerous normoblasts. MCV is more than 110 femtoliter. MCH is decreased.
- **Reticulocyte count:** Reticulocyte count is increased.
- **Hemoglobin electrophoresis:** Hemoglobin electrophoresis will reveal affected fetuses or neonates to have about 80% hemoglobin Bart (γ_4, a tetramer of γ-chains) and about 20% hemoglobin Portland ($\gamma_2\zeta_2$) or sometimes hemoglobin Gower 1 ($\zeta_2\varepsilon_2$) normally present only in embryonic life in the first trimester. There is absence of adult hemoglobin.

HEREDITARY PERSISTENCE OF FETAL HEMOGLOBIN

Hereditary persistence of fetal hemoglobin (HPFH) is characterized by continuous synthesis of fetal hemoglobin (HbF) in significant quantity since postnatal to adulthood life due to mutation of β-globin gene cluster. Fetal hemoglobin concentration ranges between 10 and 100%. These patients are most often asymptomatic and detected during screening.

SICKLE CELL DISORDERS AND OTHER HEMOGLOBINOPATHIES

Every person has combination of 2α genes and 2β genes responsible for adult hemoglobin A (HbA) synthesis.

- Persons having one normal adult hemoglobin A (HbA) gene and one sickle (HbS) gene known as heterozygous (sickle cell trait) having HbA (60%) and HbS (20–40%). These patients remain asymptomatic because sufficient HbA prevents polymerization of HbS.
- Persons having both sickle cell genes are known as *homozygous*. Such person has 85–95% of HbS. Sickle cells are demonstrated in the peripheral blood smear in sickle cell anemia in contrast to sickle cell trait. Double heterozygote state occurs as a result of interaction of HbS with thalassemia (α, β), HbC, HbD and HbE. Sickle cell disorders are given in Table 8.79.
- Structural gene mutations involving β-globin chain in HbS, HbC, HbD, HbE and α-globin chain in HbG are given in Table 8.80.

Table 8.79 Sickle cell disorders

HbSS (sickle cell anemia)	HbS/C-Harlem
HbSC disease	HbS/S Antilles
HbS/B^+ thalassemia	HbS/Lepore
HbS/B^0 thalassemia	HbC/C-Harlem
HbSD disease	HbS-Antilles (heterozygous)
HbSO-Arab	HbS-Oman (heterozygous)

- Sickle cell disease (homozygous HbS) and sickle cell trait (HbA and HbS) are given in Table 8.81.

SICKLE CELL DISEASE

Sickle cell disease is an autosomal recessive disorder, which most often affects African and Mediterranean populations. Approximately 7% of African Americans

Table 8.80 Structural gene mutations involving β-globin chain in HbS, HbC, HbD, HbE and α-globin chain in HbG

Disorder	Globin Gene Mutation and Inheritance	RBC Morphology	Laboratory Diagnosis
Sickle cell disease (HS)	▪ Change in 6th amino acid of the β-globin chain from glutamate to valine (β_6 glutamine → valine) ▪ Hemoglobin S trait (A/S) ▪ Hemoglobin S disease (S/S)	Sickle cells in peripheral blood smear	Hemoglobin electrophoresis
Hemoglobin C (HbC) disease	▪ Change in 6th amino acid of the β-globin chain from glutamate to valine (β_6 glutamine → lysine) ▪ Hemoglobin C trait (A/C) ▪ Hemoglobin C disease (C/C)	Target cells in peripheral blood smear	Hemoglobin electrophoresis
Hemoglobin D (HbD) disease (affects East Indian population)	Change in 121st amino acid of the β-globin chain from glutamate to glutamine (β_{121} glutamine → glutamine)	Mild hemolytic anemia	Hemoglobin electrophoresis (HbD migrates with HbS at pH 8.6)
Hemoglobin E (HbE) disease (affects South-East Asian population)	▪ Change in 26th amino acid of the β-globin chain from glutamate to lysine (β_{26} glutamine → lysine) ▪ Hemoglobin E trait (A/E) ▪ Hemoglobin E disease (E/E)	Microcytic hypochromic picture	Hemoglobin electrophoresis (HbE migrates with HbC and HbA_2 at pH 8.6)
Hemoglobin G (HbG) disease	Change in 68th amino acid of the α-globin chain from asparagine to lysine (α_{68} asparagine → lysine)	Mild hemolytic anemia	Hemoglobin electrophoresis

Table 8.81 Sickle cell disease (homozygous HbS) and sickle cell trait (HbA and HbS)

Sickle Cell disease (Homozygous HbS)	Sickle Cell Trait (HbA and HbS)
Red blood cells contain ≥80% HbS (rest is fetal hemoglobin)	Red blood cells contain <50% of HbS and rest HbA
Sickled red blood cells may be present in peripheral blood film: Sickling occurs at oxygen tension found in venous blood; cyclic sickling episodes	Renal papillary necrosis may occur in occasional case
Reticulocytes: Raised to 10–20%	Older persons are unable to concentrate urine
Variable hemolysis	Red blood cells do not sickle unless oxygen saturation <40% (rarely reached in venous blood)
Hand and foot syndrome (dactylitis)	Painful crises and splenic infarction have been reported in severe hypoxia such as unpressurized aircraft, anesthesia
Intermittent episodes characterized by bone pain, worsening anemia, or pulmonary or neurological disease	Sickling is more severe where HbS is present with another β-globin chain abnormality—such as HbS and HbC (HbSC) or HbS and HbD (HbSD)
Chronic leg ulcers common	Chronic leg ulcers absent
Gallbladder stones	Present

carry the hemoglobin S gene, which confers resistance to *Plasmodium falciparum* malarial infection. Prenatal diagnosis of HbS can be performed on amniotic cells or on chorionic villus samples. Hemoglobin percentage in normal adult person is given in Table 8.82.

Table 8.82 Hemoglobin percentage in normal adult person

Hemoglobin Type	Chains	Percentage
Hemoglobin A	$\alpha_2\beta_2$	97%
Hemoglobin A_2	$\alpha_2\delta_2$	1–3%
Hemoglobin F	$\alpha_2\gamma_2$	<1%

BASIC GENETIC DEFECT IN SICKLE CELL DISEASE

Sickle cell disease occurs due to partial missense point mutation in codon 6 of the **β**-globin gene resulting in substitution of valine for glutamic acid in the **β**-globin gene (Fig. 8.39A and B). Consequently, sickle cell hemoglobin (HbS) replaces normal adult HbA in the red blood cells. HbS causes polymerization of hemoglobin leading to formation of needle-like insoluble crystals within RBCs. Therefore, RBCs take shape of crescentic or holly leaf-like or boat shape, which are called sickle cells or drepanocytes. Solubility of HbS is altered by nonpolar (water insoluble) valine without affecting its structure, function and/or stability.

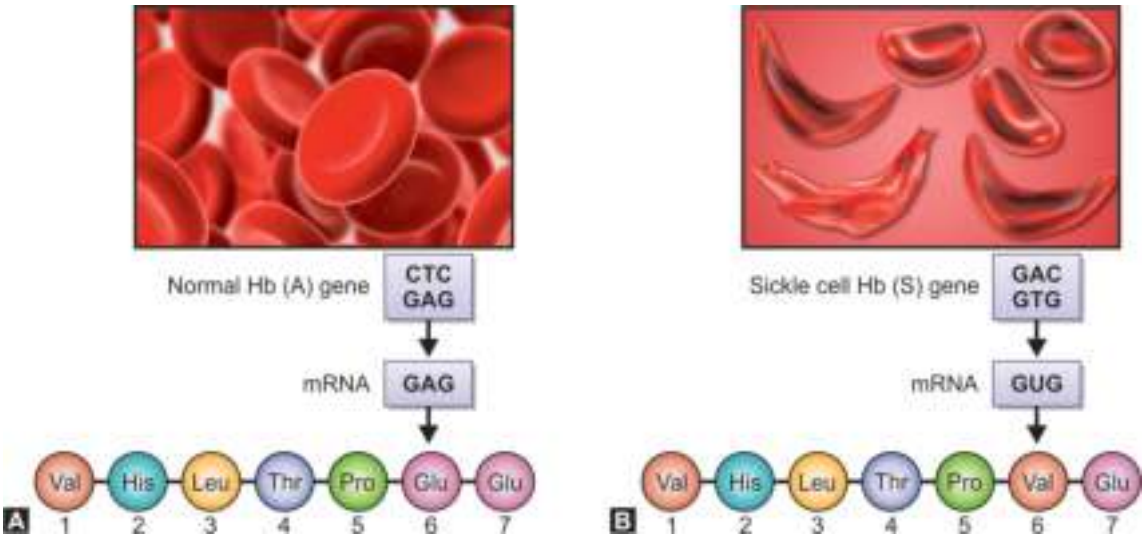


Fig. 8.39: (A) HbA peptide shows synthesis of normal hemoglobin; (B) HbS peptide shows synthesis of HbS as a result of replacement of glutamic acid (polar) by valine (nonpolar) in codon 6 of the β-globin gene.

CLINICAL FEATURES

Systemic complications of sickle cell disease are given in Table 8.83 and clinical features of sickle cell disease are shown in Fig. 8.40.

Severe Hemolytic Anemia

Deoxygenated HbS is 50 times less soluble in blood than deoxygenated HbA.

Table 8.83 Systemic complications of sickle cell disease

Systemic Complications	Comments
Hand and foot syndrome	Hand and foot syndrome occurs in infancy; painful swelling of digits
Painful crisis	Painful crisis occurs later in life, generalized bone pain; precipitated by cold, dehydration, self-limited clinical course over a few days
Aplastic crisis	Bone marrow temporarily becomes hypoplastic and may follow Parvovirus B19 infection, patient has profound anemia and reduced reticulocyte count
Splenic sequestration crisis	Splenic sequestration crisis occurs most often in infancy; progressive anemia; and splenomegaly; later autosplenectomy
Hepatic sequestration crisis	Hepatic sequestration crisis similar to splenic sequestration crisis but with sequestration of red blood cells in liver
Gallbladder stones	Pigment gallbladder stones due to hemolysis
Ischemic retinopathy	Blindness due to occlusion of retinal veins by sickle cells
Brain syndrome	Blockage of cerebral circulation and endothelial damage by sickle cells
Lung syndrome	Blockage of pulmonary circulation and endothelial damage by sickle cells
Renal function impairment	Papillary necrosis occurs due to occlusion of vasa recta by sickle cells
Infections	▪ *Streptococcus pneumoniae* ▪ *Haemophilus influenzae*
Progressive renal failure	Blockage of renal vessels by sickle cells cause progressive renal failure
Recurrent priapism	Blockage of renal vessels by sickle cells
Aseptic necrosis of humerus/femoral head	Occlusion of vessel by sickle cells supplying humerus or femur
Chronic osteomyelitis	*Salmonella typhi* in some cases
Impaired immune system	Autosplenectomy or hyposplenism increases susceptibility to infection
Chronic leg ulcers	Chronic leg ulcers around the malleoli around bilateral ankle joints due to stagnant blood flow due to occlusion of blood vessels by sickle cells
Priapism	Prolonged painful erection of penis due to venous occlusion by sickle cells

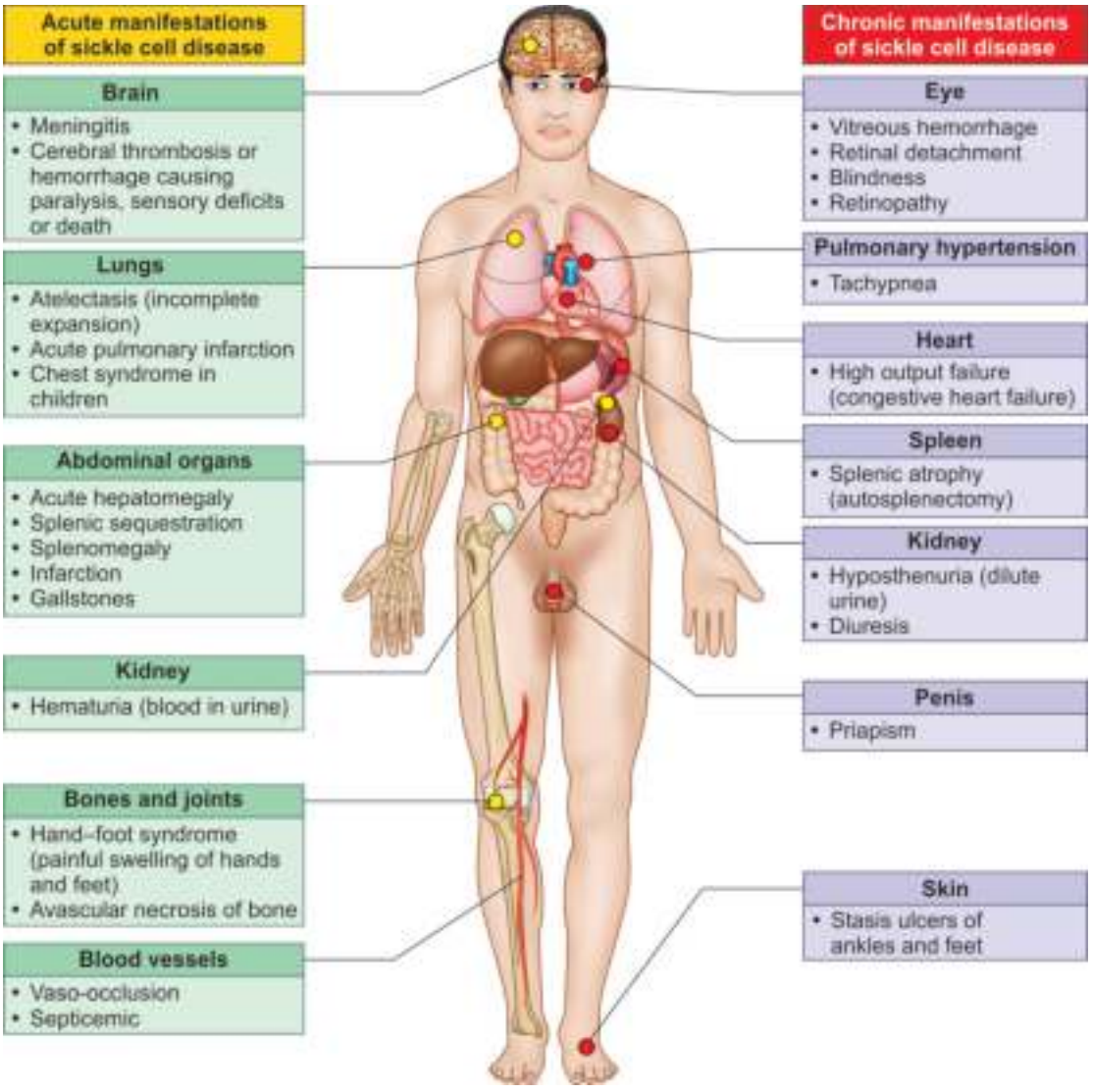


Fig. 8.40: Clinical features of sickle cell disease.

- Under low oxygen tension in capillaries, deoxygenated HbS comes out of solution forming long parallel fibers (needle-like insoluble crystals) called tactoids which distort the red blood cells to assume sickle or crescent shaped.
- Repeated episodes of red blood cell sickling lead to intravascular hemolysis and obstruction of the microvasculature.
- The life span of sickle cell becomes 10–20 days as opposed to a normal life span of red blood cell of 120 days. Sickle cell disease results in chronic hemolytic anemia with hemoglobin of around 5–8 g/dl.

Sickle Cell Crisis

Sickle cell disease leads to cell death and tissue infarction at the site of obstruction, which is termed a sickle cell crisis. Apart from hypoxia, acidosis irrespective of the prevailing oxygen tension is important reason for most sickling of red blood cells occurring in the venous circulation. Therapeutic agent is administered to increase HbF levels in these patients. Sickle cell crises are given in Table 8.84.

Vaso-occlusive Crisis

Vaso-occlusive crisis occurs at about 6 months of age after the reduction in fetal hemoglobin (HbF), which initially acts as a protective mechanism. Vaso-occlusive phenomenon is often precipitated by infection or dehydration. Patient develops infarction of bones, lungs, brain, renal papillae, spleen, and liver resulting in pain lasting for few minutes or several days.

- **Hand and foot syndrome:** Patient develops hand and foot syndrome characterized by dactylitis of small bones of hands and feet in first 2 years of life.

- **Avascular necrosis of femoral head:** Avascular necrosis of the femoral head often occurs in sickle cell patients. Aseptic necrosis is also referred to as osteonecrosis. Pain, tenderness and disability are frequently signs of avascular necrosis of femoral head.
- **Bone and joint crises:** Vascular occlusion of vertebrae, long bones such as femur, tibia, fibula and humerus undergo infarction. This can cause permanent damage to hips, shoulders or knees. Fish mouth vertebrae are demonstrated due to vaso-occlusive phenomenon. Skull shows crewcut appearance on radiographs due to expansion of bone marrow leading to bone resorption and new bone formation.
- **Acute chest syndrome:** Repeated vascular occlusive episodes result in pulmonary infarction and impaired lung functions. Patient presents with classical features of the acute chest syndrome characterized by dyspnea, cough, pleuritis (chest pain) and hemoptysis.
- **Renal papillary necrosis:** The relative hypoxia and hyperosmolarity in the renal medulla create an environment for sickling of red blood cells in the vasa recta, which leads to destruction of long loops of Henle resulting in renal papillary necrosis and renal failure. The kidney loses its ability to concentrate urine. Hematuria is also a complicating feature.
- **Priapism:** Prolonged painful penile erection occurs due to venous occlusion as a result of sickling of red blood cells is common, often requiring surgical decompression.
- **Liver manifestations:** Liver becomes congested with sickle cells. The liver is often firm and can become tender. Hepatocellular failure may supervene as a result of multiple infarcts or hemosiderosis from frequent blood transfusions. Patient may develop jaundice (yellow discoloration of sclera) and gall-bladder stones as a consequence of chronic hemolysis.
- **Autosplenectomy:** Spleen becomes congested and enlarged during childhood. Blood circulation in spleen is slow. Deoxygenation promotes sickling of red blood cells resulting in blockage of capillaries in spleen, and thus causing ischemic infarcts. Later, spleen becomes progressively smaller through repeated multiple tiny infarcts and fibrosis resulting in autosplenectomy. In autosplenectomy patients, peripheral blood smear shows numerous target cells, Howell-Jolly bodies, marked anisocytosis, poikilocytosis and thrombocytosis.

Aplastic Crisis

Patients with sickle cell anemia may undergo an aplastic crisis in 80% of cases due to infection of the bone marrow by human Parvovirus B19, which suppresses erythropoiesis leading to precipitous fall in hemoglobin concentration. Bone marrow failure (aplastic crisis) also occurs associated with a high mortality.

Splenic Sequestration Crisis and Autosplenectomy

Splenic sequestration crisis is most severe crisis in infants and children. Sudden pooling of blood in the spleen known as splenic sequestration results in severe anemia, shock, loss of consciousness with fatal outcome.

- Repeated RBCs sickling in spleen impairs blood supply, which results in autosplenectomy. Impairment of the normal function of the spleen increases the risk of infection.
- Patient presents with painful splenomegaly and hypovolemic shock. There is acute fall in hemoglobin usually secondary to infection induced hemolysis or an acute sequestration syndrome in the spleen. Blood transfusion is often essential.
- Gamna-Gandy bodies are demonstrated in the spleen due to organization of splenic hemorrhages. There is deposition of calcium and hemosiderin in Gamna-Gandy bodies.

Table 8.84 Sickle cell crises

Crisis	Comments
Vaso-occlusive crisis	Vaso-occlusive phenomenon in any tissue but especially, bone, chest and abdomen (e.g. splenic infarcts); in cerebral vessels resulting in cerebral stroke
Aplastic crisis	Aplastic crisis due to Parvovirus B19 infection
Sequestration crisis	Sequestration crisis especially occurs in infants and young children; massive pooling of red blood cells in spleen and other organs, e.g. liver resulting in precipitous drop in hemoglobin
Hemolytic crisis	Hemolytic crisis further reduces life span of red blood cells resulting in worsening anemia and features of hemolysis
Acute chest syndrome	Chest syndrome characterized by pleuritic pain and fever may mimic pneumonia or pulmonary embolism; progressive respiratory failure

Treatment of sickle cell crises includes vigorous intravenous hydration, adequate analgesia (e.g. intravenous opiates), broad spectrum antibiotics, oxygen therapy and consideration of exchange of blood transfusion.

Chronic Leg Ulcers

Patient of sickle cell disease is susceptible to chronic leg ulcers particularly around the malleoli of ankles and both sides of lower legs due to stagnant blood flow caused by sickled red blood cells. The leg ulcers are often complicated by trauma and poor hygiene.

Osteomyelitis

Damage to the spleen with increased susceptibility to bacterial infections occurs with age. Patient with autosplenectomy is prone to Salmonella infections. Salmonella is most often implicated in osteomyelitis.

Lobar Pneumonia and Meningitis

Lobar pneumonia is extremely common in children with sickle cell anemia. Patient with poor functioning of spleen is prone to *Streptococcus pneumoniae* infection. These children frequently develop meningitis due to pneumococci and *Haemophilus influenzae* bacteria.

Gallbladder Pigment Stones

Accumulated bilirubin in the liver can concentrate into crystals that build up in the gallbladder resulting in cholelithiasis.

Neurological Manifestations

Acute brain syndrome is rare but serious, complication of sickle cell disease characterized by confusion with variable neurological defects. There is an increased risk of subarachnoid hemorrhage, retinal hemorrhage (partial or complete blindness) and deafness.

Laboratory Diagnosis of Sickle Cell Disease

Following tests are useful to complement the clinical history and examination.

Hemoglobin

Normal hemoglobin level excludes sickle cell disease.

Peripheral Blood Smear Examination

- Person with sickle cell disease has mostly HbS. Peripheral blood smear examination shows sickle cells, some target cells, nucleated red blood cells, Howell-Jolly bodies and sideroblasts.
- Patients with sickle trait are usually fit and healthy. Peripheral blood film may show a few sickle cells in sickle cell trait (Figs 8.41 to 8.44).

Erythrocyte Sedimentation Rate

ESR is most often increased in all anemias in except sickle cell disease. Rouleaux formation does not occur in sickle cell anemia.

Serum Bilirubin

Unconjugated bilirubin may be raised in the blood as a result of the hemolytic anemia.

Sickling Test

- Sickling test is a screening test for sickle cell anemia. Mixing blood with the reducing agent, sodium metabisulphite on a sealed slide, will induce sickling of red blood cells.
- Sickling test is simple and quick. The results can be viewed under light microscope after 20 minutes.
- The sickle cell preparation is positive whenever hemoglobin S is present (e.g. sickle cell anemia, sickle cell trait, sickle C disease, sickle cell thalassemia). Sickling test in sickle cell disease is shown in Fig. 8.45.

High Performance Liquid Chromatography (HPLC)

HPLC is one of the commonest laboratory investigations performed for the identification of globin chain synthesis. It estimates quantity of normal and abnormal hemoglobins. It is the investigation of choice to confirm diagnosis of sickle cell anemia.

Hemoglobin Electrophoresis

- Hemoglobin electrophoresis is the second best method for diagnosing sickle cell disease. HbA moves faster than HbS towards anode.
- HbS moves faster than HbA towards cathode. Formation of 1 band indicates sickle cell anemia. Formation of 2 band is suggestive of sickle cell carrier.
- Hemoglobin moves in the S region on hemoglobin electrophoresis in alkaline pH. HbD, HbG, HbQ India and HB Lepore are also demonstrated in the same S region on hemoglobin electrophoresis. It differentiates between homozygous and heterozygous conditions.
- In the absence of electrophoresis, a positive sickling test associated with normal hemoglobin concentration indicates a patient with sickle cell disease.

Kidney Function Tests

Blood urea, serum creatinine and electrolytes are analyzed to assess renal function to rule out renal papillary necrosis.

Cardiovascular System Evaluation

ECG is performed to look for evidence of cardiac damage.

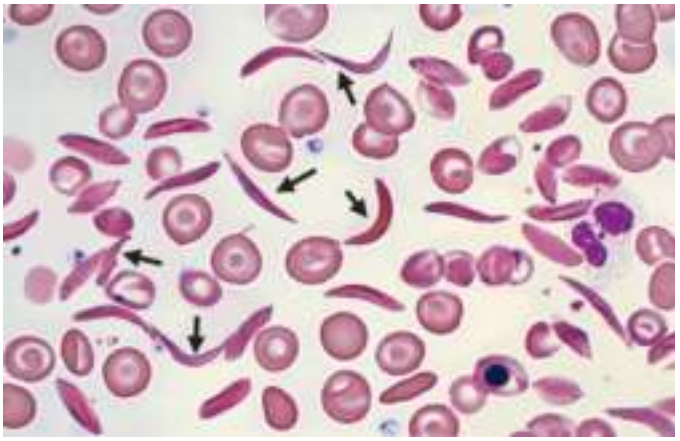


Fig. 8.41: Peripheral blood smear examination shows sickling (arrows).

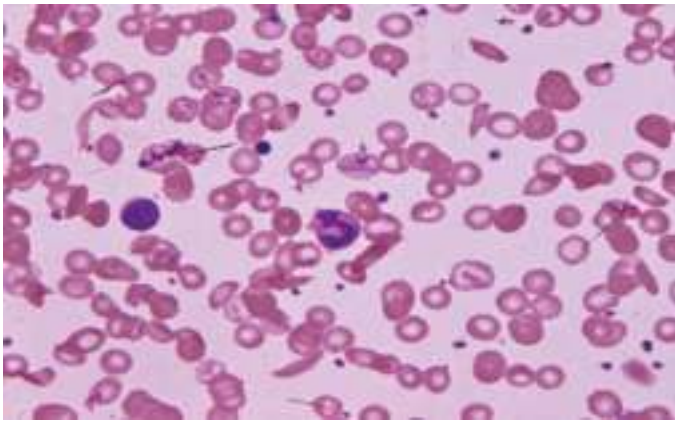


Fig. 8.42: Peripheral blood smear examination shows normocytic normochromic picture and many sickle red blood cells.

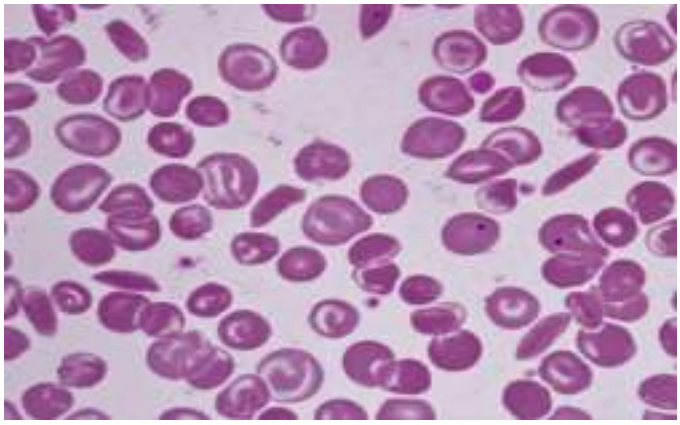


Fig. 8.43: Peripheral blood smear examination shows normocytic normochromic picture. Classic sickle red blood cells are demonstrated.

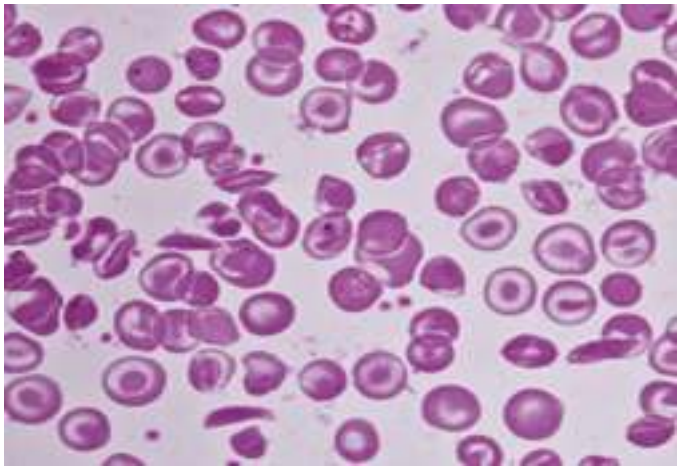


Fig. 8.44: Peripheral blood smear examination shows normocytic normochromic picture and many sickle red blood cells.

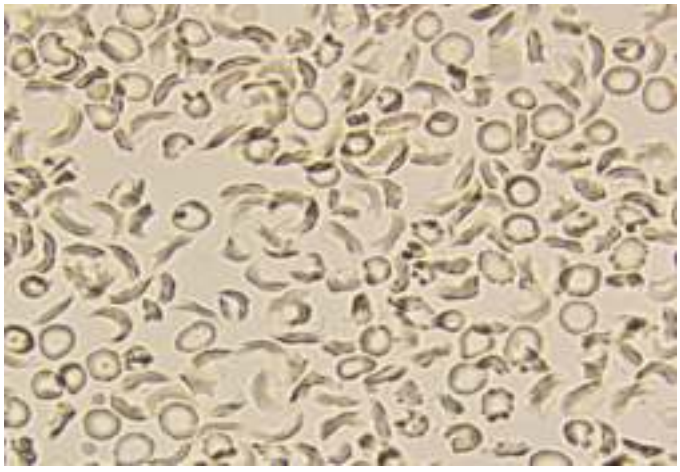


Fig. 8.45: Sickling test in sickle cell disease performed by mixing blood with the reducing agent, sodium metabisulphite on a sealed slide.

SICKLE CELL TRAIT

Sickle cell trait (HbAS) is benign disorder, which shows no hematological manifestations. It is associated with normal growth and life expectancy. It reduces the risk of falciparum malaria.

- Ratio of HbA to HbS is 60:40. Sickle cell trait affects 25–30% of the population in West Africa.
- Patients may have impaired urine concentration ability and hematuria. Pregnant women are at risk of development of urinary tract infection. There is risk of splenic infarction at high altitude and sudden death during exercise. Elevated plasma myeloperoxidase and RBC sickling have been observed during exercise with fluid restriction in sickle celltrait.
- Plasma levels of VCAM-1 are higher following exercise in sickle cell trait suggestive of subtle microcirculatory dysfunction. Anoxia, hyperosmolarity and low pH of the renal medulla predisposes to sickling of RBCs.
- Risk of venous thromboembolism is also increased in these patients.

Laboratory Diagnosis of Sickle Cell Trait

- Sickle cell trait is characterized by a laboratory profile of evidence of hemolytic anemia, hemoglobin between 5 and 11 gm/dl with increase in lactic dehydrogenase (LDH), indirect bilirubin and reticulocyte count, and decrease in plasma haptoglobin. Red blood cell density is increased with normal MCHC. Serum erythropoietin level is decreased.
- Sickle cell trait can be accurately diagnosed by high-performance liquid chromatography (HPLC) and isoelectric focusing. Polymerase chain reaction is the method of choice for prenatal diagnosis.

Peripheral Blood Smear Examination

- Peripheral blood smear examination shows normocytic normochromic picture. A few sickle red blood cells are demonstrated in sickle cell trait disease (Fig. 8.46).
- Elevated neutrophil count is observed in persistent low-grade inflammation.

Bone Marrow Smear Examination

Bone marrow smear examination shows erythroid hyperplasia.

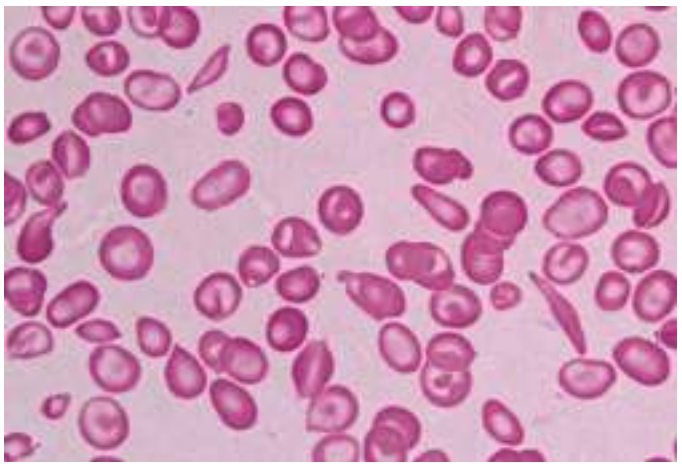


Fig. 8.46: Peripheral blood smear examination shows normocytic normochromic picture. A few sickle red blood cells are demonstrated in sickle cell trait disease.

SICKLE CELL THALASSEMIA SYNDROME

Sickle cell thalassemia syndrome results from coinheritance of hemoglobin S gene and thalassemic variant of the β-globin gene mutation (compound heterozygosity). Its clinical picture resembles thalassemia intermedia. Prognosis is better than sickle cell anemia and thalassemia.

HEMOGLOBIN C DISORDER

Hemoglobin C disorder primarily affects 2–3% of African lineage, which occurs due to point mutation in codon 6 of the β-globin gene on chromosome 11 resulting in substitution of lysine for glutamic acid in the β-globin gene. Consequently, hemoglobin C replaces normal adult hemoglobin (HbA) in the red blood cells. But it does not lead to sickling of red blood cells, but produces target cells.

HOMOZYGOUS HEMOGLOBIN C DISORDER

Homozygous person with mutation of two β-globin genes on chromosome 11 develops clinical manifestations such as anemia varying in severity, episodes of abdominal and joint pain, splenomegaly, and mild jaundice.

Laboratory Diagnosis of Homozygous HbC Disorder

- Serum bilirubin (indirect) concentration is increased.
- Peripheral blood smear examination shows numerous target cells and occasionally elongated hexagonal crystals in red blood cells particularly in splenectomized persons.
- Hemoglobin electrophoresis reveals 80–90% of HbC and 1–7% HbF. When hemoglobin C is present, the amount of hemoglobin A_2 is difficult to assess, as these two hemoglobins are difficult to distinguish by hemoglobin electrophoresis.

HETEROZYGOUS (HbS/HbC) DISORDER

Heterozygous (HbS/HbC) person develops disorder, when HbC and HbS are inherited together. Patients present with complications at about half the rate of patients with homozygous sickle cell disease. The patients are prone to avascular necrosis of the bone and proliferative retinopathy.

HEMOGLOBIN D DISORDER

Hemoglobin D is an autosomal recessive disorder that occurs when a person inherits two genes coding for the hemoglobin D, one from each parent. Parents of the affected children with hemoglobin D are usually not affected and remain healthy carriers. The parents have one copy of the hemoglobin D gene and are said to have hemoglobin D trait.

- Hemoglobin D disorder is seen most frequently among people of Asiatic Indian heritage and European descent: however, anyone can have the disorder.
- Homozygous hemoglobin D does not typically cause clinically significant symptoms. Occasionally, disorder can cause mild hemolytic anemia and mild splenomegaly. Anemia usually occurs in the first few months as fetal hemoglobin decreases (HbF) and hemoglobin D increases.
- Hemoglobin disorder-Punjab becomes significant when it is co-inherited with HbS and β-thalassemia. Genetic counseling is recommended for family planning during future pregnancies.
- Persons with hemoglobin D trait have red blood cells that contain normal hemoglobin A (HbA) more than an abnormal hemoglobin D.
- Hemoglobin D (HbD) trait is inherited from one's parent. If one parent has HbD trait and the other parent has normal hemoglobin, there is 50% chance with each pregnancy of having a child who has HbD trait. There are the possible outcomes of HbD disorder with each pregnancy: 50% of children have HbD trait and rest 50% without HbD trait.

HEMOGLOBIN E DISORDER

Hemoglobin E disorder is prevalent in South-East Asia. Clinical and laboratory manifestations are similar to those of hemoglobin C disorder. HbE disorder occurs due to point mutation in codon 26 of the β-globin gene on chromosome 11 resulting in substitution of lysine for glutamic acid in the β-globin gene. Consequently, hemoglobin E replaces normal adult HbA in the red blood cells. But it does not lead to sickling of red blood cells, but produces microcytosis.

HOMOZYGOUS HEMOGLOBIN E DISORDER

Homozygous HbE disorder in person having mutation of two β-globin genes on chromosome 11 develops mild anemia. Peripheral blood smear examination shows mild microcytic anemia with lower MCV. Hemoglobin electrophoresis demonstrates 90–95% hemoglobin E, 3–5% hemoglobin A_2, and 1–5% hemoglobin F.

HETEROZYGOUS HEMOGLOBIN E DISORDER

Heterozygous HbE disorder in person having mutation of single β-globin gene on chromosome 11 has no clinical manifestations. Peripheral blood smear examination shows microcytes with decreased MCV. Hemoglobin electrophoresis demonstrates 30–40% hemoglobin E and 60–70% hemoglobin A.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY AND PYRUVATE KINASE DEFICIENCY DISORDERS

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) DEFICIENCY DISORDER

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an X-linked recessive disorder affecting exclusively males that causes a hemolytic anemia characterized by abnormal sensitivity of red blood cells to oxidative stress (Fig. 8.47).

- Most G6PD deficient persons are clinically well until exposed to excessive oxidant stress induced by ingestion of fava beans and drugs (e.g. primaquine, chloroquine, phenacetin, nitrofurantoin, sulfonamides drugs) and infection (e.g. viral hepatitis, pneumonia and typhoid fever).
- G6PD deficiency disorder is the most common form of enzyme deficiency hemolytic anemia. Major hemolysis is of extravascular type, while minor component as intravascular hemolysis. G6PD should not be assessed during hemolytic episode, because reticulocytes contain normal G6PD. It should be assessed when reticulocyte count is within normal range.
- World Health Organization classification of G6PD deficiency disorder is given in Table 8.85.

EPIDEMIOLOGY

Full expression of G6PD deficiency disorder is seen only in males. Females are being asymptomatic carriers. Affected persons are also more prone to infection.
The highest prevalence of G6PD deficiency disorder is in Africa and the Mediterranean region in 10% of population. G6PD deficiency gives protection against malarial parasitic infection.

PATHOPHYSIOLOGY

Physiologic State

Under physiologic state, G6PD enzyme is involved in the hexose monophosphate shunt pathway. G6PD reduces nicotinamide adenine dinucleotide phosphate (**NADP**) to **NADPH**, which reduces glutathione within RBCs. Reduced glutathione aids in protecting red blood cells against oxidant injury.

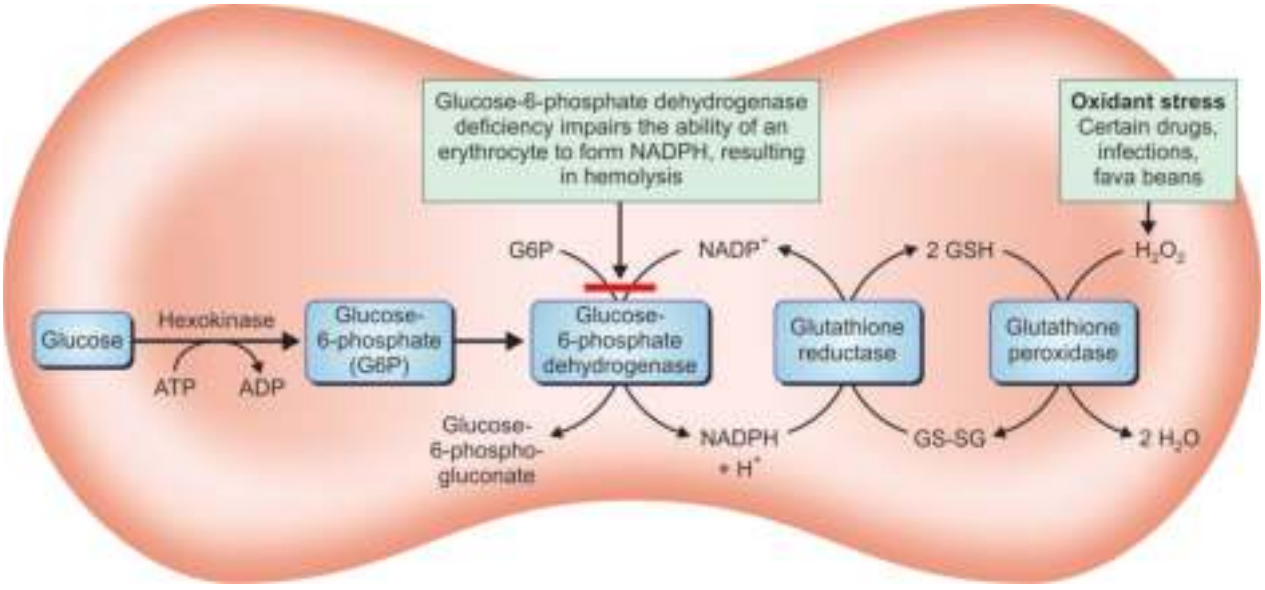


Fig. 8.47: Pathogenesis of glucose-6-phosphate dehydrogenase (G6PD) deficiency.

Table 8.85 World Health Organization classification of G6PD deficiency

Class of G6PD Deficiency Disorder	G6PD Enzyme Activity (% of Normal)	Examples	Clinical Consequences
Class I G6PD deficiency disorder	▪ Severe disease ▪ G6PD activity <2% of normal	Santigo de Cuba (Gly447Arg)	Congenital nonspherocytic hemolytic anemia
Class II G6PD deficiency disorder	G6PD activity <10% of normal	▪ Mediterranean Ser188Phe) Orissa (Ala44Gly)	▪ Favism and drug-induced acute intravascular hemolysis ▪ Neonatal jaundice
Class III G6PD deficiency disorder	G6PD activity >10% and >60% of normal	▪ A (Val68Met) ▪ Asn126Asp	Acute intravascular hemolysis (drug-induced), neonatal jaundice
Class IV G6PD deficiency disorder	G6PD activity 100% of normal	▪ B-Wild type ▪ A (Ana126Asp)	Nonclinical consequences
Class V G6PD deficiency disorder	G6PD activity 150% of normal	▪ B-Wild type ▪ A (Ana126Asp)	Nonclinical consequences

Table 8.86 WHO classification of mutant G6PD isoenzymes

Class	G6PD Deficiency	Hemolysis
I	Severely deficient G6PD seen in African American population (<10%)	Chronic hemolysis is self-limited course because younger RBCs are not affected
II	Mediterranean form with severely deficient G6PD	More severe acute episode of hemolysis. Patients are protective against *Plasmodium falciparum*
III	Mild to moderate deficient (10–60%)	Acute hemolysis (episodic)
IV	Mild to normal deficient	Hemolysis absent
V	Increased G6PD	Hemolysis absent

Pathologic State

- **Gene mutations:** Red blood cells deficient in G6PD are less resistant to oxidant injury.
  - Mutant gene on X chromosome results in either impaired G6PD enzyme synthesis or impaired enzyme stability due to loss of normal folding of G6PD proteins. The unfolding form of G6PD proteins undergo proteolytic degradation.
  - Any exposure to oxidants results in hemolysis, which mainly occurs in the older red blood cells due to reduced amount of G6PD, hence prone to hemolysis. WHO classification of mutant G6PD isoenzymes is given in Table 8.86.
- **Oxidant stress:** Under oxidant stress, inadequate G6PD activity leads to oxidation of sulfhydryl groups of hemoglobin resulting in precipitation of denatured hemoglobin is known as Heinz bodies.
  - Heinz bodies can cause direct injury to RBCs. Cell rigidity and increased membrane permeability cause erythrocyte injury leading to intravascular and extravascular hemolysis in spleen.
  - Splenic macrophages remove Heinz bodies and form bite cells demonstrated in peripheral blood smear.

CLINICAL FEATURES

Red blood cells are more prone to hemolysis in G6PD deficiency disorder. Patient presents with acute self-limited intravascular hemolytic anemia with intravascular hemoglobinemia and hemoglobinuria caused by oxidative stress. Patient may present with malaise, severe lethargy, nausea, vomiting, abdominal pain, fever with chills. Due to intermittent hemolysis, there is no splenomegaly and cholelithiasis. Characteristic features of hemolytic episode in G6PD deficiency disorder are given in Table 8.87.

Laboratory Diagnosis of G6PD Deficiency Disorder

Peripheral Blood Smear Examination

- Peripheral blood smear examination shows bite cells, Heinz bodies and polychromasia, fragmented RBCs.
- When RBCs containing Heinz bodies pass through the spleen, reticuloendothelial cells of spleen take a bite of red blood cells and remove Heinz bodies and result in formation of bite cells (Fig. 8.48).

Supravital Staining

- Heinz bodies are inclusions of denatured hemoglobin present near red blood cell membrane demonstrated by supravital

staining (brilliant cresyl blue or methylene blue) and not with Romanowsky stains.
- Failure of disposal of oxygen-derived free radicals converts hemoglobin to methemoglobin resulting in condensation to form Heinz bodies.

Fluorescent Spot Test

Principle of the fluorescent spot test is based on generation of NADPH by normal G6PD in the lysate of red blood cells to fluorescence under ultraviolet light. In G6PD deficiency, no fluorescence spot is observed as a result of lack of generation of NADPH.

Reticulocyte Count

Reticulocyte count is increased (8–10%).

Serum Bilirubin

Serum bilirubin is increased (indirect bilirubin) due to hemolysis.

Serum Haptoglobin

- Haptoglobin is α_2 glycoprotein synthesized by liver, which combines with any hemoglobin in plasma and prevents its excretion in urine.
- Haptoglobin–hemoglobin molecule is removed by liver, spleen and bone marrow. Hence, plasma haptoglobin levels are decreased with episode of hemolysis, which come to normal within 3 days.

Serum Lactic Dehydrogenase

Values of plasma lactic dehydrogenase are increased in hemolytic anemia (normal range is 125–270 microliter).

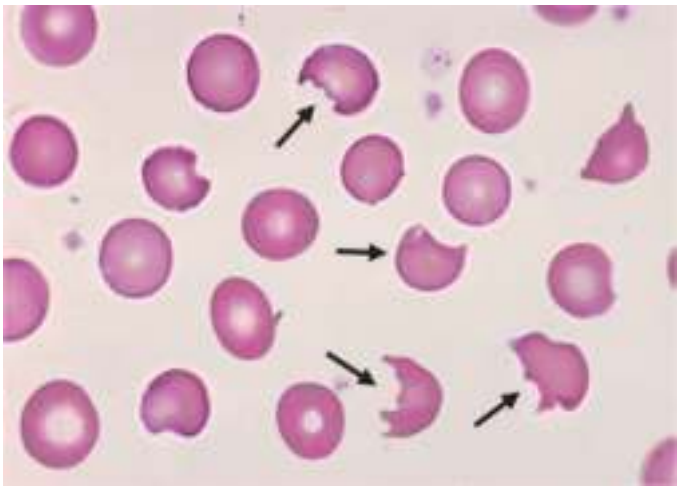


Fig. 8.48: Peripheral blood smear examination shows bite cells (arrows).

PYRUVATE KINASE DEFICIENCY DISORDER

Pyruvate kinase deficiency disorder is the second most common enzyme deficiency hemolytic anemia. Pyruvate kinase deficiency disorder is an autosomal recessive disorder characterized by hereditary nonspherocytic hemolytic anemia. In contrast to the more common G6PD deficiency disorder, in which the anemia is episodic and self-limited, the anemia in pyruvate kinase deficiency disorder is chronic and sustained. Peripheral blood smear examination shows acanthocytes in pyruvate kinase deficiency (Fig. 8.49A and B).

Table 8.87 Characteristic features of hemolytic episode in G6PD deficiency

Clinical Manifestations	Hematologic Findings
Acute phase of hemolysis	
▪ Abrupt onset of jaundice, pallor, malaise and prostration ▪ Abdominal pain, fever and passage of dark-colored urine (hemoglobinuria) and may progress to renal failure	▪ Peripheral blood smear examination shows features of anemia, and Heinz bodies ▪ Reticulocyte count increased and peak level in 5–8 days ▪ Hemoglobinemia ▪ Methemalbuminemia ▪ Hemoglobinuria ▪ Serum bilirubin increased ▪ Serum haptoglobin decreased/absent ▪ Serum creatinine increased ▪ Blood urea increased
Recovery phase	
▪ Gradual but rapid cessation of hemolysis ▪ Urine clears in a few days ▪ Jaundice clears in 1–2 weeks	Concentration of G6PD increases but rarely to normal range

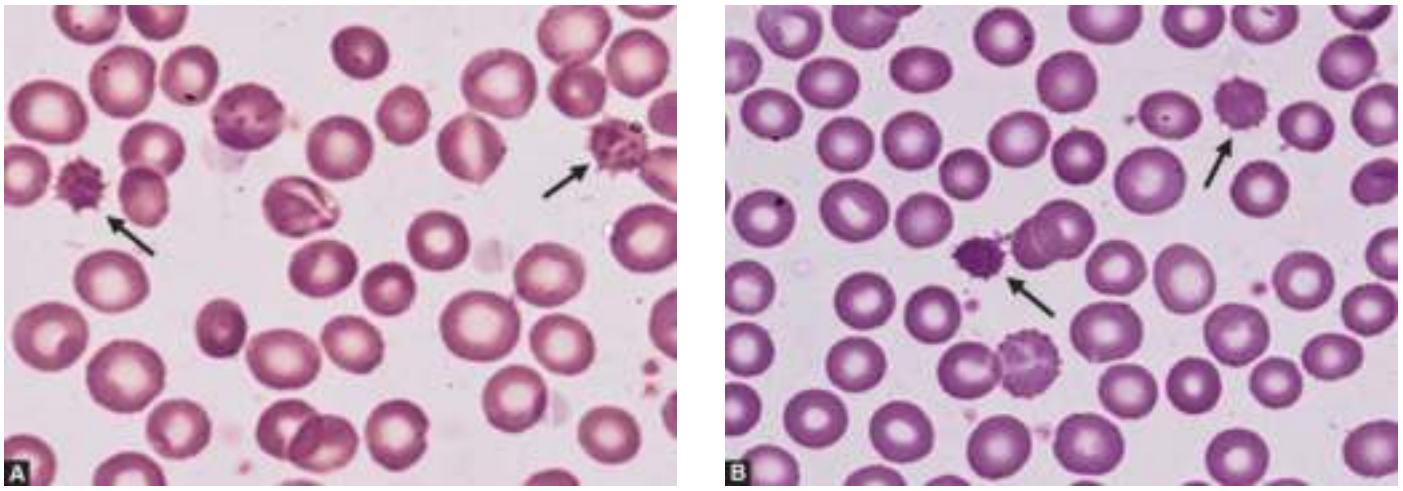


Fig. 8.49A and B: Peripheral blood smear examination shows acanthocytes in pyruvate kinase deficiency (arrows).

IMMUNE-MEDIATED HEMOLYTIC ANEMIA

In immune-mediated hemolytic anemias, hemolysis of red blood cells is mediated by autoantibodies or alloantibodies, which occurs in both intravascular and extravascular compartments. Autoantibodies produced by the patient's immune system are directed against antigens expressed on red blood cells leading to their premature destruction. These patients are diagnosed by demonstration of antibodies and/or complement by Coombs' test. Classification of immune hemolytic anemia is given in Table 8.88.

Table 8.88 Classification of immune hemolytic anemia

Immune Hemolytic Anemia	Mechanism	Causes
Autoimmune hemolytic anemia		
Warm antibody-mediated immune hemolytic anemia	RBCs coated by IgG (most common) or IgM autoantibodies bind to Fc receptors of macrophages in spleen at 37°C leading to RBCs hemolysis in spleen	▪ Primary or idiopathic (50%) ▪ Chronic lymphocytic leukemias ▪ Systemic lupus erythematosus ▪ Viral infections ▪ Drug-induced hemolysis (benzylpenicillin, α-methyldopa and quinidine)
Cold agglutinin disease	IgM class antibody agglutinate RBCs at 0°–4°C (<37°C) leading to extravascular destruction of RBCs in spleen by macrophages	▪ It is primary disorder ▪ Lymphoma-related disorder ▪ *Mycoplasma pneumoniae* ▪ Paroxysmal cold hemoglobinuria ▪ Infectious mononucleosis (EB virus)
Drug-induced (benzylpenicillin) autoimmune hemolytic anemia	Antibodies directed against surface antigens on RBCs. Opsonization and phagocytosis of RBCs and platelets	Benzylpenicillin, cephalosporins and quinidine
Alloimmune hemolytic anemia		
Hemolytic disease of newborn	Antibodies directed against RBCs membrane proteins (Rh blood group antigen, I antigen) leading to opsonization and phagocytosis and destruction of red blood cells in newborn	Hemolytic disease of newborn (rhesus incompatibility)
Hemolytic mismatch blood transfusion	IgG or IgM antibodies attach to the foreign cells of donor, resulting in complement fixation leading to formation of membrane attack complex lysing the donor cells	Hemolytic transfusion reactions

AUTOIMMUNE HEMOLYTIC ANEMIA

Two types of autoantibodies (warm and cold) react with antigens expressed on red blood cells leading to their destruction by spleen.

- Warm autoantibodies (IgG) being active at 37°C bind to antigens expressed on red blood cells without activation of complement system leading to their destruction by splenic macrophages.
- On the other hand, cold autoantibodies (IgM) being active at 30°C bind to antigens expressed on red blood cells and activates complement system leading to RBCs destruction by splenic macrophages. These splenic macrophages possess Fc receptor for Fc fragment of immunoglobulin. Clinical features and laboratory findings in a case of autoimmune hemolytic anemia are given in Table 8.89. Pathogenesis of immune hemolytic anemia is shown in Fig. 8.50.

Pathology Pearls: Autoimmune Hemolytic Anemias

- Warm autoimmune hemolytic anemia (WAIHA) is mediated by IgG autoantibodies that optimally bind red blood cells at body timperature (37°C).
- Cold autoimmune hemolytic anemia (CAIHA), also called cold agglutinin disease, is mediated by IgM autoantibodies that bind red blood cells at lower temperature ranges (<30°C).
- Drug-induced immune hemolytic anemia occurs through several pathophysiologic mechanisms: (a) drug absorption (hapten) induced extravascular hemolysis, (b) immune-complex mediated intravascular hemolysis, and (c) autoimmune hemolytic anemia by drug-induced extravascular hemolysis.
- If a pregnant woman has antibodies against a fetal antigen, the fetus is at risk for hemolytic disease of newborn.

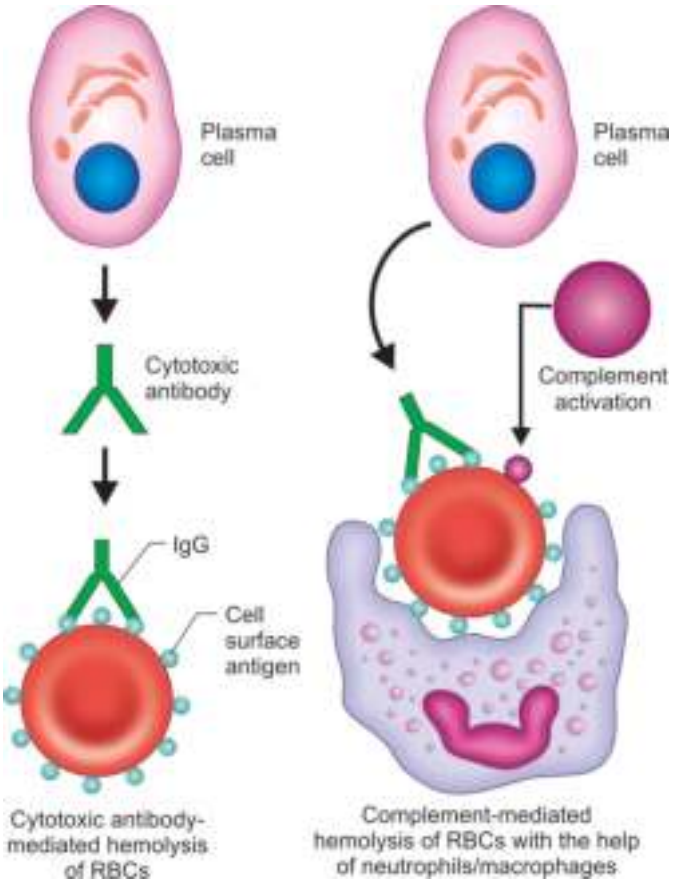


Fig. 8.50: Pathogenesis of immune hemolytic anemia by type 2 hypersensitivity reaction complement-mediated phagocytosis by macrophage resulting in RBCs destruction by spleen.

WARM ANTIBODY-MEDIATED HEMOLYTIC ANEMIA

Warm antibody-mediated hemolytic anemia is the most common form of immune hemolytic anemia. Disorder is mediated by IgG class antibodies that react with red blood cell surface antigens (often Rh antigens) at 37°C. IgM autoantibody is active at 4°C, which becomes less active at higher temperature; but is still

Table 8.89 Clinical features and laboratory findings in a case of autoimmune hemolytic anemia (AIHA)

Parameters	Warm Autoimmune Hemolytic Anemia (AIHA)	Cold Autoimmune Hemolytic Anemia (AIHA)
Onset of disease	Sudden onset	Gradual onset
Jaundice	Present	Usually absent
Splenomegaly	Present	Absent
Immunoglobulin-mediated	IgG-mediated AIHA (rarely IgM or IgA)	IgM-mediated (except PCH-IgG)
Temperature at which antibody reacts	Antibody reacts at 37°C	Antibody reacts below 30°C
Red blood cells morphology	Spherocytes	Agglutinated RBCs
Clinical association	Usually associated with autoimmune disorders	Usually due to infections
Direct antiglobulin test (DAT)	IgG-mediated positive test	C3-mediated positive test
Mechanism of hemolysis	Extravascular hemolysis by reticuloendothelial system	Intravascular hemolysis mediated by complement system

able to bind complement and agglutinate red blood cells at 30°C temperature of the peripheral tissues (e.g. hands, feet, nose and ears). Sometimes, disorder is mediated by IgA.

Pathophysiology

Immune-mediated hemolytic anemia disorder is idiopathic (primary) in majority of patients. Secondary immune-mediated hemolytic anemia is associated with systemic lupus erythematosus, rheumatoid arthritis, chronic lymphocytic leukemia, Hodgkin disease, or non-Hodgkin lymphoma and drug-induced hypersensitivity.

- RBCs coated by IgG or IgM autoantibodies bind to receptors on macrophages because they have receptors for the Fc fragments of immunoglobulins.
- Once activated, the complement system cascade leads to the destruction of the RBCs through formation of C5b–C9 membrane attack complex (MAC).
- IgM antibodies, which are powerful agglutinins, may agglutinate red blood cells (RBCs) in the red pulp of the spleen, resulting in RBCs destruction. IgM may also activate complement system cascade, resulting in RBCs lysis. Such autoantibodies are detected by Coombs' test (agglutination test).

Clinical Features

Patient presents with progressive anemia, jaundice and mild splenomegaly. History of drug intake such as benzylpenicillin, cephalosporins, methyldopa and quinidine should be taken into consideration.

Laboratory Diagnosis of Warm Antibody-mediated Hemolytic Anemia
Hemoglobin
Hemoglobin and PCV become low.
Peripheral Blood Smear Examination
Peripheral blood smear examination shows polychromasia and spherocytes due to progressive loss of membrane protein by serial passage of antibody-coated red cells through the spleen (Fig. 8.51).
Reticulocyte Count
Reticulocyte count is increased between 5 and 20%. Higher reticulocyte count is demonstrated in acute hemolytic process.
Serum Bilirubin
There is mild to moderate increase in unconjugated serum bilirubin.
Serum Lactic Dehydrogenase (LDH)
Hemolysis of red blood cells increases serum LDH level.
LE Cell Phenomenon
LE cell test is significant in immune hemolytic anemia induced by systemic lupus erythematosus.

Coombs' Test

Positive direct Coombs' test (also known as direct antiglobulin test or DAT) is reflecting the binding of IgG autoantibody to the red cell surface (Table 8.90).

- **Direct Coombs' test (direct antiglobulin test):** In direct Coombs' test, RBCs are coated *in vivo* and washed to remove unbound globulins. Addition of anti-human globulin (AHG) promotes agglutination after centrifugation.
  - Direct Coombs' test (DAT) is performed for the demonstration *in vivo* sensitization of red blood cells with immunoglobulin and/or complement. It can occur after antigen–antibody complexes have been formed in certain diseases such as autoimmune hemolytic anemia or hemolytic disease of the fetus and newborn.
  - Direct Coombs' test (DAT) may also be positive following blood transfusion of antigen-positive, to which the recipient possesses the antibody.
- **Indirect Coombs' test (indirect antiglobulin test):** In indirect Coombs' test (DAT), serum with specific antibody is mixed with reagent red blood cells.
  - Anti-human globulin (AHG) is added to promote agglutination on centrifuge. Indirect antiglobulin test (IAT) is performed for the detection of antibodies of the IgG class. In spin-tube techniques, unbound immunoglobulins or other proteins are washed from the test system and anti-human globulin (AHG) is added to the sensitized red blood cells. During centrifugation, anti-human globulin (AHG) promotes agglutination and thus visible hemagglutination.
  - Indirect antiglobulin test (IAT) is used for identification of antibody in the serum/plasma of a potential transfusion recipient or as part of antenatal testing, to help assess the likelihood of the antibody promoting fetal red blood cell destruction. It can also be used with known antibodies for typing antigens on the red blood cells.

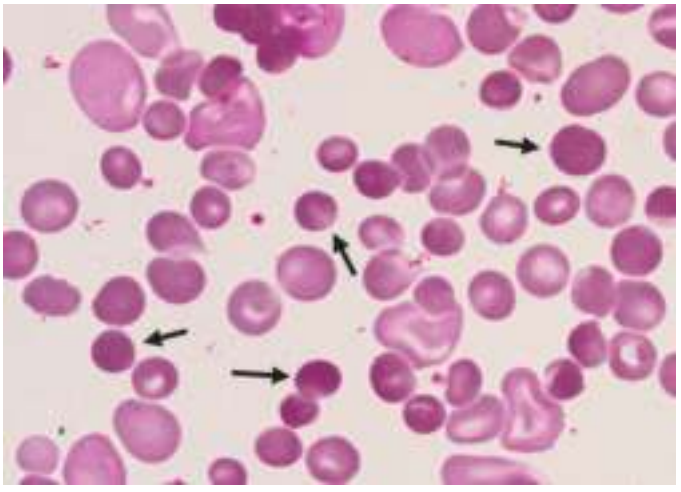


Fig. 8.51: Peripheral blood smear examination from a case of autoimmune hemolytic anemia showing polychromasia and spherocytes (arrows).

Table 8.90 Direct and indirect Coombs' test (Antiglobulin test)

Parameters	Coombs' Test	
	Direct Coombs' Test (Direct Antiglobulin Test)	Indirect Coombs' Test (Indirect Antiglobulin Test)
Principle	Detects antibodies or complement bound to surface of RBCs antigens *in vivo*	Detects *in vitro* antigen–antibody reactions or antibodies in serum
Utility in clinical practice	▪ Immunomediated hemolytic anemia ▪ Drug-induced hemolytic anemia ▪ Hemolytic disease of newborn ▪ ABO hemolytic disease of newborn ▪ Mismatched blood transfusion	▪ Detection of low concentration of antibodies in patient's blood prior to blood transfusion ▪ Detection of antibodies in antenatal women that may cause hemolytic disease of newborn ▪ Compatibility testing in blood banking ▪ RBC phenotyping ▪ Identification of antibody in mother's serum ▪ Titration studies

COLD AGGLUTININ DISEASE

Cold agglutinin disease is caused by IgM antibodies, which agglutinate RBCs at low temperature (0°–4°C). Cold agglutinin disease is mediated by IgM antibodies optimally active at low temperatures at 4°C against the red blood cells I antigen.

- IgM antibodies cause agglutination of red blood cells at low temperature at especially during winter season in the capillaries of nose and fingers.
- Complement coated RBCs are phagocytozed by macrophages in spleen leading to extravascular hemolysis.

Pathophysiology

Cold agglutinin disease disorder occurs due to formation of anti-I antibodies in EB virus associated infectious mononucleosis. These anti-I antibodies frequently complicate *Mycoplasma pneumoniae* infection. This disorder also occurs in patient with HIV infection. Chronic hemolysis may be primary or associated with B cell neoplasms.

Clinical Features

Patient with cold agglutinin disease presents with chronic hemolytic anemia exacerbated by cold weather, episodes of jaundice, sometimes with hemoglobinemia and hemoglobinuria. Disorder has self-limited course, which rarely induces significant extravascular hemolysis.

Laboratory Diagnosis of Cold Agglutinin Disease

Peripheral Blood Smear Examination

- The peripheral blood smear from this patient shows clumped RBCs caused by cold agglutinins during especially winter.
- During winter, it is difficult to prepare peripheral blood smear, because agglutination occurs at low temperature (Fig. 8.52).

Hematocrit Values

MCH and MCHC values are very high due to agglutination of red blood cells. Red blood cell value is very low.

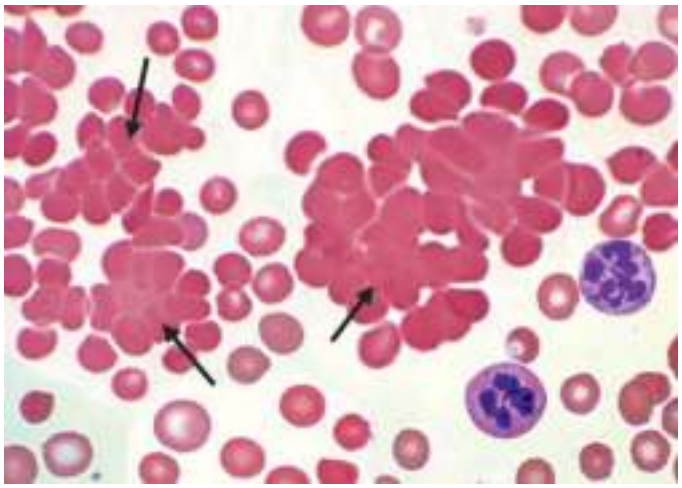


Fig. 8.52: Cold agglutinin disease. Peripheral blood smear examination shows agglutination of red blood cells (arrows).

PAROXYSMAL COLD HEMOGLOBINURIA

Paroxysmal cold hemoglobinuria (PCH) most often occurs in children associated with viral infections. IgG autoantibodies are formed against P antigen on surface of RBCs at low temperature. These antibodies fix complement and induce intravascular hemolysis. IgG antibodies are also known as Donath-Landsteiner antibody.

- **Hemoglobinuria:** Hemoglobin in urine is demonstrated by benzidine test.
- **Hemosiderinuria:** Hemosiderin is demonstrated by Perls Prussian blue reaction.
- **Hemoglobinemia:** Presence of hemoglobin in the plasma gives red discoloration. Hemoglobinemia is demonstrated by hemoglobin blood test.

DRUG-INDUCED HEMOLYTIC ANEMIA

Most antigens have high molecular weight in excess of 10,000. Small molecules cannot act as antigen by

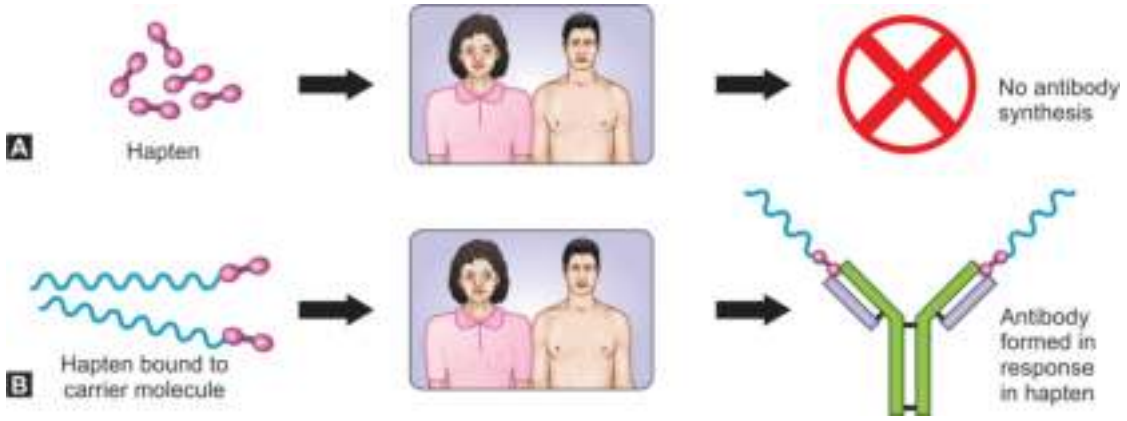


Fig. 8.53: The hapten-carrier phenomenon. (A) Hapten molecule is too small to trigger an immune response, (B) binding of hapten with carrier triggers an immune response.

themselves. Binding of antigen with small carrier hapten molecule, which induces immune response.

- Hapten molecule is too small (MW <1000) to trigger an immune response alone but can be immunogenic when it attaches to a larger carrier molecule such as host serum protein.
- Drug-induced hemolytic anemia occurs by two mechanisms: antigenic drugs and tolerance-breaking drugs. The hapten-carrier phenomenon is shown in Fig. 8.53A and B.
  - Antigenic drugs are benzylpenicillin, cephalosporins and quinidine, which bind to the surface of RBCs leading to formation of antibodies against drug. These antibodies react with drugs and form complex on the surface of RBCs leading to hemolysis.
  - Example of tolerance breaking drug is α-methyldopa drug, which induces antibodies against intrinsic antigens.

ALLOIMMUNE HEMOLYTIC ANEMIA

Alloimmune hemolytic anemia occurs when immune system produces antibodies against foreign or non-self antigens, which include hemolytic disease of newborn and hemolytic blood transfusion reactions.

HEMOLYTIC DISEASE OF NEWBORN

Hemolytic disease of newborn most commonly occurs with Rh blood group incompatibility between mother and fetus. It occurs when the mother is Rh –ve and baby is Rh +ve, having inherited an allele for one of the Rh antigens from the father (Fig. 8.54).

- **During first pregnancy:** At birth, a small quantity of fetal blood usually leaks across the placenta into the maternal bloodstream. Upon exposure to Rh antigen, the mother's immune system responds by making anti-Rh antibodies. Because the baby is already born, it suffers no damage.
- **During subsequent pregnancy:** The maternal antibodies cross the placenta into the fetal blood. If the second fetus is Rh +ve, the ensuring antigen–antibody reaction causes hemolysis of fetal RBCs. The result is hemolytic disease of newborn (HDN).

Clinical Features

Newborn develops anemia, jaundice, hepatosplenomegaly, cardiac failure and kernicterus (staining of the basal ganglion and other structures of CNS by unconjugated bilirubin). Kernicterus with resultant neurological damage is the most significant long-term consequence of hemolytic disease of newborn. In addition, consequences include stillbirth or in hydrops fetalis, fetal heart failure with massive generalized edema.

Management

Administration of anti-D antiserum to D-negative mother at the time of delivery of D-positive child prevents maternal alloimmunization by removing fetal red blood cells from the maternal circulation.

Preventive Measures

Routine administration of anti-D IgG antiserum to D-negative mothers at 28 weeks gestation and at the time of first delivery (or at the time of termination of pregnancy) of D-positive child results in the antibody-mediated removal of fetal red blood cells from

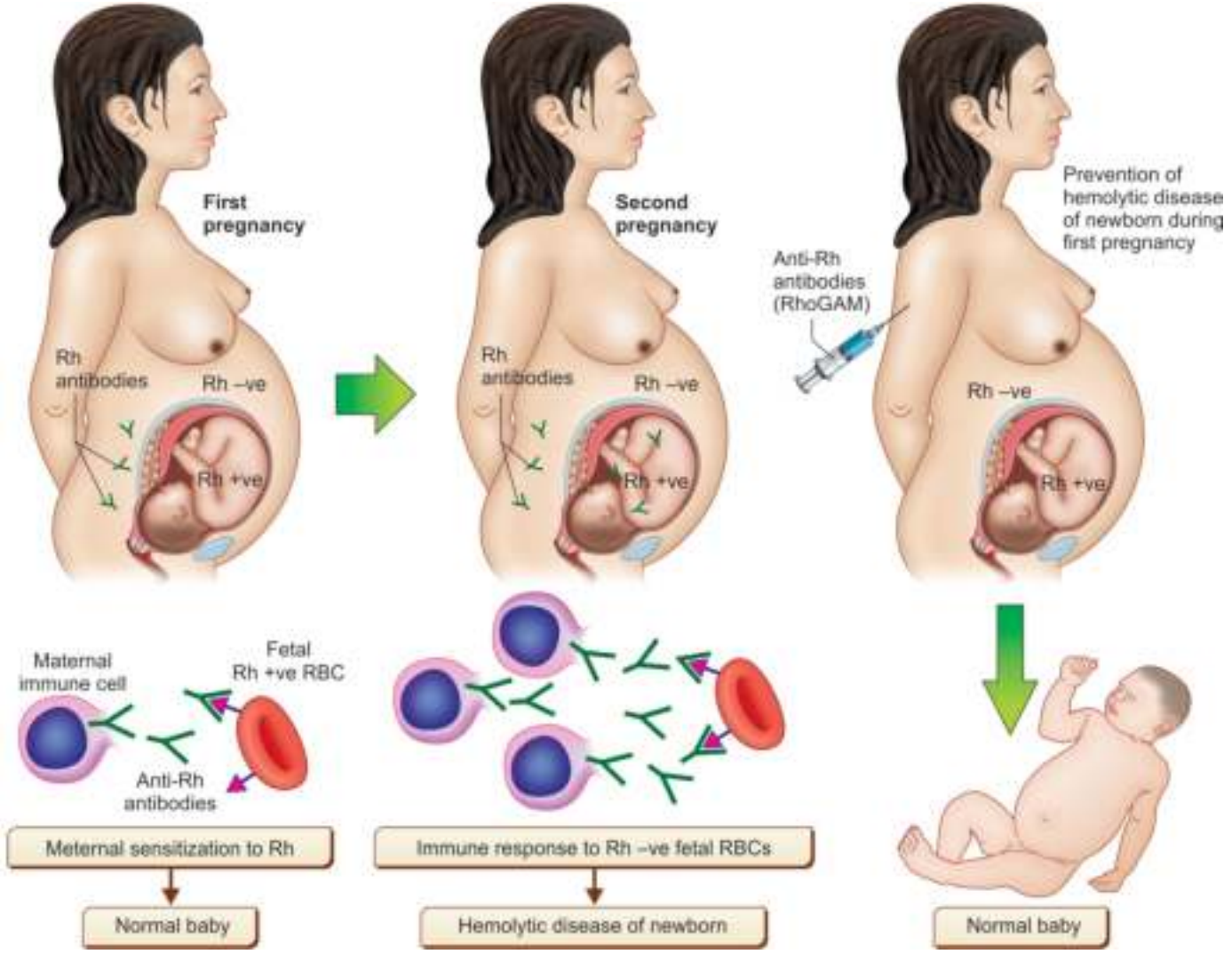


Fig. 8.54: Hemolytic disease of newborn. Rh factor incompatibility can cause lysis of red blood cells. A naturally occurring red blood cell incompatibility results when a fetus Rh +ve develops within Rh –ve mother. Initial sensitization of the maternal immune system occurs when fetal blood passes the placental barrier. In most cases, the fetus develops normally. However, a subsequent pregnancy with Rh +ve fetus results in a severe fetal red blood cells hemolysis. Control of incompatibility is done by administration of anti-Rh antibodies (RhoGAM) to Rh –ve mothers during pregnancy to inactivate and remove any Rh factor that may be transferred from the fetus in maternal circulation. In some cases, anti-Rh antibody (RhoGAM) is administered before sensitization occurs.

the maternal circulation, preventing maternal alloimmunization.

Laboratory Diagnosis of Hemolytic Disease of Newborn

- Hemolytic disease of newborn (HDN) can be diagnosed during pregnancy or after the baby is born by amniocentesis, blood and percutaneous umbilical cord blood sampling to analyze serum bilirubin, anemia, and Rh +ve antibodies (direct antiglobulin test/direct Coombs' test).
- Peripheral blood smear examination shows evidence of hemolysis such as anemia and erythroid percursors.

HEMOLYTIC BLOOD TRANSFUSION REACTIONS

Hemolytic blood transfusion reactions occur immediately or within days of a red blood cell transfusion. The transfusion recipient is exposed to a foreign red blood cell antigen on the donor red blood cells, which stimulates an immune response.

In ABO mismatch blood transfusion, preformed IgM antibodies present in the serum of recipient are active at 37°C. These antibodies and complement coated red blood cells and activate complement system resulting in intravascular hemolysis.

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA: OVERVIEW

Paroxysmal nocturnal hemoglobinuria may develop as a primary disorder or evolve from preexisting etiology.

- Paroxysmal nocturnal hemoglobinuria (PNH) occurs due to an acquired (somatic) mutation in the PIG-A gene of a hematopoietic stem cell, the major consequence of which is decreased synthesis of glycosylphosphatidylinositol (GPI) anchor proteins. PNH is characterized by passage of hemoglobin in urine, pancytopenia and mild jaundice due to complement mediated recurrent bouts of intravascular hemolysis.
- During hemolytic episodes, patient develops normocytic or macrocytic anemia accompanied by an appropriate increased reticulocyte response.
- Patient may develop acute myelogenous leukemia (AML), or myelodysplastic syndrome or aplastic anemia.
- Thrombosis in portal and hepatic veins is the most common cause of death in these cases.

PATHOPHYSIOLOGY

Physiologic State

PIG-A gene located on X chromosome encodes GPI (glycosylphosphatidylinositol) anchor regulatory proteins, which bind to various red blood cell membrane proteins like membrane inhibitor of reactive lysis (MIRL) (CD59), decay accelerating factor (DAF) (CD55), CD8, CD14, CD16a and CD52 required for the protection of red blood cells, granulocytes, and platelets from complement-mediated lysis. DAF cleaves C3b. MIRL participates in cleaving C5–C9. These complement regulatory proteins are required for the protection of red blood cells, granulocytes, and platelets from complement-mediated lysis.

Pathologic State

Paroxysmal nocturnal hemoglobinuria occurs due to PIG-A gene located on X chromosome resulting in deficient cytoskeleton anchoring proteins, i.e. GPI (glycosylphosphatidylinositol), MIRL (CD59), DAF (CD55) and CD8, CD16a and CD52.

- Normally, these cytoskeleton anchoring proteins are expressed on red blood cells, granulocytes and platelets from complement-mediated lysis.
- Acidic pH causes uncontrolled activation of complement pathway to form membrane attack complex (C5b–C9), which attacks RBCs, granulocytes and platelets leading to recurrent bouts of complement-mediated intravascular hemolysis.
- In normal persons, membrane attack complex (C5b–C9) formation is neutralized by C-reactive protein (CPR). Pathogenesis of paroxysmal nocturnal hemoglobinuria is shown in Fig. 8.55A and B.

CLINICAL FEATURES

Paroxysmal nocturnal hemoglobinuria (PNH) is often marked by the passage of hemoglobin-containing urine on awakening. Patient presents with recurrent bouts of complement-mediated intravascular hemolysis, mild jaundice, abdominal pain and pancytopenia. There is increased risk of thrombosis in the portal vein, hepatic veins and cerebral veins. Most common cause of death is thrombosis in portal vein and hepatic veins. PNH may be associated with aplastic anemia and myelodysplastic syndrome progressing to acute myelogenous leukemia.

Laboratory Diagnosis of Paroxysmal Nocturnal Hemoglobinuria (PNH)

Diagnostic tests commonly used in detection of paroxysmal nocturnal hemoglobinuria include hematologic analysis (hemoglobin, hematocrit values, reticulocyte count, peripheral blood smear and bone marrow examination; NAP score), serum bilirubin and serum haptoglobin levels, flow cytometric analysis for decay accelerating factor DAF (CD55) and MIRL (CD55) expressed on RBCs, granulocytes and platelets; modified gel diffusion analysis, fluorescein labeled proaerolysin (FLAER) test, Ham's test *in vitro*, and sucrose hemolysis test. Markers for paroxysmal nocturnal hemoglobinuria testing by flow cytometry are given in Table 8.91.

Hematologic Findings

- Hemoglobin and hematocrit values are decreased.
- Reticulocyte count is increased.
- Peripheral blood smear examination shows normocytic normochromic picture with polychromasia.
- Bone marrow examination shows hypercellular marrow due to erythroid hyperplasia.
- Neutrophil alkaline phosphatase (NAP) score is decreased.

Biochemical Alterations

- Serum bilirubin level is increased.
- Serum haptoglobin level is decreased.
- Hemoglobinemia is present.
- Hemoglobinuria is demonstrated by benzidine test.

Flow Cytometric Analysis

Flow cytometry is **most sensitive** diagnostic technique to demonstrate diminished DAF (CD55) and MIRL (CD59) expressed on red blood cells, ganulocytes and platelets.

Modified Gel Diffusion Analysis

Modified gel diffusion analysis by using gelcard demonstrates deficient DAF (CD55) and MIRL (CD59) expressed on red blood cells.

Fluorescein-labeled Proaerolysin (FLAER) Test

- FLAER test is a diagnostic tool to demonstrate clones on neutrophils and monocytes.
- FLAER binds to GPI-AP and increases the detection accuracy of flow cytometry for paroxysmal nocturnal hemoglobinuria, granulocyte and monocyte clones.

Ham Test *in vitro*

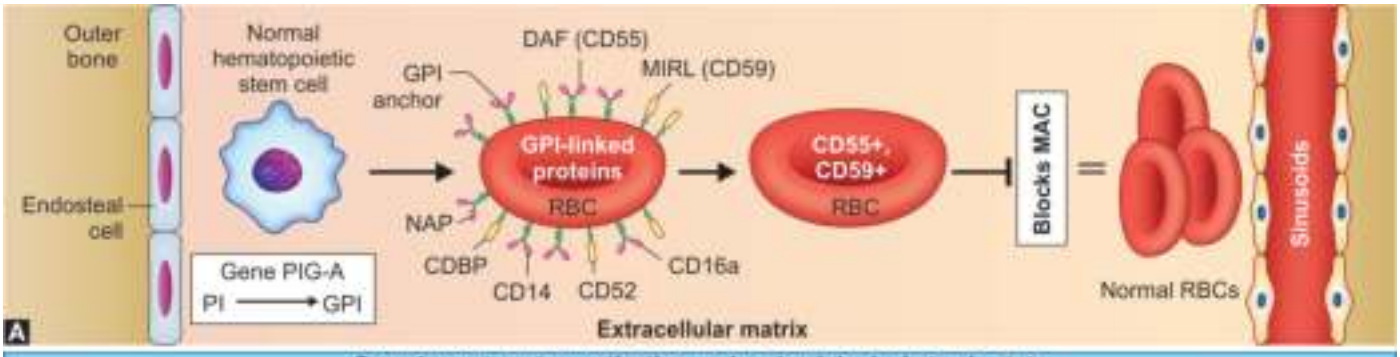
The Ham test is used in the diagnosis of paroxysmal nocturnal hemoglobinuria (PNH) by using acidified serum. Acidic pH of serum activates complement by alternate pathway resulting in hemolysis of red blood cells. This is now an obsolete test for diagnosis of PNH due to its low sensitivity and specificity.

Sucrose Hemolysis Test *in vitro*

Sucrose hemolysis test is a screening test for paroxysmal nocturnal hemoglobinuria (PNH). Sucrose reduces pH and thus activates complement pathway resulting in hemolysis of red blood cells. Hemolysis of RBCs >10% is diagnostic of PNH.

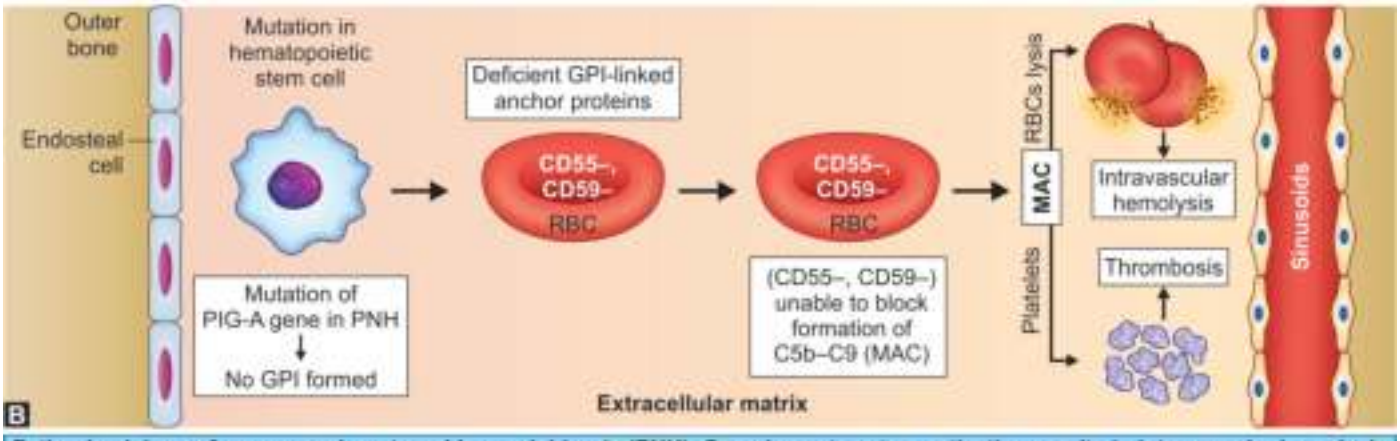
MANAGEMENT

Paroxysmal nocturnal hemoglobinuria patients are managed by blood transfusions, oral anticoagulants and antibiotics. Bone marrow transplantation is curative of disorder.



Complement system activation under physiological steady state

- Normal red blood cells (CD55+, CD59+) can withstand the hazard of complement system activation.
- Under physiological state, alternative, classic and lectin pathways of complement system activation, C3 convertase enzyme takes over that converts C3 to C3b. Then C3b is attached to red blood cells (CD55+, CD59+).
- The C3b + C3 convertase + C5 convertase participate in conversion of C5 to C5b. Then C5b activates C6, C7, C8, C9 and forms C5b–C9 (MAC—membrane attack complex).
- Red blood cells (CD55+, CD59+) block the action of membrane attack complex (MAC) and thus prevent intravascular hemolysis.



Pathophysiology of paroxysmal nocturnal hemoglobinuria (PNH): Complement system activation results in intravascular hemolysis

- Abnormal paroxysmal nocturnal hemoglobinuria (PNH) red blood cells (CD55–, CD59–) will undergo intravascular hemolysis sooner or later by activated complement system (C5b–C9 membrane attack complex).
- In paroxysmal nocturnal hemoglobinuria patient with PIG-A gene mutation, alternative, classic and lectin pathways of complement system activation, C3 convertase enzyme takes over that converts C3 to C3b. Then C3b is attached to red blood cells (CD55–, CD59–).
- The C3b + C3 convertase + C5 convertase participate in conversion of C5 to C5b. Then C5b activates C6, C7, C8, C9 and forms C5b–C9 (MAC—membrane attack complex).
- Red blood cells (CD55–, CD59–) are unable to block the action of membrane attack complex (MAC) and thus leading to intravascular hemolysis sooner or later.

Fig. 8.55: Pathogenesis of paroxysmal nocturnal hemoglobinuria. (A) Under physiologic state, PIG-A gene encoding GPI membrane anchoring proteins, which bind to CD55, CD59, CD8, CD14, CD16a and CD52 resulting in maintenance of red blood cell membrane, (B) under pathologic state, PIG-A gene mutation causes deficiency of membrane anchoring proteins leading to red blood cell destruction.

Table 8.91 Markers for paroxysmal nocturnal hemoglobinuria testing by flow cytometry

Study of Cells	Lineage Defining Markers	Glycosylphosphatidylinositol (GPI) Markers	Other Markers Used
Red blood cells	CD235a (glycophorin)	CD59	CD55
Granulocytes	CD15	▪ FLAER* (fluorescein-labeled proaerolysin) test ▪ CD24	▪ CD16 ▪ CD66b
Monocytes	CD64	▪ FLAER* (fluorescein-labeled proaerolysin) test ▪ CD157	CD14

**FLAER (fluorescein-labeled proaerolysin) test increases the detection accuracy of flow cytometry for paroxysmal nocturnal hemoglobinuria to demonstrate clones on neutrophils and monocytes.*

RED BLOOD CELL FRAGMENTATION SYNDROMES

Turbulent blood flow over prosthetic cardiac valves or synthetic vascular graft can cause shearing of red blood cells (RBCs) leading to intravascular hemolysis, hyperbilirubinemia and mild to moderate anemia.

- Red blood cell fragmentation syndromes occur in the settings of are prosthetic heart valves or synthetic vascular graft, disseminated intravascular coagulation and thrombotic thrombocytopenic purpura, malignant hypertension, vasculitis syndrome, hemolytic uremic syndrome, long distance running or walking and prolonged vigorous exercise. Vascular endothelial alterations in capillaries cause generalized thrombosis of capillary vessels leading to turbulent blood flow.
- These conditions can cause repetitive trauma to red blood cells in the microcirculation leading to intravascular hemolysis. Causes of red blood cell fragmentation syndrome are given in Table 8.92. Red blood cell fragmentation syndromes with or without thrombocytopenia is given in Table 8.93.

CARDIAC HEMOLYTIC ANEMIA

Hemolytic anemia occurs due to prosthetic valve, aortic stenosis, mitral valvular disease, coarctation of aorta and ruptured chordae tendineae. Turbulence of blood flow within cardiac chambers induces mechanical injury to red blood cells resulting in anemia.

Laboratory Diagnosis of Cardiac Hemolytic Anemia

- **Peripheral blood smear examination:** Periheral blood smear examination shows fragmented triangular-shaped red blood cells known as schistocytes or helmet cells (Fig. 8.56).
- **Reticulocyte count:** Reticulocyte count is increased.
- **Serum bilirubin:** Serum bilirubin level is increased.

MICROANGIOPATHIC HEMOLYTIC ANEMIA

Red blood cells undergo destruction, when these pass through arterioles, capillaries and venules in cases of hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, mucin secreting metastatic carcinoma, eclampsia and cavernous hemangioma. Cancers associated with microangiopathic hemolytic anemia are given in Table 8.94.

HEMOLYTIC UREMIC SYNDROME

Hemolytic uremic syndrome (HUS) is a triad characterized by acute renal failure, microangiopathic hemolytic anemia and thrombocytopenia. Bacterial toxins

Table 8.92 Causes of red blood cell fragmentation syndrome

Categories	Causes	
Microangiopathic hemolytic anemia	▪ Thrombotic thrombocytopenic purpura (TTP) ▪ Hemolytic uremic syndrome (HUS) ▪ Disseminated intravascular coagulation (DIC) ▪ Eclampsia of pregnancy	▪ Metastatic mucin-secreting carcinoma ▪ Hemolysis with elevated liver enzymes and low platelets (HELLP)
Cardiac hemolytic anemia	▪ Prosthetic valve ▪ Aortic stenosis ▪ Mitral valvular disease	▪ Coarctation of aorta ▪ Ruptured chordae tendineae
March hemoglobinuria	Athletes and soldiers	

Table 8.93 Red blood cell fragmentation syndrome with or without thrombocytopenia

Red Blood Cell Fragmentation with Thrombocytopenia
Disseminated intravascular coagulation (DIC)
Thrombotic thrombocytopenic purpura (TTP)
Hemolytic uremic syndrome (HUS)
Pre-eclampsia/eclampsia of pregnancy
HELLP syndrome occurs during later stage of pregnancy characterized by hemolysis, elevated liver enzymes and low platelet count
Malignant hypertension
Disseminated mucin secretory carcinoma
Systemic lupus erythematosus (SLE)
Sepsis
Antiphospholipid antibody crisis
Vascular corporeal circulation device
Red Blood Cell Fragmentation without Thrombocytopenia
Damaged cardiac valves
Renal transplant rejection
Renal cortical necrosis
Vasculitis
Drugs (cyclosporine, tacrolimus)

play role in the pathogenesis of HUS, which cause vascular endothelial damage resulting in activation of coagulation system. Patient presents with bloody diarrhea, anemia, jaundice, oliguria, hemoglobinuria and proteinuria.

Laboratory Diagnosis of Hemolytic Uremic Syndrome
Hematologic Findings
Hematologic findings are low hemoglobin, microcytic hypochromic picture with fragmented red blood cells, increased serum bilirubin, increased blood urea and serum creatinine.
Urine Analysis
Urine analysis shows oliguria, hemoglobinuria and proteinuria.

THROMBOTIC THROMBOCYTOPENIC PURPURA

Thrombotic thrombocytopenic purpura (TTP) may affect young adults. There is widespread thrombi formation in arterioles, capillaries and venules. Patient presents with fever, central nervous system manifestations and renal failure. There are three variants of TTP: congenital, primary and secondary. Schistocytes are demonstrated in peripheral blood smear examination.

- **Pathogenesis:** Congenital and primary TTP occur due to deficiency of ADAMTS 13 enzyme. The disorder is initiated as a result of vascular endothelial injury resulting in release of von Willebrand factor (vWF) and other procoagulant substances. Deficient cleaving protease activity leads to accumulation of vWF multimers resulting in platelets aggregation and microthrombi formation in microcirculation.
- **Management:** Thrombotic thrombocytopenic purpura is treated by plasma exchange. Corticosteroids are commonly administered in these patients. In cases of thrombocytic thrombocytopenic purpura refractory to plasma exchange, administration of anti-CD20 monoclonal antibody rituximab resolves acute disease and prolongs remission.

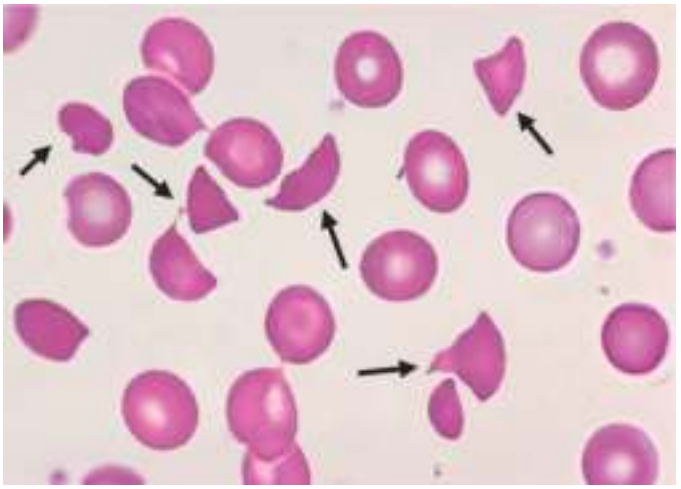


Fig. 8.56: Peripheral blood smear examination shows fragmented red blood cells (arrows) in a case of mitral valvular disease.

Table 8.94 Cancers associated with microangiopathic hemolytic anemia

Organs	Malignant Tumor
Gastrointestinal tract	▪ Gastric adenocarcinoma (55%) ▪ Colon adenocarcinoma
Breast	Breast carcinoma (13%)
Lung	▪ Lung adenocarcinoma (10%) ▪ Small cell lung carcinoma
Gallbladder	Gallbladder adenocarcinoma
Liver	Hepatocellular carcinoma
Pancreas	Pancreatic adenocarcinoma
Male genital system	Prostatic carcinoma
Female genital system	Ovarian carcinoma
Thymus	Thymoma
Oropharynx	Squamous cell carcinoma
Hematology	Erythroleukemia
Skin and other sites	Melanoma

MARCH HEMOLYTIC ANEMIA

March hemolytic anemia is seen in athletes and soldiers following strenuous exercise and marching. Mechanical destruction of red blood cells occurs in the microvessels of the feet. There is transient hemoglobinemia and hemoglobinuria, which persist for a few days.

DIAGNOSTIC APPROACH OF HEMOLYTIC ANEMIA

HEMOLYTIC ANEMIA: LABORATORY DIAGNOSIS

Hemolytic anemia is produced due to increased red blood cell destruction that cannot be compensated by bone marrow. RBCs destruction may be due to defects within red blood cells or due to extrinsic causes. Family history of hemoglobinopathy or hereditary spherocytosis, recent blood transfusions, malaria and drug intake provide clues to proper diagnosis. Diagnostic approach of hemolytic anemia is shown in Fig. 8.57.

PERIPHERAL BLOOD SMEAR EXAMINATION

Peripheral blood smear should be examined for the presence of microspherocytes, fragmented red blood cells and malarial parasite. Hemolytic anemia in which spherocytes are not present includes G6PD deficiency, hemoglobinopathies (other than HbC and thalassemia), unstable hemoglobin, drugs, snakebite and malaria. RHAG gene located on chromosome 6p21.1–p11 encodes rhesus antigen. RHAG gene mutation causes total loss of rhesus antigens leading to chronic nonspherocytic hemolytic anemia.

- Microspherocytes appear small dense and spherical. These are demonstrated in hereditary spherocytosis and immune hemolytic anemia. These are also demonstrated in HbC and hypersplenism. When hereditary spherocytosis is considered as a possible cause, peripheral blood smear obtained from family members supports the diagnosis.
  - Presence of microspherocytes and numerous target cells are highly suggestive of hemoglobin C disease. Hemoglobin electrophoresis should be performed to establish hemoglobin C.
  - Hereditary spherocytosis is diagnosed by osmotic fragility and acidified glycerol tests.
- Ovalocytes are demonstrated in hereditary ovalocytosis.
- Acanthocytes are demonstrated in pyruvate kinase deficiency disorder.
- Fragmented RBCs: Presence of fragmented red blood cells (burr cells, schistocytes and helmet cells) indicate microangiopathic hemolytic anemia.
- Sickle cells are demonstrated in cases of sickle cell disease and trait.
- Bite cells are demonstrated in peripheral blood smear of G6PD deficiency disorder. The diagnosis of G6PD deficiency disorder is done by methemoglobin reduction test and fluorescent assay.

PLASMA HAPTOGLOBIN

Haptoglobin is a glycoprotein that is synthesized in the liver, which consists of two pairs of α-chains and β-chains. With hemolysis, free hemoglobin readily dissociates into dimers of α- and β-chains, which bind with haptoglobin in plasma or serum. The complex is removed by liver. Plasma haptoglobin level is decreased in hemolytic anemia.

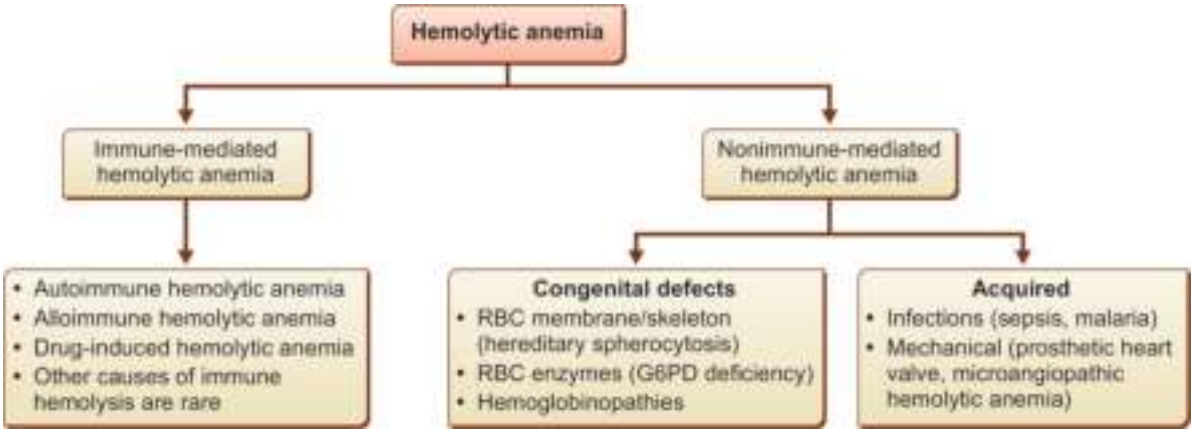


Fig. 8.57: Diagnostic approach of hemolytic anemia.

PLASMA HEMOPEXIN

Hemopexin is a β_1-glycoprotein of molecular weight 70,000 synthesized in the liver, which directly binds to ferriheme rather hemoglobin. In severe intravascular hemolysis, when haptoglobin is depleted, hemopexin is low or absent and elevated plasma methemalbumin. With less severe hemolysis, although haptoglobin is likely to be reduced or absent, hemopexin may be normal or only slightly lowered.

PLASMA HEMOGLOBIN

In plasma hemoglobin analysis, the catalytic action of heme-containing proteins brings about the oxidation of benzidine by H_2O_2 to give a green color, which changes to blue and finally to reddish violet. The intensity of reaction may be compared in a spectrophotometer with that produced by solutions of known hemoglobin. When the plasma hemoglobin is >50 mg/L, it can be measured as hemoglobin cyanide (HiCN) or oxyhemoglobin by a spectrometer at 540 nm.

HEMOSIDERINURIA

Hemosiderinuria is a sequel to the presence of hemoglobin in the glomerular filtrate. Hemosiderinuria is a valuable sign of intravascular hemolysis because the urine will be found to contain iron-containing granules even if there is no hemoglobinuria at the time.

- However, hemosiderinuria is not found in the urine at the onset of a hemolytic attack even if this is accompanied by hemoglobinemia and hemoglobinuria. Because the hemoglobin has first to be absorbed by renal tubular cells.
- The intracellular breakdown of hemoglobin liberates iron, which is then re-excreted. Hemosiderinuria may persist for several weeks after a hemolytic episode.

SICKLING TEST

Hemoglobin S is less soluble in a reducing agent than other forms of hemoglobin. Mixing blood with the reducing agent, sodium metabisulphite on a sealed slide, will induce sickling in susceptible sickle cells.

- Sickling test is simple and quick. The results can be viewed under light microscope after 20 minutes.
- Sickling test is positive whenever hemoglobin S is present (e.g. sickle cell anemia, sickle cell trait, sickle C disease, sickle cell thalassemia).
- False positivity of sickling test is seen in exceptionally high hematocrit, unstable hemoglobin, elevated plasma proteins and lipids.

ANTIHUMAN GLOBULIN TEST (COOMBS' TEST)

Direct Coombs' Test

Direct Coombs' test also known as antihuman globulin test is performed to detect autoantibodies.

- Patient's RBCs are mixed with antibodies directed against human immunoglobulin or complement. Presence of RBCs agglutination indicates positive direct Coombs' test.
- Direct Coombs' test detects antibodies or complement bound to surface of RBCs antigens *in vivo* in immune-mediated hemolytic anemia, hemolytic disease of newborn, mismatched blood transfusion.
- Drug-induced hemolytic anemia may also be associated with direct positive Coombs' test. If direct Coombs' test is negative in the presence spherocytes, one should consider hereditary spherocytosis as a possible cause of hemolysis.

Indirect Coombs' Test

Indirect Coombs' test is performed to detect *in vitro* antigen–antibody reactions, which detects low concentration of antibodies in patient's blood prior to blood transfusion and antenatal Rh –ve with Rh +ve fetus. Direct and indirect Coombs' test is also performed for compatibility testing in blood banking, and RBCs phenotyping (Fig. 8.58A and B).

HEMOGLOBIN ELECTROPHORESIS

Hemoglobin electrophoresis is a diagnostic tool to measure different types of hemoglobin in the blood in suspected disorder associated with abnormal hemoglobin such as sickle cell anemia and thalassemia.

Principle

The principle behind hemoglobin electrophoresis is that when proteins are exposed to charge gradient, they separate from each other by forming bands that separate toward one end or the other end in the electric field. Cellulose acetate membrane, starch gel, agarose gel, citrate agar gel and filter paper are the commonly used. Cellulose acetate electrophoresis is performed at alkaline pH of 8.2–8.6. Citrate agar or agarose gel electrophoresis is done in acidic pH of 6.0–6.2. High-performance liquid chromatography (**HPLC**) is rapidly taking its place in many laboratories.

Blood Sampling

Blood transfusions given within the previous 12 weeks may alter hemoglobin electrophoresis test results. Hemoglobin electrophoresis is performed on venous

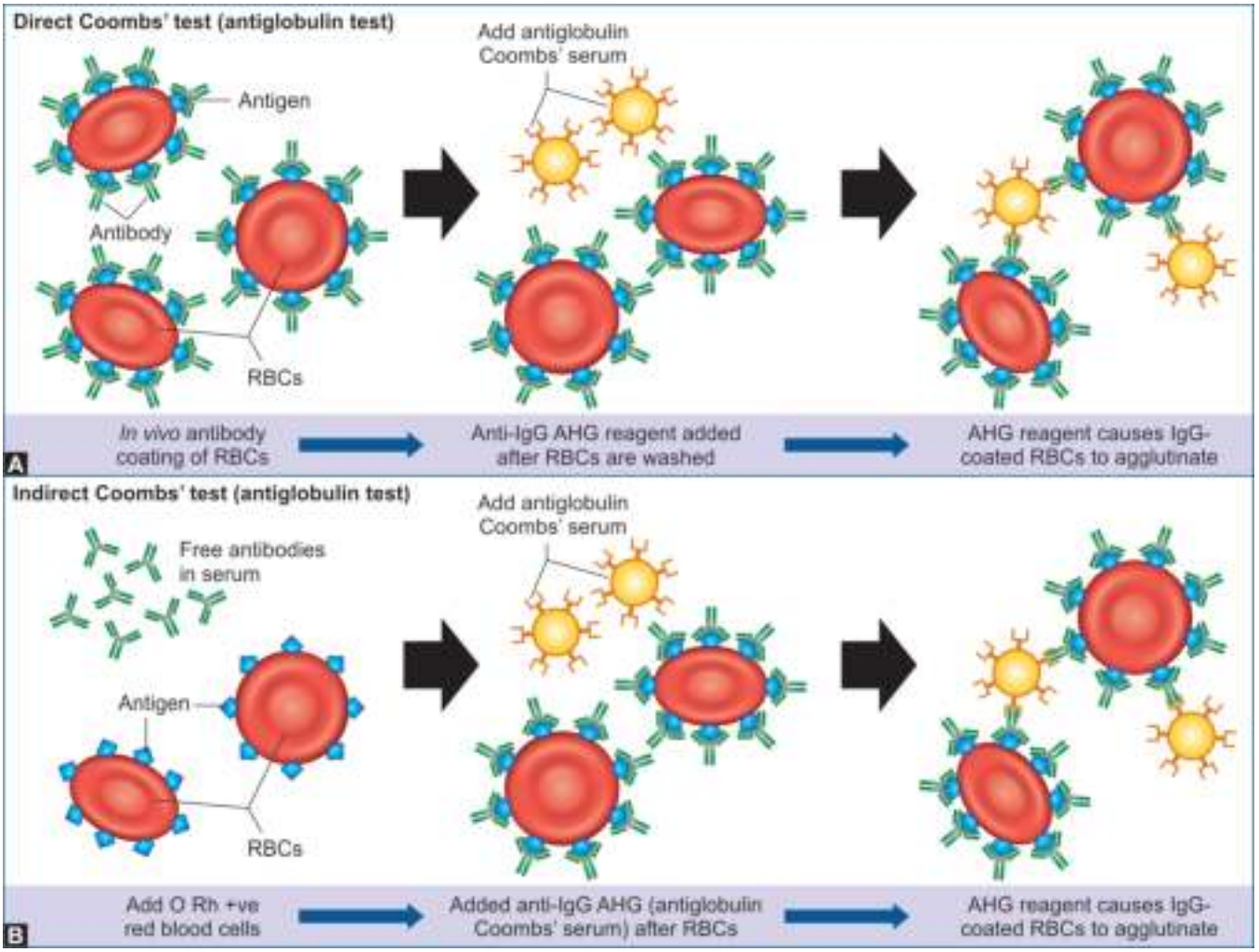


Fig. 8.58A and B: Direct and indirect Coombs' test.

blood samples taken in anticoagulant EDTA container. Red blood cell lysate is prepared using a lysing reagent (Na_4EDTA and KCN).

Interpretation

Each of the major hemoglobin types has an electrical charge of a different degree. Hemoglobin electrophoresis separates and measures normal and abnormal hemoglobin. The components then move away from each other at different rates, and when separated from a series of distinctly pigmented bands.

- These pigmented bands are then compared with those of a normal sample. Each band is further assessed as a % of the total hemoglobin, thus indicating the severity of any abnormality.
- Description of hemoglobin (Hb) is comprising of many different types, the most common being HbA_1, HbA_2, HbF, HbS, and HbC. Normal reference values can vary by laboratory but are generally within the following ranges.

Hemoglobin Electrophoresis Patterns

In normal adults, HbA_1 is the major component of hemoglobin in the normal red blood cell. HbA_2 is a minor component of normal hemoglobin, comprising approximately 2–3% of the total hemoglobin levels.

- In children, HbF is the major hemoglobin component in the fetus, but usually exists only in minimal quantities in the normal adult.
- Levels of fetal hemoglobin (HbF) greater than 2% in patients over three years of age are considered abnormal. Hemoglobin electrophoresis pattern in normal persons and hemoglobin disorders is given in Tables 8.95 and 8.96.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography (HPLC) is one of the commonest laboratory investigations performed for the identification of globin chain synthesis.

Table 8.95 Hemoglobin electrophoresis pattern in normal persons

Age Group	Hemoglobin	Percentage
Adults	▪ HbA_1 ▪ HbA_2 ▪ HbF ▪ HbS ▪ HbC	▪ 95–98% ▪ 2–3% ▪ 0.8–2.0% ▪ 0% ▪ 0%
Children	▪ HbF (newborn) ▪ HbF (6 months age) ▪ HbF (>6 months age)	▪ 50–80% ▪ 8% ▪ 1–2%

Principle

Cation exchange HPLC is a process by which mixture of hemoglobins with net positive charge are separated by their adsorption onto a negatively charged stationary phase in a chromatography column. This process is then followed by their elution by a liquid mobile phase containing increasing concentrations of cations flowing through the column. Hemoglobins separated by this process are optically identified in the elute. The quantification is done by computing the area under the corresponding peak in the elution profile.

Interpretation

Different hemoglobins have different affinity for the stationary phase. Hemoglobin with strong positive charge has higher affinity for stationary phase and appears in elute later than other hemoglobins. Retention time is the time taken by normal and variant hemoglobin to appear in the elute. Retention time of normal and variant hemoglobins is given in Table 8.97.

Advantages

High performance liquid chromatography is performed on very small venous sample, which estimates quantity of normal and abnormal hemoglobins. HPLC is used to estimate quantity of HbA_2 in diagnosing β-thalassemia trait. Overall, HPCL has more advantages than hemoglobin electrophoresis and routinely performed in many laboratories.

Disadvantages

HPLC instrument and its reagents are very costly.

ISOELECTRIC FOCUSING

Isoelectric focusing (IEF) is a technique for separating different molecules by differences in their isoelectric point.

- Hemoglobins in isoelectric focusing (IEF) are separated in agarose gel according to their isoelectric points at which these have no net charge.
- Bands in IEF are sharper as compared to electrophoresis. But this diagnostic tool is more expensive than electrophoresis.

MICROCOLUMN CHROMATOGRAPHY

Microcolumn chromatography is an anion exchange chromatography used to estimate quantity of **HbA_2 in α-thalassemia**.

Table 8.97 Retention time of normal and variant hemoglobins

Window	Retention Time (Minutes)	Hemoglobins that Appear in the Window
H	Peak within first minute	HbH, Bart's hemoglobin, bilirubin, acetylated HbF
F	1.10	HbF
A	2.50	HbA, glycosylated S
A_2	3.60	HbA_2, D-Iran, E-Lepore
D	4.10	D-Punjab
S	4.50	S
C	5.10	C-constant spring

Table 8.96 Hemoglobin electrophoresis pattern in hemoglobin disorders

Hemoglobin	Hemoglobin Disorder	Percentage
HbA_2	▪ β-thalassemia ▪ HbH disease	▪ 4.00–5.8% ▪ <2%
HbF	▪ β-thalassemia major ▪ β-thalassemia minor ▪ Homozygous hereditary persistence of fetal hemoglobin (HPFH) ▪ Heterozygous hereditary persistence of fetal hemoglobin (HPFH) ▪ Heterozygous HbS	▪ 10–90% ▪ 2–5% ▪ 100% ▪ 5–35% ▪ 15%
HbS	Sickle cell disease (homozygous)	70–98%
HbC	Hemoglobin C disease (homozygous)	90–98%

IMMUNOASSAY

Immunoassay is used to evaluate various hemoglobins by commercially available kits such as hemocards for specific hemoglobins.

KLEIHAUER-BETKE TEST

Kleihauer test (acid elution test) is a blood test to measure the amount of fetal hemoglobin transferred from a fetus to a mother's bloodstream. Test is based on principle that red blood cells containing HbF become resistant to lysis as compared to red blood cells containing HbA. It is most often performed on Rh –ve mothers to determine the required dose of Rho (D) immune globulin (RhIg) to inhibit formation of Rh antibodies in the mother and prevent Rh disease in future Rh positive children.

FLOW CYTOMETRY TECHNIQUE

Flow cytometry technique is done by using fluorochrome conjugated monoclonal antibody to HbF (kindly *refer* to flow cytometry technique disscuss in detail in Ch 14: Cellular–Molecular Diagnostic Techniques in Clinical Practice).

2,6-DICHLOROPHENOLINDOPHENOL TEST

Hemoglobin E is an abnormal hemoglobin with a single point mutation in the β-chain due to change in the amino acid, from glutamic acid to lysine at position 26. HbE disorder is very common in South-East Asia but has a low frequency amongst other ethnicities HbE can be detected on electrophoresis. 2,6-dichlorophenolindophenol (**DCIP**) test is used to screen patient with HbE.

SUPRAVITAL STAINING

Supravital staining is used to demonstrate HbH inclusions. Peripheral blood smears are stained with brilliant cresyl blue stain, that acts as a mild oxidant and precipitates HbH.

GLOBIN CHAIN ELECTROPHORESIS

In globin chain electrophoresis DL-dithiothreitol and urea added to red blood cell lysate to dissociate heme and globin chains. Globin chain electrophoresis is then carried out on cellulose acetate membrane with both acid and alkaline buffer systems, which method permits the distinction between α- and β-chain abnormalities.

BONE MARROW FAILURE SYNDROMES

Bone marrow participates in hematopoiesis. Bone marrow failure is characterized by marrow hypoplasia leading to pancytopenias (erythroid, myeloid and megakaryocytes) and decreased reticulocyte count as a result of failure of bone marrow to produce precursor cells. Bone marrow failure occurs due to inherited or acquired causes. Summary of inherited bone marrow failure syndrome is given in Table 8.98.

Table 8.98 Summary of inherited bone marrow failure syndrome

Inherited Disorder	Inheritance	Cellular Pathway Abnormality	Associated Risk of Malignant Neoplasms
Fanconi syndrome	Autosomal recessive disorder (except X-linked FNCB)	DNA repair abnormality	▪ Myelodysplastic syndrome ▪ Acute myelogenous leukemia
Diamond-Blackfan anemia	Autosomal dominant (except GATA1 related X-linked disorder)	Ribosomal biogenesis abnormality	▪ Myelodysplastic syndrome ▪ Acute myelogenous leukemia ▪ Colon adenocarcinoma ▪ Osteosarcoma
Shwachman-Diamond syndrome	Autosomal recessive disorder	Ribosomal assembly abnormality	▪ Myelodysplastic syndrome ▪ Acute myelogenous leukemia ▪ Acute lymphoblastic leukemia
Congenital dyserythropoietic anemia (CDA)	▪ Autosomal recessive disorder (CDA I, CDA II) ▪ Autosomal dominant (CDA III, CDA IV)	Abnormality of unfolded protein response, cytokinesis and erythroid transcription factor	Multiple myeloma related to CDA III
Congenital amegakaryocytic thrombocytopenia	Autosomal recessive disorder	Abnormality of megakaryopoiesis	▪ Myelodysplastic syndrome ▪ Acute lymphoblastic leukemia

Contd...

Table 8.98 Summary of inherited bone marrow failure syndrome (*Contd...*)

Inherited Disorder	Inheritance	Cellular Pathway Abnormality	Associated Risk of Malignant Neoplasms
Thrombocytopenia with absent radii	Autosomal recessive disorder	Abnormality of mRNA processing	▪ Acute myelogenous leukemia ▪ Acute lymphoblastic leukemia
Severe congenital neutropenia with ELANE gene mutation	Autosomal dominant disorder	Abnormality of unfolded protein response	▪ Myelodysplastic syndrome (MDS) ▪ Acute myelogenous leukemia
Dyskeratosis congenita	X-linked disorder, autosomal dominant disorder, autosomal recessive disorder	Abnormality of maintenance of telomere	▪ Myelodysplastic syndrome (MDS) ▪ Acute myelogenous leukemia ▪ Squamous cell carcinoma ▪ Other malignant tumors
GATA deficiency	Autosomal dominant disorder	Abnormality of hematopoietic stem cell differentiation	▪ Myelodysplastic syndrome (MDS) ▪ Acute myelogenous leukemia
Familial platelet disorder with a propensity to myeloid malignancy (RUNX-1)	Autosomal dominant disorder	Abnormality of hematopoietic stem cell differentiation	▪ Myelodysplastic syndrome (MDS) ▪ Acute lymphoblastic leukemia

APLASTIC ANEMIA

Aplastic anemia is pluripotent stem cell disorder results in bone marrow failure, which adversely affects erythroid, myeloid and megakaryocytic series.

- Bone marrow shows variably reduced cellularity, depending on the clinical stage of the disease.
- Severe aplastic anemia is characterized by <25% cellularity of bone marrow, platelet count <20,000/cu mm and increased fat spaces in the bone marrow.
- Bone marrow pluripotent stem cell failure occurs due to primary or secondary causes. Causes of aplastic anemia are given in Table 8.99.

PATHOGENESIS

Primary aplastic anemia occurs in 65% of cases. Four inherited aplastic anemia syndromes include Fanconi anemia, dyskeratosis congenita, Shwachman-Diamond syndrome and congenital megakaryocytic thrombocytopenia. Fanconi anemia is characterized by pancytopenia, congenital abnormalities and increased risk of cancers. Secondary aplastic anemia may be caused by total body irradiation, drugs, chemical agents, viral infections, and Fanconi anemia.

- **Physical agent:** Ionizing radiation causes aplastic anemia.
- **Exposure to chemical agents:** Bone marrow suppression due to drugs and chemicals may be dose related (e.g. benzene, alkylating agents, vincristine).
- **Exposure to drugs:** Drugs causing bone marrow suppression in unpredictable manner (idiosyncrasy) are streptomycin, chloramphenicol and chlorpromazine.

Table 8.99 Causes of aplastic anemia

Primary/Idiopathic Aplastic Anemia	
Constitutes 70% cases of aplastic anemia	
Secondary Aplastic Anemia	
▪ **Cytotoxic drugs** • Anti-inflammatory drugs (e.g. phenylbutazone, indomethacin) • Antibiotics (e.g. chloramphenicol) • Anticonvulsants (e.g. carbamazepine, phenytoin, valproic acid)	
▪ **Exposure to industrial chemical agents** • Benzene	• Pesticides
▪ **Exposure to radiation** • Therapeutic radiation	• Accidental radiation
▪ **Viral infections** • Parvovirus B19 • Hepatitis virus A • Hepatitis virus B • Cytomegalovirus	• Human immunodeficiency virus (HIV) syndrome • Epstein-Barr virus
▪ **Immune including autoimmune disorders** • Systemic lupus erythematosus (SLE) • Graft-versus-host disease	• Transfusion related graft-versus-host disease • Eosinophilic fasciitis
▪ **Miscellaneous disorders** • Paroxysmal nocturnal hemoglobinuria (PNH) • Pregnancy	• Thymoma • Thymic carcinoma

- **Viruses:** Aplastic anemia may be caused by human Parvovirus B19 or HCV.
- **Hematologic disorders:** Aplastic anemia can occur in the course of hereditary spherocytosis or sickle cell anemia. Some cases of paroxysmal nocturnal

hemoglobinuria are associated with aplastic anemia, while some may progress to myelodysplastic syndrome resulting in acute myelogenous leukemia. Fanconi anemia is inherited disease due to defects in telomerase activity.

Laboratory Diagnosis of Aplastic Anemia

Diagnostic Criteria

Diagnosis and assessment of severe aplastic anemia defined by Camitta's criteria include as bone marrow cellularity <30% and severe pancytopenia with at least two of the following peripheral blood count findings:

- Absolute neutrophil count is $<0.5 \times 10^9$/L
- Platelet count is $<20 \times 10^9$/L
- Corrected reticulocyte count is <1%

Reticulocyte Count

The lack of an appropriate reticulocyte response to the anemia indicates ineffective hematopoiesis as the mechanism for the pancytopenia.

Bone Marrow Smear Examination

Hematopoietic elements are replaced by fat cells lacking normal hematopoietic activity. There is relative increase in lymphocytes and plasma cells. As bone marrow cellularity decreases, there is a corresponding increase in bone marrow fat. One must look for metastatic deposits in bone marrow (Fig. 8.59).

CLINICAL FEATURES

Patients with aplastic anemia present with weakness, fatigue, progressive anemia, recurrent infections due to neutropenia, and bleeding tendencies as a result of thrombocytopenia. Withdrawal of potential inciting agent may sometimes lead to recovery. Bone marrow transplantation is recommended in these cases.

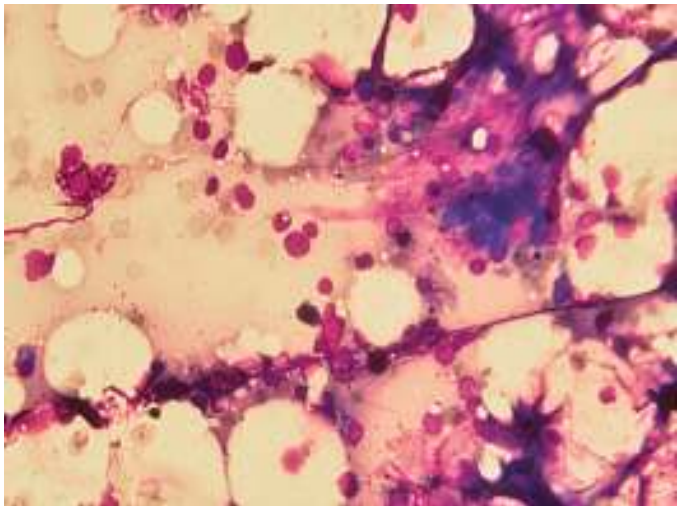


Fig. 8.59: Bone marrow in aplastic anemia shows marked reduction in the erythroid, myeloid and megakaryocytic precursor cells with increased adipose cells.

PURE RED CELL APLASIA (ERYTHROBLASTOPENIA)

Pure red cell aplasia (PRCA) is an uncommon disorder in which maturation arrest occurs in the formation of red blood cells. Erythroblasts are virtually absent in the bone marrow. Bone marrow shows normal myelopoiesis and megakaryopoiesis.

CLASSIFICATION

Pure red cell aplasia may be congenital or acquired. Congenital pure red cell aplasia may be inherited (Diamond-Blackfan anemia) or nonherited (Pearson syndrome). Diamond-Blackfan anemia belongs to one of the rare group of genetic disorders known as the inherited bone marrow failure syndromes (IBMFSs). These disorders share a predilection to bone marrow failure, birth defects, and cancer are characterized by proapoptotic hematopoiesis. Classification of pure red cell aplasia (erythroid hypoplasia) is given in Table 8.100.

PATHOGENESIS

In some cases, the IgG seems to be cytotoxic to CFU-E in the presence of complement; in others, its inhibitory activity is independent of the presence of complement but its presence is continuously required during the 7-day period of maturation of CFU-E to erythroblasts.

Table 8.100 Classification of pure red cell aplasia (erythroid hypoplasia)

Inherited Pure Red Cell Aplasia
Diamond-Blackfan syndrome
Thymoma (T cell-mediated suppression of erythropoiesis)
Acquired Transient Pure Red Cell Aplasia
Transient erythroblastopenia of childhood
Parvovirus infection cytotoxic to CFU-E in children usually transient affects erythroid precursors in bone marrow showing few giant proerythroblasts with basophilic cytoplasm, intranuclear inclusions in sieve-like nucleus
Acquired Sustained Pure Red Cell Aplasia
Thymoma (T cell-mediated suppression of erythropoiesis)
Large granular lymphocytic T cell leukemia
Chronic lymphocytic leukemia
Clonal myeloid disease especially 5q syndrome
Viral infection other than Parvovirus
Solid malignant tumors
Drugs (phenytoin, procainamide, isoniazid)
Collagen vascular diseases

- The bone marrow arrest occurs at any level between CFU-E and basophilic erythroblasts.
- The stage of erythropoiesis at which the bone marrow arrest occurs has been studied by assaying PRCA bone marrow cells in semisolid media for erythroid progenitors.

CLINICAL FEATURES

Adult patients of inherited pure red cell aplasia present with symptoms of anemia that may be quite severe at the time of diagnosis. Complete arrest of erythropoiesis in bone marrow leads to a decline in red cell count averaging about 1% per day, so the development of anemia is slow and progressive, allowing for physiologic compensatory changes. Physical examination in primary pure red cell aplasia (PRCA) is usually negative except for pallor and signs of anemia. In secondary pure red cell aplasia cases, patients may have physical findings related to the underlying disease. Hepatosplenomegaly and lymphadenopathy findings are not consistent with primary PRCA.

Laboratory Diagnosis of Pure Red Cell Aplasia (PRCA)

Peripheral Blood Smear Examination

- Peripheral blood smear examination shows normochromic and normocytic.
- The white cell count and the differential leukocyte count are normal. Occasionally, mild leukopenia, lymphocytosis, and/or eosinophilia may be present.
- There is a complete absence of polychromatophilic red blood cells on the peripheral blood smear, and the reticulocyte count is between 0 and 1%.

Bone Marrow Smear Examination

- The hallmark of PRCA is the absence of erythroblasts from an otherwise normal bone marrow.
- The cellularity of the bone marrow is normal or slightly increased. High cellularity with elimination of fat spaces should lead away from the diagnosis of PRCA. In typical cases, the erythroblasts are either totally absent, or these constitute <1% on the marrow differential count.
- The myeloid cells and the megakaryocytes in the bone marrow are normal and exhibit full maturation.
- An increased number of lymphocytes on bone marrow smear, or an increased number of lymphoid aggregates in bone marrow trephine biopsy, or a mild increase in plasma cells, eosinophils, or mast cells may be seen.

Bone Marrow Iron Stores

Bone marrow iron stores are increased and normally distributed, but during recovery or the phase of ineffective erythropoiesis, a few ring sideroblasts may be seen.

SIDEROBLASTIC ANEMIA

Sideroblastic anemias (SA) are heterogeneous group of disorders characterized by presence of ring sideroblasts in the bone marrow, ineffective erythropoiesis, increased levels of tissue iron and microcytic hypochromic picture. Sideroblastic anemia may be hereditary or secondary (more common). Classification of sideroblastic anemia is given in Table 8.101.

- Sideroblasts are erythroblasts containing aggregates of iron which are demonstrable by Perls Prussian blue reaction. There are three types of sideroblasts: normal sideroblast, abnormal sideroblast and ring sideroblast (Fig. 8.60). Types of sideroblastic anemia according to WHO are given in Table 8.102.
- Under physiologic state, iron present in mitochondria combines with protoporphyrin to synthesize heme in erythroid precursors. Under pathologic state, iron present in mitchondria fails to bind with protoporphyrin affecting heme synthesis. Iron remains in the mitochondria of developing erythroblasts and forms ring sideroblasts, which do not mature into RBCs leading to sideroblastic anemia. Ineffective erythropoiesis increases iron overload.

HEREDITARY SIDEROBLASTIC ANEMIA

Hereditary sideroblastic anemia may be X-linked affecting males or autosomal recessive disorder, which occurs due to defective ALA synthatase resulting in defective synthesis of hemoglobin. Patient presents with anemia, hepatomegaly, splenomegaly, failure to thrive and cardiac arrhythmias.

Laboratory Diagnosis of Hereditary Sideroblastic Anemia

Peripheral Blood Smear Examination and Other Hematological Tests

- Peripheral blood smear examination shows anisopoikilocytosis and microcytic hypochromic anemia. Leukocytes and platelets are within normal ranges.
- Hematocrit values (MCH and MCHC) are decreased.
- Serum iron and ferritin levels are increased.

Bone Marrow Examination

- Bone marrow examination shows erythroid hyperplasia and dyserythropoiesis. Megakaryocytes are normal.
- Bone marrow iron stores are increased demonstrated by Perls Prussian blue reaction.

Table 8.101 Classification of sideroblastic anemia

Sideroblastic Anemia: Classification	Comments
Genetic sideroblastic anemia	
X-linked sideroblastic anemia (XLSA)	▪ Erythroid δ-aminolevulinic acid synthase (ALAS2) gene mutation inactivates hematopoietic component resulting in mild anemia ▪ Sideroblastic anemia with ataxia (ABCB7, an ATP-binding cassette protein)
Mitochondrial DNA genetic sideroblastic anemia	▪ Mitochondrial amino acid carrier protein SIC25A38, MCV low, severe transfusion dependent anemia ▪ Mitochondrial respiratory enzyme mutations, e.g. Pearson syndrome*; Kearns-Sayre syndrome* ▪ YARS2 gene encoding for mitochondrial tyrosyl-tRNA synthase
Autosomal genetic sideroblastic anemia	▪ Glutaredoxin 5 (GLRX5) gene mutation, MCV low, rarely transfusion dependent anemia ▪ Erythropoietic protoporphyria (EPP) congenital sideroblastic anemia (ferrochelatase gene mutation), mild anemia in children with photosensitivity ▪ Thiamine-responsive megaloblastic anemia and sideroblastic anemia [SLC19A2 gene encoding the thiamine transporter THTR-1, DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy, and deafness) syndrome]
Acquired sideroblastic anemia	
Primary genetic sideroblastic anemia	Myelodysplastic syndrome (MDS)
Secondary genetic sideroblastic anemia	▪ Hematological disorders • Acute myelogenous leukemia (AML) • Multiple myeloma • Polycythemia vera • Myelofibrosis • Porphyria • Megaloblastic anemia • Hemolytic anemia ▪ Vitamin deficiency • Pyridoxine deficiency ▪ Autoimmune disorders • Systemic lupus erythematosus • Rheumatoid arthritis ▪ Drugs • Isoniazid • Pyrazinamide • Chloramphenicol • Cycloserine • Penicillamine • Fusidic acid ▪ Gastrointestinal disorder • Celiac disease

**Pearson syndrome (neutropenia, thrombocytopenia, sideroblastic anemia, exocrine pancreatic dysfunction and hepatic dysfunction); Kearns-Sayre syndrome (neutropenia, thrombocytopenia, sideroblastic anemia, exocrine pancreatic dysfunction and hepatic dysfunction, and involvement of brain, spinal cord and peripheral nerve).*

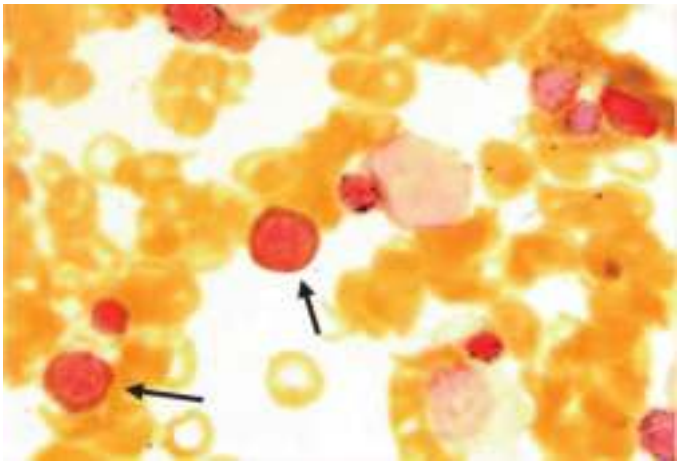


Fig. 8.60: Bone marrow aspirate in sideroblastic anemia shows ring sideroblasts (arrows).

ACQUIRED SIDEROBLASTIC ANEMIA

Acquired sideroblastic anemia occurs more frequently affecting adults than hereditary type. Ineffective erythropoiesis leads to iron overload. Patient presents with progressive anemia, signs of congestive heart failure and mild hepatosplenomegaly.

Laboratory Diagnosis of Acquired Sideroblastic Anemia

Peripheral Blood Smear Examination

- Peripheral blood smear examination shows dimorphic picture (microcytic hypochromic and macrocytic anemia).
- RBCs show basophilic stippling and target cells.
- RBCs also show iron deposits known as Pappenheimer bodies demonstrated by Perls Prussian blue reaction. Red blood cell distribution width (RDW) is increased.

Bone Marrow Smear Examination

- Bone marrow smear examination is hypercellular showing ringed sideroblasts demonstrated by Perls Prussian blue reaction.
- Dyserythropoiesis is characterized by nuclear budding.
- Howell-Jolly bodies are demonstrated in the normoblasts.
- Megakaryopoiesis is normal.

Biochemical Tests

- Serum iron, serum ferritin, % transferrin saturation and free protoporphyrin are increased.
- TIBC is decreased.

CONGENITAL DYSERYTHROPOIETIC ANEMIA

Congenital dyserythropoietic anemia is a heterogeneous disorder characterized by markedly ineffective erythropoiesis with dysplastic multinucleated erythroblasts and reduced reticulocyte count.

- Congenital dyserythropoietic anemias are designated as types 1 to 3 defined by the presence of distinctive morphologic abnormalities in erythroblasts demonstrated by light and electron microscopy.
- Dyserythropoiesis refers to abnormalities in the morphology or function or both of erythroid precursors. Features of congenital dyserythropoietic anemia (CDA) are given in Table 8.103.
  - Congenital dyserythropoietic anemia type 1 is characterized by moderate to severe macrocytic anemia in neonates or intrauterine growth retardation.
  - Congenital dyserythropoietic anemia type 2 is characterized by moderate anemia, splenomegaly and hepatomegaly.
  - Congenital dyserythropoietic anemia type 3 is characterized by mild anemia and retinal degeneration.
  - Congenital dyserythropoietic anemia type 4 is characterized by severe anemia at birth.

CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE 1

Congenital dyserythropoietic anemia (CDA) type 1 is an autosomal recessive disorder characterized by megaloblastic erythropoiesis due to CDAN1 or C150RF41 gene mutation (15q15).

Table 8.102 Types of sideroblastic anemia according to World Health Organization

Type of Sideroblastic Anemia	Bone Marrow	Iron Contents Demonstrated by Prussian Blue Reaction
Type 1	Intermediate or late normoblasts	1–2 pinpoint size iron granules in normoblasts randomly distributed
Type 2	Intermediate or late normoblasts	1–5 pinpoint size iron granules in normoblasts seen in megaloblastic anemia and thalassemia
Type 3	Intermediate or late normoblasts	Nonferritin iron, pinpoint-sized granules forming partial or complete ring seen in primary/secondary sideroblastic anemia

Table 8.103 Features of congenital dyserythropoietic anemia (CDA)

CDA Type	Gene Mutation	Inheritance	Bone Marrow Findings	Associated Clinical Findings
CDA type 1	▪ CDAN1 gene mutation (15q15) ▪ CD150RF4 gene mutation	Autosomal recessive	Megaloblastic erythropoiesis, erythroblasts with internuclear bridging	Skeletal abnormalities
CDA type 2	▪ CDAN2 gene mutation ▪ SEC23B gene mutation (20p11.2)	Autosomal recessive	Numerous binucleated erythroblasts and occasional multinucleated erythroblasts	Variable clinical findings
CDA type 3 (sporadic)	Unknown	Unknown	Giant multinucleated normoblasts containing 12 nuclei	Variable
CDA type 3 (familial)	KIF23 gene mutation (19p13.13)	Autosomal dominant	Giant multinucleated normoblasts	Plasma cell dyscrasias and angioid streaks
CDA type 4	KLF1 gene mutation (p13.12)	Autosomal dominant	Erythroblasts with internuclear bridging	Mild to moderate splenomegaly
CDA other variants	▪ KLF1, GATA1 gene mutation ▪ XLR gene mutation	▪ Autosomal dominant ▪ X-linked disorder	▪ Erythroblasts with internuclear bridging to numerous binucleated erythroblasts ▪ Erythroblasts with internuclear bridging to numerous binucleated erythroblasts	▪ Not applicable ▪ Not applicable

- On electron microscopy, erythroblasts in bone marrow show spongy nuclear chromatin giving Swiss cheese appearance.
- A few patients with CDA type 1 have been treated with interferon-α_2, with a good response.

CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE 2

CDA type 2 is most common autosomal recessive disorder due to mutation of CDAN2 gene located on chromosome 20q11.2. Geographic distribution—higher frequency of the gene mutation in North-West Europe, Italy and North Africa. Both sexes are equally affected. CDA type 2 patients suffer from lifelong anemia.

- **Pathogenesis:** CDA type 2 disorder results from a combination of the death of erythroblasts in the bone marrow (ineffective erythropoiesis) and hemolysis of red blood cells is known as peripheral hemolysis. The red blood cell glycosylation defects are responsible for both mechanisms.
- **Clinical features:** Patient during infancy or early childhood presents with anemia, jaundice and hepatosplenomegaly. There may be evidence of extramedullary hematopoiesis.
- **Laboratory diagnosis:** CDA type 2 is defined by serological abnormalities. It is also known as hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS), because RBCs most often show positive HEMPAS result on the acidified serum lysis test. RBC membrane shows marked reduction in membrane glycoprotein and band 3 detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). On electron microscopy, RBCs show double cytoplasmic membrane.

CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE 3

CDA type 3 is an autosomal dominant disorder in which erythroblasts show multiple nuclei up to 12 due to KIF23 gene mutation mapped on 19p13.13.

ANEMIA OF CHRONIC DISEASE

Anemia of chronic disease can be secondary to acute and chronic inflammation, viral infections including HIV, hematologic solid malignant tumors, autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, and sarcoidosis), chronic rejection of solid organ transplantation and chronic renal disease.

- Anemia of chronic disease is second in incidence to iron deficiency anemia, which occurs due to impaired utilization of iron from macrophage stores in the bone marrow.

Table 8.104 Causes of anemia of chronic inflammation, autoimmune and malignant disorders

Infectious Causes	
▪ Tuberculosis	▪ Pneumonia
▪ Pulmonary abscess	▪ Bacterial endocarditis
Noninfectious Causes	
▪ Systemic lupus erythematosus (SLE)	▪ Sarcoidosis
▪ Rheumatoid arthritis	▪ Crohn's disease
	▪ Gaucher's disease
Malignant Tumors	
▪ Carcinoma	▪ Lymphoma
▪ Sarcoma	

- Anemia of chronic disease is a functional iron deficiency, although storage iron is either normal or even increased. Causes of anemia of chronic inflammation, autoimmune and malignant disorders are given in Table 8.104.

PATHOGENESIS

In chronic inflammation, activated macrophages synthesize cytokines like IL-6, IL-1 and TNF-α. IL-6 activates synthesis of master regulator of iron protein hepcidin by liver.

- Hepcidin inhibits release of iron from storage sites resulting in decreased erythropoiesis.
- The cytokines inhibit CFU-E (colony forming units-erythroid) resulting in decreased production of red blood cells.
- Anemia in chronic kidney disease shares some of the characteristics of anemia of chronic disease. Anemia in chronic kidney disease occurs due to decreased production of erythropoietin, renal insufficiency and circulating the antiproliferative effects of accumulating uremic toxins.
- Interleukin 1 (IL-1) cytokine synthesized by activated macrophages inhibits synthesis of erythropoietin by kidney. IL-1 also increases uptake of iron by reticuloendothelial cells.
- Tumor necrosis factor α (TNF-α) cytokine synthesized by activated macrophages participates in phagocytosis and degradation of red blood cells.
- Interferon γ (IFN-γ) cytokine synthesized by lymphocytes inhibits erythropoiesis and increases uptake of iron by reticuloendothelial cells.
- Erythropoiesis may be decreased by infiltration of bone marrow by microorganisms and cancer stem cells. Tumor cells can produce proinflammatory cytokines and oxygen-derived free radicals that damage developing erythroid progenitor cells in the bone marrow.

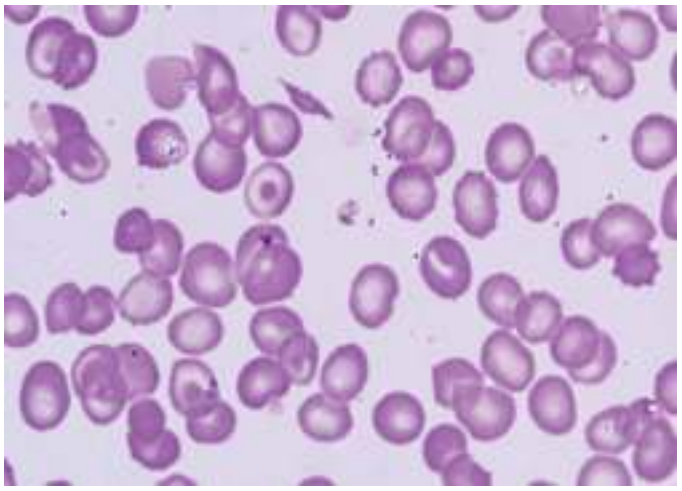


Fig. 8.61: Anemia of chronic disease. Peripheral blood smear examination shows mild to moderate microcytic hypochromic picture.

Laboratory Diagnosis of Anemia of Chronic Disease

Peripheral Blood Smear Examination

- Peripheral blood smear examination shows mild normocytic or mild to moderate microcytic hypochromic anemia.
- In chronic renal disease, anemia is most often normocytic and normochromic.
- Anemia in liver disease is moderately macrocytic. Peripheral blood smear findings are shown in Fig. 8.61.

Bone Marrow Smear Examination

- Increased iron is demonstrated in reticuloendothelial cells, e.g. bone marrow
- Defective iron transfer to red blood cell precursors
- Iron is reduced in red blood cell precursors
- Increased red cell protoporphyrin is observed
- Hematologic findings in anemia of chronic disease are given in Table 8.105.

MYELOPHTHISIC ANEMIA

Myelophthisic anemia is caused by bone marrow infiltration by metastatic tumor (e.g. lymphoma, breast, prostate, lung, stomach, kidney, thyroid gland) or extracellular material, bone marrow fibrosis (e.g. hairy cell leukemia, myelodysplastic syndrome), granulomatous disease, storage disease (e.g. Gaucher's disease, Niemann-Pick disease), and bone marrow necrosis.

Myelophthisic anemia is most often accompanied by a leukoerythroblastic peripheral blood picture characterized by increased dacrocytes (tear drop cells), nucleated red blood cells (normoblasts) and granulocyte precursors (shift to the left). Disorders causing myelophthisic anemia are given in Table 8.106.

- **Clinical features:** Patient presents with anemia and symptoms related to underlying cause, abdominal pain and massive splenomegaly.

Laboratory Diagnosis of Myelophthisic Anemia

Peripheral Blood Smear Examination

Peripheral blood smear examination shows severe anemic picture with leukoerythroblastic picture.

- Peripheral blood smear examination also shows increased dacrocytes (tear drop cells), nucleated red blood cells (normoblasts) and precursor granulocytic picture (shift to left).

Bone Marrow Smear Examination

- Bone marrow may demonstrate findings related to underlying etiology such as metastatic deposits (carcinomas, lymphoma), granulomatous disease, Gaucher's cells, Niemann-Pick cells.
- Bone marrow may show necrosis.

- **Prognosis and treatment:** Patient should be treated the underlying cause of myelophthisic anemia. Prognosis of myelophthisic anemia depends on underlying pathology.

Table 8.105 Hematologic findings in anemia of chronic disease

Features	Results
Peripheral blood smear examination	Mild normocytic or mild to moderate microcytic hypochromic anemia
Serum iron	Decreased
Serum total iron binding capacity (TIBC). In contrast to iron deficiency anemia, total iron binding capacity is decreased	Decreased
Transferrin saturation	Decreased
Serum ferritin	Normal or increased
Iron deposition in reticuloendothelial cells of bone marrow	Increased
Iron transfer to red blood cell precursors	Defective
Iron in red blood cell precursors	Decreased
Red blood cell protoporphyrin level	Increased

Table 8.106 Disorders causing myelophthisic anemia

Metastatic Solid Malignant Tumors and Lymphoma
▪ Breast carcinoma ▪ Prostatic carcinoma ▪ Lung carcinoma ▪ Gastric carcinoma ▪ Renal cell carcinoma ▪ Thyroid carcinoma ▪ Lymphomas
Granulomatous Inflammation
Tuberculosis
Hematologic Disorders
▪ Myeloproliferative neoplasms causing myelofibrosis ▪ Hairy cell leukemia causing myelofibrosis
Storage Disorders
▪ Gaucher's disease ▪ Niemann-Pick disease
Bone Marrow Necrosis
▪ Sickle cell disease crisis ▪ Infective etiology/sepsis ▪ All-*trans* retinoic acid therapy of acute promyelocytic leukemia
Disseminated Intravascular Coagulation (DIC)
▪ Blood transfusion reactions ▪ Infection by bacteria or fungi ▪ Liver disease ▪ Pregnancy-related complication due to retained placenta after delivery
Pulmonary Hypertension causing Myelofibrosis
Persistent pulmonary hypertension
Hematophagocytic Syndrome
Triggered by viral infections, e.g. Epstein-Barr virus or cytomegalovirus

FANCONI ANEMIA

Fanconi anemia (FA) is a rare autosomal recessive disorder characterized by bone marrow suppression (pancytopenia), multiple congenital anomalies, e.g. hypoplasia of bones of thumbs, radius, kidney or spleen, developmental delay and hyperpigmentation.

- **Molecular genetics:** Fanconi anemia occurs due to defects in a cluster of proteins responsible for DNA repair via homologous recombination.
  - Cells lack ability to excise UV-induced pyrimidine dimers from cellular DNA.
  - At least 13 gene mutations are known to cause Fanconi anemia, e.g. FANCD1/BRCA2, GANCG (XRCC9), FANCI, FANCJ, FANCL, FANCM and FANCN.
  - Patient with BRCA2 gene mutation is move prone to develop breast carcinoma.
- **Clinical features:** Fanconi anemia affects all bone marrow elements. Patient develops pancytopenia, bone marrow failure and growth retardation. There is increased risk for development of myelodysplasia (MDS), acute myelogenous anemia (AML) and solid tumors, e.g. brain tumors, hepatocellular carcinoma and breast carcinoma.

Laboratory Diagnosis of Fanconi Anemia (FA)

Peripheral Blood Smear Examination

- After attaining 10 years of age, patient develops pancytopenia defining abnormalities in two or more blood cell lineages in contrast to Diamond-Blackfan anemia, which affects only erythrocytes.
- Peripheral blood smear examination shows pancytopenia and thrombocytopenia.

Bone Marrow Smear Examination

- Bone marrow aspiration and trephine biopsy are essential to assess cellularity and morphology of residual marrow cells.
- Bone marrow shows a few hematopoietic cells replaced by fat cells, stromal cells and lymphocytes. Residual erythroid precursors may show evidence of dysplasia with nuclear-cytoplasmic maturation dissociation.

- **Treatment:** Hematopoietic stem cell (HSC) transplantation is curative. After bone marrow transplantation, patient may still develop myelodysplasia, acute myelogenous leukemia, brain tumors, hepatocellular carcinoma, and head and neck cancers. Surveillance for early detection of cancer is essential in these patients.

CHAPTER 9

White Blood Cell Disorders

Vinay Kamal, Anubhav and Vigyat

LEARNING OBJECTIVES

LEUKOPOIESIS, QUANTITATIVE AND QUALITATIVE DISORDERS OF LEUKOCYTES

- Leukopoiesis
- Leukocytes: granulocytes and agranulocytes
  - Neutrophils
  - Eosinophils
  - Basophils
  - Monocytes
  - Lymphocytes
- Quantitative variations in leukocytes
  - Leukocytosis
  - Leukopenia
- Qualitative variations in leukocytes
  - Alder-Reilly anomaly
  - Chédiak-Higashi syndrome
  - May-Hegglin anomaly
  - Pelger-Hüet anomaly
  - Chronic granulomatous disease of the childhood
  - Myeloperoxidase deficiency
  - Leukocyte adhesion deficiency disorder
- Infectious mononucleosis
  - Mode of transmission
  - Clinical features
  - Pathophysiology
  - Complications
  - Laboratory diagnosis

LEUKEMIAS

- Leukemias: overview
  - Classification of leukemia
    - French-American-British (FAB) classification of leukemias
    - Revised 2024 WHO classification of leukemias
  - Pathogenesis
  - Laboratory diagnosis

ACUTE LYMPHOBLASTIC LEUKEMIA

- Acute lymphoblastic leukemia: overview
  - Predisposing factors
  - Molecular pathogenesis
  - Clinical features
  - Life-threatening complications of ALL and management
  - Laboratory diagnosis
    - Lymphoblast morphology in ALL subtypes
    - Immunophenotying in ALL subtypes
    - Cytogenetic/molecular genetic alterations in acute lymphoblastic leukemia
  - Prognosis
  - Treatment
  - Minimal residual disease

ACUTE MYELOGENOUS LEUKEMIA

- Acute myelogenous leukemia: overview
  - Pathogenesis
  - Clinical features
    - French-American-British (FAB) and revised 2024 WHO classification of acute myelogenous leukemia
  - Laboratory diagnosis
- Acute myelogenous leukemia: entities
  - AML-M0 with minimal differentiation
  - AML-M1 without maturation
  - AML-M2 with maturation
  - Acute promyelocytic leukemia (AML-M3, FAB)
  - Acute myelomonocytic leukemia (AMML/ AML-M4, FAB)
  - Acute monoblastic/monocytic leukemia (AML-M5A and AML-M5B, FAB)
  - Acute erythroid leukemia (AML-M6)
  - Acute megakaryoblastic leukemia (AMKL-M7)
- Myeloid sarcoma
- Acute myelogenous leukemia, not otherwise specified (AML-NOS)
  - Diagnostic approach
  - Prognostic factors
  - Treatment

MYELODYSPLASTIC SYNDROMES

- Myelodysplastic syndromes: overview
  - French-American-British (FAB) and revised 2024 WHO classification of myelodysplastic syndrome (MDS)
  - Pathogenesis
    - Molecular genetic alterations
    - Cytogenetic abnormalities
  - Clinical features
  - Laboratory diagnosis
  - Prognostic criteria
  - Treatment

MYELODYSPLASTIC/MYELOPROLIFERATIVE NEOPLASMS

- Myelodysplastic/myeloproliferative neoplasms: entities
  - Chronic myelomonocytic leukemia (CMML)
  - Juvenile myelomonocytic leukemia (JMML)
  - Atypical chronic myelogenous leukemia (aCML), BCR-ABL1 negative

CHRONIC MYELOPROLIFERATIVE NEOPLASMS

- Chronic myelogenous leukemia, BCR-ABL1 positive
  - Pathophysiology
  - Molecular genetic analysis
  - Clinical features
  - Clinical course
  - Differential diagnosis
  - Targeted therapy
  - Allogeneic hematopoietic stem cell transplantation
- Chronic neutrophilic leukemia
  - Pathophysiology
  - Clinical features
  - Laboratory diagnosis
  - Targeted therapy
  - Clinical course
  - Differential diagnosis
- Essential thrombocythemia
  - Pathophysiology
  - Clinical features
  - Laboratory diagnosis
  - Management
  - Differential diagnosis
- Polycythemia vera
  - Pathophysiology
  - Clinical features

- Primary myelofibrosis
  - Pathophysiology
  - Clinical features
  - Prognosis and treatment
  - Differential diagnosis
- Clonal hypereosinophilia
  - Myeloid and lymphoid neoplasms associated with eosinophilia and PDGFRA, PDGFRB, or FGFR1
- Idiopathic hypereosinophilic syndrome
- Chronic eosinophilic leukemia, not otherwise specified
- Mastocytosis (mast cell disease)

CHRONIC LYMPHOPROLIFERATIVE NEOPLASMS

- Chronic lymphocytic leukemia
  - Pathogenesis
  - Clinical features
  - Diagnosis
  - Clinical history and staging system
  - Prognosis
  - Treatment
- Hairy cell leukemia
  - Pathophysiology
  - Clinical features
  - Laboratory diagnosis
  - Differential diagnosis
  - Treatment
- B cell prolymphocytic leukemia
- Adult T cell leukemia
- Non-Hodgkin's lymphoma: spillover in blood
- Hodgkin's disease
- Mantle cell lymphoma: spillover in blood
- Splenic lymphoma with villous lymphocytes

MULTIPLE MYELOMA AND OTHER MONOCLONAL GAMMOPATHIES

- Multiple myeloma
  - Terminology
  - Molecular classification
  - Molecular genetic alterations
  - Molecular pathogenesis
  - Clinical features
  - Laboratory diagnosis
  - Differential diagnosis
  - Prognostic factors
  - Treatment
- Waldenström macroglobulinemia
  - Clinical features
  - Predictive poor prognostic factors
  - Management
- Monoclonal gammopathy of undetermined significance

MONONUCLEAR PHAGOCYTIC SYSTEM DISORDERS

- Lysosomal storage disorders
  - Gaucher's disease
  - Niemann-Pick disease
  - Sea-blue histiocytosis
- Langerhans' cell histiocytosis
  - Letterer-Siwe disease (acute disseminated LCH)
  - Hand-Schüller-Christian disease (multifocal LCH)
  - Eosinophilic granuloma

HEMATOPOIETIC STEM CELL TRANSPLANTATION

- Hematopoietic stem cell transplantation: overview
  - Hematopoietic stem cell harvesting
- Selection of donor for obtaining HSCs
  - Techniques to isolate HSCs
- Types of HSC transplantation
  - Allogeneic HSC transplantation
  - Autologous HSC transplantation
  - Umbilical cord HSC transplantation
- Hematopoietic stem cell transplantation in the recipients
  - Conditioning
  - Infusion of HSCs
  - Supportive measures
- Complications of HSC transplantation

LEUKOPOIESIS, QUANTITATIVE AND QUALITATIVE DISORDERS OF LEUKOCYTES

LEUKOPOIESIS

Hematopoiesis occurs from a succession of anatomic sites during embryonic and fetal development, beginning outside the embryo in the yolk sac. Soon afterward, hematopoiesis begins in the embryo proper, then in the fetal liver, spleen and finally the fetal bone marrow. With each change of anatomic site, the range of hematopoiesis becomes more complex and similar to that of the adult.

- Hematopoietic growth factors may stimulate proliferation of early bone marrow cells, direct differentiation to one or other cell type, stimulate cell maturation, suppress apoptosis, or affect the function of mature non-dividing cells. Hematopoietic growth factors can act on stromal cells, pluripotent stem cells, multipotential progenitor cells and committed progenitor cells.
- Hematopoietic cells comprise hematopoietic stem cells (HSCs; CD34+), progenitor cells (committed cells) and mature red blood cells, white blood cells and platelets.
- Leukocytes originate from pluripotent hematopoietic stem cells (CD34+) in the bone marrow common myeloid and lymphoid hematopoietic stem cells.
- Hematopoietic stem cells (HSCs) possess self-renewal property. Hematopoietic growth factors are a family of regulatory molecules that play important roles in the growth, and differentiation of blood progenitor cells, as well as functional activation of mature cells.
- Granular leukocytes (neutrophils, eosinophils, basophils) have their origin in the myeloid stem cells and develop through a sequence involving myeloblast, promyelocyte, myelocyte, metamyelocyte, band forms and mature granular cells such as neutrophils, eosinophils, and basophils.
- Neutrophils are predominant cells in the first few weeks in the newborn. Lymphocytes are predominant cells in the postnatal period between 4 weeks and 4 years. After 4 years of age, neutrophils are predominant cells.
- Monocytes like granulocytes have their origin from myeloid stem cell, but develop along a pathway involving monoblast, promonocyte and monocytes. The monocytes migrate to tissues and become tissue macrophages (called reticuloendothelial cells). Monocyte-macrophages phagocytose bacteria and particulate material and play a pivotal role in the inflammatory reactions.
- Lymphocytes originate from lymphoid stem cell lineage, which develop through sequence involving

Table 9.1 Hematopoietic growth factors and their actions on bone marrow cells

Hematopoietic Growth Factors	Actions on Bone Marrow Cells
TNF (tumor necrosis factor), IL-1	Stromal cells
SCF (stem cell factor), FLT3-L (FLT-ligand), VEGF (vascular endothelial growth factor)	Pluripotent stem cells
IL-3, GM-CSF (granulocyte-macrophage colony-stimulating factor), IL-6, G-CSF (granulocyte colony-stimulating factor), Thrombopoietin	Multipotential progenitor cells
G-CSF (granulocyte colony-stimulating factor), M-CSF (macrophage colony-stimulating factor), IL-5 (maturation and release of eosinophils), erythropoietin, thrombopoietin	Committed progenitor cells

Hematopoietic growth factors also act synergistically with early acting growth factors on pluripotential progenitors.

Table 9.2 Normal percentage and differential white blood cell count

White Blood Cells	Percentage
Neutrophils	50–70%
Eosinophils	1–4%
Basophils	0–1%
Lymphocytes	20–40%
Monocytes	2–10%

lymphoblasts, prolymphocytes, lymphocytes, which undergo further differentiation in the lymphoid organs. T progenitor cell migrates to thymus. B progenitor cell is transformed into plasma cell. Natural killer progenitor cell gives rise to natural killer cell.

- Life span of leukocytes is relatively short, so the constant renewal is essential to maintain normal blood levels. Any conditions that decrease the availability of hematopoietic stem cells or hematopoietic growth factors lead to decrease in white blood cells.
- Hematopoietic growth factors and their actions on bone marrow cells are given in Table 9.1. Normal percentage and differential white blood cell count are given in Table 9.2.

LEUKOCYTES: GRANULOCYTES AND AGRANULOCYTES

Leukocytes are the major cellular components of the inflammatory and immune response that protect against infection and neoplasia and assist in the tissue repair.

- Like all blood cells, leukocytes are produced primarily in the bone marrow, which develop from hematopoietic stem cells that mature into one of the five major types of white blood cells (i.e. neutrophils, lymphocytes, monocytes, eosinophils, and basophils).
- Normally, persons produce approximately 100 billion white blood cells. The number of white blood cells in a given volume of blood is expressed as cells per microliter of blood. The total leukocyte count normally ranges between 4,000 to 11,000 cells per microliter (**4×10^9/L to 11×10^9/L**).

NEUTROPHILS

Neutrophils are the dominant circulating myeloid-lineage phagocytes involved in self-defense in humans, which constitute 50–70% of leukocytes.

- Neutrophils measure 12–15 µm in diameter and have 2–5 lobed nucleus. Cytoplasm contains small purple azurophilic and specific granules rich in hydrolytic enzymes and other potentially toxic oxygen-derived free radicals leading to elimination of pathogens.
- Neutrophils are recruited to the site of tissue injury within first 24 hours of acute inflammation and provide essential defense against acute bacterial infections. Following bacterial infections, neutrophils are attracted by chemotactic factors. Neutrophils eliminate injurious agent by phagocytosis of antibody and complement-coated bacteria and degradation occurs through activation of hydrolytic proteases and other potentially toxic oxygen-derived free radicals.
- Neutrophils are derived from hematopoietic stem cell (HSC). The development of neutrophils occurs in the bone marrow in 14 days. Subsequent differentiation of myeloid stem cell occurs through the stages of myeloblast, promyelocyte, myelocyte, metamyelocyte, band form neutrophil and mature neutrophil. Neutrophils are terminally differentiated, which neither divide nor alter their morphologic phenotype after their release from the bone marrow.
- Several myeloid factors are required for the transcription regulation of neutrophils including lymphoid enhancer-binding factor 1 (LEF1), growth factor independent 1 (GFI-1) and CCAAT/enhancer-binding proteins (C/EBPs).
- Principal among the hematopoietic growth factors/cytokines regulating granulopoiesis is granulocyte colony-stimulating factor (G-CSF) derived from monocyte-macrophage, T cells, fibroblasts, vascular

endothelium. G-CSF binds to its cognate G-CSFR participates in myeloid differentiation, proliferation of granulocyte precursors, and release of mature neutrophils from the bone marrow. Other hematopoietic cytokines such as **IL-6** and **IL-3** also contribute to granulopoiesis.

- Mature neutrophils exit the bone marrow through the pores of sinusoidal endothelium and enter in blood circulation, a process called transcellular migration. Neutrophils released from the bone marrow have a bloodstream half-life of 4–10 hours and tissue half-life of two days.
- Neutrophils undergo apoptosis through soluble signaling molecules such as tumor necrosis factor (TNF) and FAS (CD95) ligand.
- Recently, the SDF-1/CXCR4 has been implicated in clearance of neutrophils. Mature neutrophils have low levels of CXC chemokine receptors. As neutrophils undergo aging, these alter their phenotype and upregulate CXCR4.

EOSINOPHILS

Eosinophil is a specialized cell of the innate immune system linked with allergic state and parasitic infections. It constitutes 1–4% of leukocytes. This pro-inflammatory white blood cell measures 12–15 µm in diameter and has a nucleus with two lobes and large coarse, orange-colored granules in cytoplasm.

- Eosinophil arises from the hematopoietic stem cells (HSCs) in the bone marrow and take about 8 days to mature through various stages such as myeloblast, eosinophilic myelocyte, metamyelocyte, band form cell and mature eosinophil. The eosinophil migrates from the bone marrow into blood circulation and target tissues/organs. The migration and movement of eosinophils is promoted by **c**hemokines such as CCL11, CCL24 and CCL26 and chemokine receptors such as CCR3.
- Eosinophils are terminally differentiated, which neither divide nor alter their morphologic phenotype after their release from the bone marrow.
- Eosinophilic granulopoiesis appears to be regulated by T cells through the secretion of hematopoietic growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5 and IL-3. GM-CSF and IL-3 also increase the production of other myeloid cells. Interleukin-5 increases eosinophil production exclusively. These proteins and cytokines are involved in the maturation, survival and persistence of eosinophils. Eosinophils are recruited by chemokine (eotaxin) at injury site in IgE-mediated allergic reactions.
- Eosinophilic granules contain four major proteins: major basic protein (**MBP**), eosinophilic cationic protein (**ECP**), eosinophil peroxidase (**EPO**) and eosinophil-derived neurotoxin **(EDN)**. These proteins are involved in phagocytosis, killing invasive helminths, antigen presentation to other cells and platelet interaction. The action of eosinophil peroxidase (EPO) also leads to oxidative burst, a crucial step in phagocytosis.
- Eosinophils may modulate immediate hypersensitivity reactions by degrading or inactivating chemical mediators released by mast cells such as histamine, leukotrienes (which may induce vasoconstriction and bronchoconstriction), lysophospholipids and heparin.
- Eosinophilic granules contain crystalline material in cytoplasm, which become 'Charcot-Leyden crystals' demonstrated in the sputum of bronchial asthmatic patients.

BASOPHILS

Basophils are the least abundant circulating granulocytes, which account for less than 1% of circulating leukocytes. The basophil measures 14–16 µm in diameter and contains large cytoplasmic dark blue to black granules which obscure the cell nucleus in Romanowsky stained peripheral blood smears examined by light microscopy. However, when unstained nucleus of basophil is visible, and it usually has two lobes.

- The cytoplasmic granules inside the basophils contain histamine, heparin and tryptase. Histamine is a vasodilator near site of infection, which prevents blood coagulation at the site of infection.
- Basophils, like other blood cells, arise from hematopoietic stem cells in the bone marrow, develop, differentiate and mature under the influence of hematopoietic growth factors such as IL-3 through various stages including myeloblast, basophilic myelocyte, metamyelocyte, band form cell and mature basophil.
- Basophils are released from the bone marrow into the blood circulation as mature basophil. Mature basophils have an estimated life span of 60–70 hours.
- Basophils play a role in immediate hypersensitivity disorders as well as atopy, allergic contact dermatitis and possibly autoimmune disorders such as systemic lupus erythematosus. Basophils bind to and may trigger production of immunoglobulin E (IgE), an antibody that helps in protection against parasites.

MONOCYTES

Monocytes are the largest cells of the blood with average 15–18 µm in diameter and make up 2–8% of the leukocytes. The nucleus is relatively big and tends to be indented and reniform (kidney) shape with open

chromatin without nucleoli. The cytoplasm contains large number of fine granules, which often appear to be more numerous near the cell membrane of the monocyte.

- Primary stimulators of monocytopoiesis and granulopoiesis are granulocyte colony-stimulating factor (G-CSF), IL-1, IL-3, IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF). In general, hematopoietic growth factors and cytokines are produced by various inflammatory cells, with or without contribution from stromal cells. Monocytes are derived from monoblasts in the bone marrow, which have life span of 12 hours.
- Monocytes are actively motile and phagocytic capable of ingesting infectious agents, red blood cells and other large particles. Monocytes cannot replace the function of neutrophils in the removal and destruction of bacteria, which usually reach the site of inflamed tissue later than the granulocytes. Monocytes are found at the sites of chronic inflammation.

LYMPHOCYTES

Lymphopoiesis is a tightly regulated sequence of events that leads to expression of a functional antigen receptor on the surface of lymphocytes. B cell expresses cell surface immunoglobulin receptors (BCRs) and T cell receptors (TCRs) are expressed on T cells.

- Lymphopoiesis begins in the bone marrow with committed lymphoid stem cells. The initial step of differentiation of the committed lymphoid stem cells appears to be the separation of B progenitor cells from T progenitor cells, and natural killer T cells.
- Several hematopoietic regulatory cytokines including IL-1, IL-2, IL-4, IL-10 and interferon-γ (IFN-γ) influence the development of B progenitor cells. T cells are derived from the precursor lymphoid cells in the bone marrow under the influence of several hematopoietic cytokines including IL-1, IL-2 and IL-9 and migrate to the thymus gland for further maturation.
- Natural killer cells (NK cells), large granular lymphocytes, appear to share a common progenitor cell with T cells in the bone marrow. Natural killer cells production is under the influence of regulatory cytokine IL-5. NK cells demonstrate HLA non-restricted cytotoxicity and release a variety of hematopoietic regulatory cytokines, such as IL-1, IL-2, IL-4 and interferons.
- Some of lymphocytes enter bloodstream and most move through lymphatic system, which is the group of tissues and organs, like tonsils, lymph nodes, spleen, and mucosa-associated lymphoid tissue (MALT) organs that provide protection from infection.
- About 25% of the newly formed lymphocytes remain in the blood marrow and become B cells. The remaining 75% of lymphocytes travel to thymus and become T cells. It is well established that the earliest progenitor cells enter the thymus gland receives critical signals delivered through the NOTCH1 receptor, which leads to specification and commitment toward the T cell fate.
- There are different kinds of B cells and T cells, which include: (a) effector cells that are activated by antigens to fight an active infection, and (b) memory cells that recognize and remember past infections and go into action quickly on reinfection with an antigen. B cells and T cells work together to fight infection.

Pathology Pearls: Different Types of Lymphocytes and their Functions

B Cells

- B cells recognize antigens and become plasma cells that produce immunoglobulins (e.g. IgG, IgA, IgM, IgD and IgE) to fight infections.
- B cells are distinguished by the presence of cell surface immunoglobulin receptors. Plasma cells are terminally differentiated B cells that produce immunoglobulins.

T Cells

- T cells migrate from bone marrow and mature in the thymus gland. T cells differentiate into CD8+ cytotoxic T cells, CD4+ helper T cells and CD4+ regulatory T cells, natural killer T cells and memory T cells.
- Once T cells are released into the blood circulation, they have a long life. T cells can discern between healthy and abnormal cells. healthy cells have abundant peptide major histocompatibility complex on their surface.
- T cells participate in cell-mediated immunity. T cells possess cell surface receptors (TCR), that bind antigens prepared by antigen-presenting cells (APCs). Activated T cells recruit monocytes from the circulation with IFN-γ, a powerful activator of macrophages. Each of these cells performs different functions.
  - **CD4+ regulatory T cells:** CD4+ regulatory T cells suppress immune system to keep its immune response in check through the transcription factor FOXP3, so that it does not overreact, as does in autoimmune disorders. Mutations in FOXP3 gene can cause the autoimmune disorder (X-linked immunodysregulation polyendocrinopathy, enteropathy).
  - **CD4+ helper T cells:** CD4+ helper T cells express glycoproteins on their cell surface, which activate in the presence of peptide antigens on the surface of invading pathogens. These cells respond immediately to protect the immune system by recruiting other immune cells. These cells secrete different cytokine proteins according to the immune response.

- **CD8+ cytotoxic T cells:** CD8+ cytotoxic T cells express CD8 glycoproteins on their cell surface, which activate in the presence of antigens that bind to MHC class I molecules present on all nucleated cells. These cells patrol the immune system and become memory T cells upon battle with invading pathogens. These cells find and directly attack cells infected with an antigen, cancer stem cells and foreign cells like transplanted organs.
- **Memory T cells:** Memory T cells are antigen-specific T cells that remain long-term after an infection has been eliminated, which remember markers on the surface of bacteria, viruses or cancer cells that have seen before. Memory T cells are quickly converted into large numbers of effector T cells upon re-exposure to specific invading antigen, thus providing a rapid immune response to past infection.
- **Natural killer T cells:** Natural killer T cells bridge the adaptive immune system to the innate immune system that recognizes pathogens with the glycolipid antigen molecule CD1d protein, which is human antigen encoded by CD10 gene. Natural killer T cells kill a variety of infected and cancer cells by lysing their cell membrane in the absence of prior exposure or priming.

QUANTITATIVE VARIATIONS IN LEUKOCYTES

Causes of quantitative variations (increase or decrease in number) in normal leukocytes include: bone marrow dysfunction, premature destruction of leukocytes in the circulation, and alterations in leukocytes that originate in the circulation or lymphoid organs in response to invasion by infectious microorganisms.

- Leukocytosis is defined as increase in the white blood cell count to more than 11,000 per microliter of blood (11×10^9/L) in adults. It is most often a normal protective response of physiologic stressors involving specific subtype of leukocyte. Leukocytosis occurs in pathologic conditions like leukemias.
- Leukopenia is defined as decrease in the white blood cells to fewer that 4000 cells per microliter (4×10^9/L), which frequently makes persons most susceptible to infections.
  - Leukopenia can be caused by radiation, certain chemotherapeutic agents, immunodeficiency states and autoimmune disease (i.e. systemic lupus erythematosus).
  - Neutropenia is abnormally low number of neutrophils. Lymphopenia is abnormally low number of lymphocytes.

LEUKOCYTOSIS

Leukocytosis is defined as increase in the white blood cell count to more than 11,000 per microliter of blood (11×10^9/L) in adults. It is most often caused by normal response to the body to help fight an infection or some drugs such as corticosteroids.

- Neutrophil leukocytosis is an abnormally high number of neutrophils. Lymphocyte leukocytosis is an abnormally high number of lymphocytes.
- However, an increase in the number of leukocytes can also occur in leukemias with the release of immature or atypical leukocytes from the bone marrow into the blood circulation.

Neutrophilia

Neutrophilia refers to neutrophil count that exceeds the reference range for age on a complete and differential blood count. The absolute neutrophil count (ANC) is not measured directly, but calculated by multiplying the total number of WBC count by the percentage of neutrophils dividing by 100. The percentage of neutrophils consists of the mature segmented neutrophils and band form neutrophils. The normal range of absolute neutrophil count is 1.5×10^9/L to 8×10^9/L.

- During established bacterial infection, absolute neutrophil count and band form neutrophils remain elevated with equal numbers in the marginal and circulatory granulocyte pool. During the recovery phase, the flow of neutrophils from the bone marrow decreases, with a resultant in the number of neutrophils in the circulation. During pregnancy and parturition, neutrophil count is raised.
- Neutrophilia may result from a shift of neutrophils from the marginal pool to the circulating pool or because of increase in number of neutrophils.
- Some bacterial infections do not cause neutrophilia, rather induce neutropenia in the settings of typhoid fever, tuberculosis, and brucellosis.
- Pathologic disorders induced neutrophilia are given in Table 9.3. Polymorphonuclear leukocytosis in Giemsa-stained peripheral blood smear is shown in Fig. 9.1. Reactive neutrophilia in Giemsa-stained bone marrow aspirate smear is shown in Fig. 9.2.

Eosinophilia

Eosinophilia is defined as a peripheral blood eosinophil count exceeding 500 per microliter of blood (>0.5×10^9/L). Peripheral eosinophilia is characterized as mild: 500–1500 per microliter of blood (0.5 to 1.5×10^9/L, moderate: 1500–5000 per microliter of blood (1.5 to 5×10^9/L) and severe: more than 5000 per microliter of blood (>5 to 10×10^9/L).

- Peripheral blood eosinophil count may be increased above 30×10^9/L in allergic disorders. Persistent eosinophilia may cause organs damage because of tissue inflammation and reaction to the cytokines and chemokines released by eosinophils as well as immune cells that are recruited to the site tissue injury.

Table 9.3 Pathologic disorders induced neutrophilia

Acute Bacterial Infections
▪ Lobar pneumonia ▪ Bronchopneumonia ▪ Pyogenic meningitis ▪ Acute appendicitis ▪ Infected burns ▪ Diphtheria
Acute Stress State
▪ Acute myocardial infarction ▪ Posthemorrhagic state ▪ Post-surgery state
Myeloproliferative State
▪ Chronic myelogenous leukemia, BCR-ABL1 positive ▪ Polycythemia vera (PV)
Miscellaneous Disorders
▪ Leukemoid reactions ▪ G-CSF therapy induction ▪ Corticosteroid therapy ▪ Rheumatoid arthritis ▪ Gout

Although any organ may be involved including heart, lungs, spleen, skin and nervous system.

- Eosinophilia may be primary or secondary. Primary eosinophilia is a clonal proliferation of eosinophils associated with hematologic disorders such as leukemias and myeloproliferative neoplasms. Secondary eosinophilia is associated with nonclonal and non-hematologic disorders.
- Eosinophilia in Giemsa-stained peripheral blood smear is shown in Fig. 9.3. Causes of primary and secondary eosinophilia are given in Table 9.4.

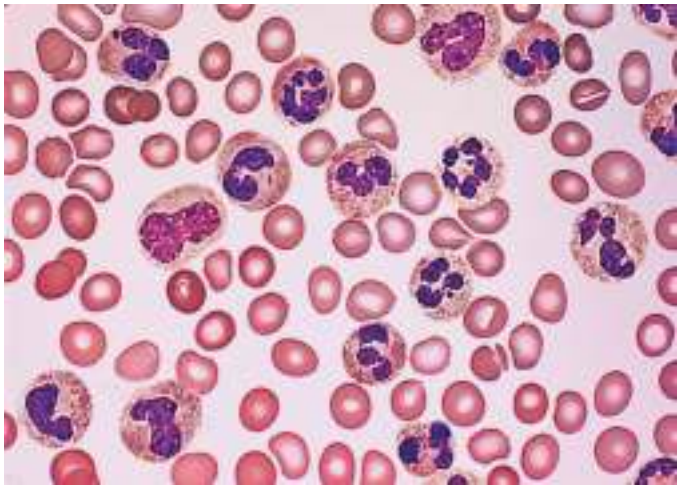


Fig. 9.1: Polymorphonuclear leukocytosis in Giemsa-stained peripheral blood smear. Polymorphonuclear leukocytosis is characterized by increased levels of polymorphonuclear leukocytes in the blood above 11,000 WBCs in a cubic millimeter of blood in the settings of infections, tissue damage, inflammatory diseases and diabetic ketoacidosis (1000X).

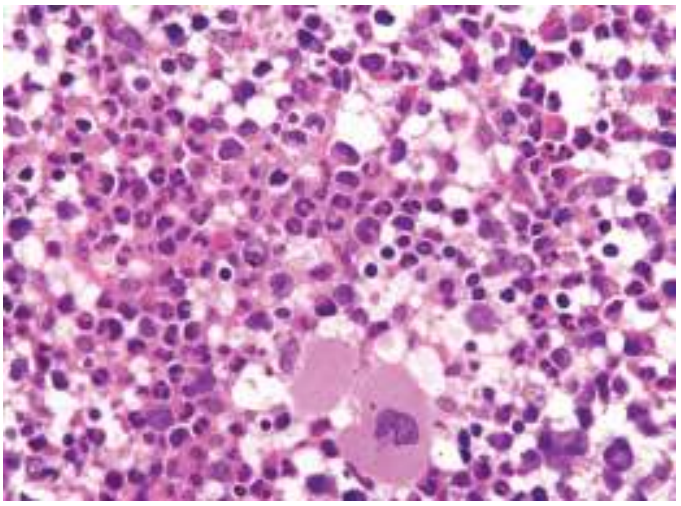


Fig. 9.2: Reactive neutrophilia in Giemsa-stained bone marrow aspirate smear. Bone marrow aspirate smear examination shows increased cellularity and replaced by numerous neutrophils (1000X).

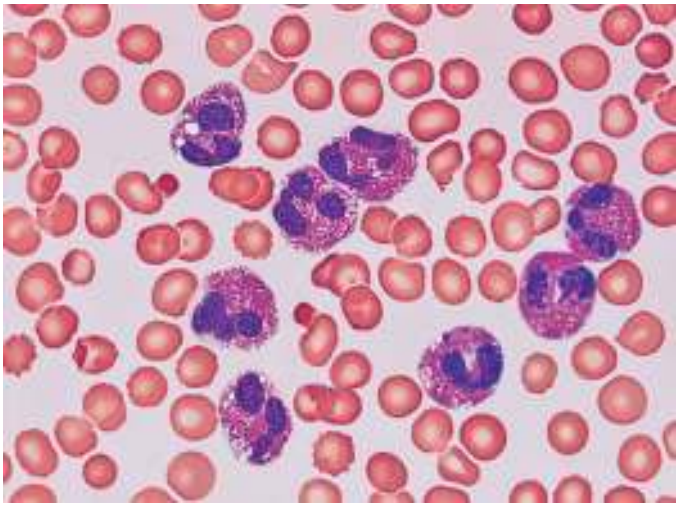


Fig. 9.3: Eosinophilia in Giemsa-stained peripheral blood smear. Eosinophilia is an increase in the number of eosinophils in the blood circulation in the setting of response to some allergens, drugs and parasites and in some types of leukemia. Eosinophils release granules of enzymes to fight some foreign substances and infections (1000X).

Clonal Hypereosinophilic Syndrome

- Hypereosinophilic syndrome is defined as peripheral blood eosinophilia more than 1500/cu mm ($\geq 1.5 \times 10^9$/L) persisting ≥6 months, which most often affects persons of 20–50 years of age.
- Only some patients with persistent eosinophilia develop organ dysfunction (heart, lungs, spleen, skin, bone marrow and nervous system).
- Cardiac involvement can cause significant morbidity and mortality.
- Two categories of clonal hypereosinophilic syndrome include myeloproliferative and lymphoproliferative variants are as follows.

Table 9.4 Causes of primary and secondary eosinophilia

Primary Clonal Eosinophilia Associated with Clonal Disorders		
Myeloid neoplasms	▪ Chronic myelogenous leukemia (BCR-ABL1 positive) ▪ Acute myelogenous leukemia with inv(16) (p13.1q22) or t(16;16) (p13.1; q22) ▪ Acute myelogenous leukemia with t(8;21) (p22; q22.1) ▪ Chronic myelomonocytic leukemia (CMML)	
Myeloid and lymphoid neoplasms associated with rearrangement and fusion genes	▪ Myeloid and lymphoid neoplasms associated with PDGFRA, PDGFRB, or FGFR1 rearrangements ▪ Myeloid and lymphoid neoplasms associated with PCM1-JAK2, ETV6-JAK2, or BCR-JAK2 fusion genes	
Lymphoid neoplasms	▪ T cell lymphoma ▪ Hodgkin disease (mixed cellularity variant) ▪ ALL (acute lymphoblastic leukemia) ▪ Lymphoproliferative variant of hypereosinophilic syndrome	
Other disorders	▪ Eosinophilic granuloma ▪ Mastocytosis	
Eosinophilia Associated with Nonclonal Reactive Disorders		
Allergic state	▪ Bronchial asthma ▪ Urticaria	▪ Hay fever ▪ Allergic rhinitis
Parasitic infections	▪ *Ancylostoma duodenale* ▪ *Necator americanus*	▪ *Ascaris lumbricoides* ▪ *Wuchereria bancrofti*
Skin disorders	▪ Eczema ▪ Pemphigus vulgaris ▪ Psoriasis	▪ Bullous pemphigoid ▪ Dermatitis herpetiformis
Collagen vascular diseases	▪ Churg-Strauss disease ▪ Wegener granulomatosis	
Pulmonary diseases	▪ Idiopathic acute or chronic eosinophilic pneumonia	▪ Tropical pulmonary eosinophilia ▪ Allergic bronchopulmonary aspergillosis
Miscellaneous disorders	▪ Gastroenteritis ▪ Adrenal insufficiency	▪ Immunologic disorders

Myeloproliferative variant of clonal hypereosinophilic syndrome

- The myeloproliferative variant of hypereosinophilic syndrome is most often associated with a small interstitial deletion of chromosome at the **CHIC2 site** that induces the FIP1L1/PDGFRA-associated fusion gene, which possess receptor tyrosine kinase activity that can transform hematopoietic stem cells. Other cytogenetic abnormalities include rearrangement of the gene for fibroblast growth factor 1 (FGFR1) or Janus kinase 2 (PCM1-JAK2).
- Patients present with splenomegaly, thrombocytopenia, anemia, elevated serum vitamin B_{12} levels, hypogranular or vacuolated eosinophils and myelofibrosis, who often develop endomyocardial fibrosis, but rarely develop acute myelogenous leukemia or acute lymphoblastic leukemia. Patients are treated by imatinib and other receptor tyrosine kinase inhibitors.

Lymphoproliferative variant of clonal hypereosinophilic syndrome

- The lymphoproliferative variant of hypereosinophilic syndrome is associated with a clonal population of T cells with aberrant phenotype.
- Clonal T cell receptor rearrangement is demonstrated by polymerase chain reaction (PCR) in lymphoproliferative variant of hypereosinophilic syndrome.
- Patient presents with angioedema, skin abnormalities or both, hypergammaglobulinemia (especially IgE), circulating immune complexes (sometimes with serum sickness) and rarely T cell lymphoma.

Basophilia

Basophilia is defined as an absolute increase in the number of basophils in blood. Reference values of basophilia vary from laboratory, but an absolute count

of basophils greater than 0.2×10^9/L in blood is considered a true basophilia.

- Basophilia is associated with chronic myelogenous leukemia BCR-ABL1 positive, basophilic leukemia, Hodgkin's disease, polycythemia vera, hypothyroidism, IgE-mediated allergic reactions, ulcerative colitis, mastocytosis and tuberculosis.
- Elevated basophil count (>20%) in the peripheral blood in a patient with chronic myelogenous leukemia BCR-ABL1 positive, often indicates a transition of chronic myelogenous leukemia BCR-ABL1 chronic phase to CML blast phase.
- Polycythemia vera is also associated with mild basophilia. A subset of acute myelogenous leukemias are associated with basophilia including acute myelogenous leukemia with t(6;9) (p23;q34); DEK-NUP214 and acute basophilic leukemia.
- Conditions associated with reactive basophilia include IgE-mediated allergic and type 1 hypersensitivity reactions, collagen vascular disorders, myxedema, irradiation, tuberculosis, and certain viral infections.

Lymphocytosis

Lymphocytosis is defined as an increase in absolute lymphocyte count (ALC) to $>4 \times 10^9$/L in adult patients, $>7 \times 10^9$/L in children and $>9 \times 10^9$/L in neonates. Lymphocytes constitute around 20–40% of white blood cells (WBCs).

- Absolute lymphocyte count calculates total white blood cell (WBC) multiplied by the percentage of lymphocytes in the peripheral blood. Different lymphocyte subsets (B cells, T cells or natural killer cells) may be elevated depending on the etiology.
- The definition of relative lymphocytosis refers to an increase in WBC count >40% in the presence of a normal absolute white blood cell count. Distinguishing reactive lymphocytosis from malignant lymphocytosis can be challenging and may vary depending on the age and other demographics.
- Reactive lymphocytosis may be caused by viruses (EBV, HIV, influenza, hepatitis, mumps, rubella, HTLV-1, measles, chickenpox, adenovirus), bacteria (*Bartonella henselae*, *Bordetella pertussis*, *Brucellosis*, *Treponema pallidum*, Mycobacterium bacilli), and parasites (mlarial parasite, *Toxoplasma gondii*).
- Lymphocytosis occurs in lymphoproliferative disorders (chronic lymphocytic leukemia, NHL-mantle cell lymphoma, marginal zone lymphoma, hairy cell leukemia, follicular lymphoma, Sézary syndrome, large granular lymphocytic leukemia).
- Congenital B cell lymphocytosis occurs due to germline heterozygous missense mutation in CARD11 gene that encodes a scaffolding protein required for nuclear factor kappa B (NF-κB) in both B and T lymphocytes. This entity typically progresses to chronic lymphocytic leukemia (CLL) by the fourth decade of life.
- Persistent B cell polyclonal lymphocytosis is characterized by binucleated lymphocytes predominantly in young tobacco smoker women, and associated with IgM polyclonal gammopathy and HLA-DR7, which exhibits a stable clinical and biological course. Lymphocytosis may also occur in post-splenectomy patients, that persists stable over years. Lymphocytosis in peripheral blood smear is shown in Fig. 9.4. Causes of lymphocytosis are given in Table 9.5.

Monocytosis

Monocytosis is defined by an absolute circulating monocyte count exceeding upper limit of the reference range of $\geq 1.0 \times 10^9$/L (1000/cu mm) in adults and $\geq 3.5 \times 10^9$/L (3500/cu mm) in neonates.

- Monocytosis usually occurs in the setting of chronic inflammation resulting from infections like tuberculosis, syphilis, brucellosis or subacute bacterial endocarditis, autoimmune disorders, granulomatous diseases, and sarcoidosis. Monocytosis is often the first sign of recovery after myelosuppression.
- Monocytosis is severe in chronic myelomonocytic leukemia, juvenile myelomonocytic leukemia. Monocytosis may be mild to moderate in acute monocytic leukemia.
- Monocytosis, especially monocyte : lymphocyte ratio greater than 0.8–1.0, may indicate active progression of tuberculosis and poor prognosis. Normal monocyte : lymphocyte ratio of ≤0.3 is restored when tissue healing process is complete. Causes of monocytosis are given in Table 9.6.

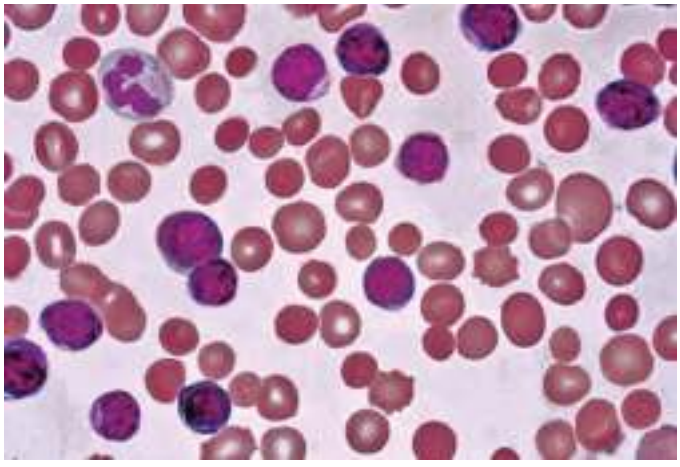


Fig. 9.4: Lymphocytosis in peripheral blood smear. Lymphocytosis is an increase in number of lymphocytes in blood higher than 5000 lymphocytes in a microliter of blood circulation (1000X).

Table 9.5 Causes of lymphocytosis

Bacterial Infections	
▪ Mycobacterium bacilli	▪ *Bartonella henselae*
▪ *Treponema pallidum*	▪ *Bordetella pertussis*
▪ Brucellosis	
Viral Infections	
▪ Infectious mononucleosis (Epstein-Barr virus)	▪ Adenovirus
▪ Hepatitis B virus (HBV)	▪ Mumps virus
▪ Hepatitis C virus (HCV)	▪ Measles virus
▪ Rubella virus	▪ Chickenpox virus
	▪ Cytomegalovirus
Parasitic Infestations	
▪ Malarial parasite	▪ *Toxoplasma gondii*
Hematological Malignancies	
▪ Chronic lymphocytic leukemia (CLL)	▪ NHL spillover
▪ Adult T cell leukemia/ lymphoma	▪ Hairy cell leukemia (HCL)
▪ Prolymphocytic leukemia (PML)	▪ Sezary syndrome
	▪ Large granular lymphocytic leukemia

Table 9.6 Causes of monocytosis

Bacterial Infections	
▪ Tuberculosis	▪ Bacterial endocarditis
▪ Syphilis	▪ Brucellosis
Parasitic Infestations	
▪ Kala-azar	▪ Trypanosomiasis
Hematologic Malignancies	
▪ Acute myelogenous leukemia (AML-M4 and AML-M5)	
▪ Hodgkin's disease	
▪ Chronic myelogenous leukemia, BCR-ABL1 positive	
▪ Chronic myelomonocytic leukemia (CMML)	
▪ Myelodysplastic syndrome (MDS)	
Autoimmune Disorders	
▪ Ulcerative colitis	▪ Systemic lupus erythematosus (SLE)
▪ Crohn's disease	
▪ Sarcoidosis	▪ Rheumatoid arthritis (RA)

LEUKOPENIA

Leukopenia refers to the decrease in the number of white blood cells in blood due to exposure to certain chemical agents, certain drugs (antiepileptic sodium valproate, immunosuppressive cyclosporin, antithyroid thiouracil, and interferon), chemotherapy and radiation for cancer treatment leading to bone marrow failure or suppression.

- Leukopenia can be caused by certain hematologic disorders such as myelodysplastic syndrome, myeloproliferative syndrome, myelofibrosis, aplastic anemia, and megaloblastic anemia.
- Cancers metastasizing to bone marrow result in leukopenia. Leukopenia is demonstrated in systemic lupus erythematosus (SLE). Hypersplenism is caused by splenomegaly, that destroys the red blood cells and white blood cells leading to anemia and leukopenia. Leukopenia leads to decreased immunity.

Pancytopenia

Pancytopenia is defined as deficiency of all three cellular components of the red blood cells, white blood cells and platelets leading to simultaneous presence of anemia, leukopenia and thrombocytopenia. Therefore, pancytopenia exists when hemoglobin is less than 13.5 g/dl in males or 11.5 g/dl in females, the leukocyte count is less than 4000 per microliter and platelet count is less than 1,500,000 per microliter.

- Most common cause of pancytopenia is megaloblastic anemia followed by acute myelogenous leukemia and aplastic anemia. Pancytopenia is induced by infections, some drugs, chemotherapeutic agents, radiation therapy, and exposure to radiation, arsenic and benzene.
- Patients present with fever, pallor, fatigue, dizziness, weight, anorexia, night sweats, bleeding tendencies and organomegaly.
- Bone marrow examination is a single useful investigation, which reveals the underlying etiology in patients with pancytopenia. Severity of pancytopenia and underlying pathology determine the management and prognosis. Thus, pancytopenia of the correct cause will help in implementing appropriate therapy. Pancytopenia in Giemsa-stained peripheral blood smear is shown in Fig. 9.5.

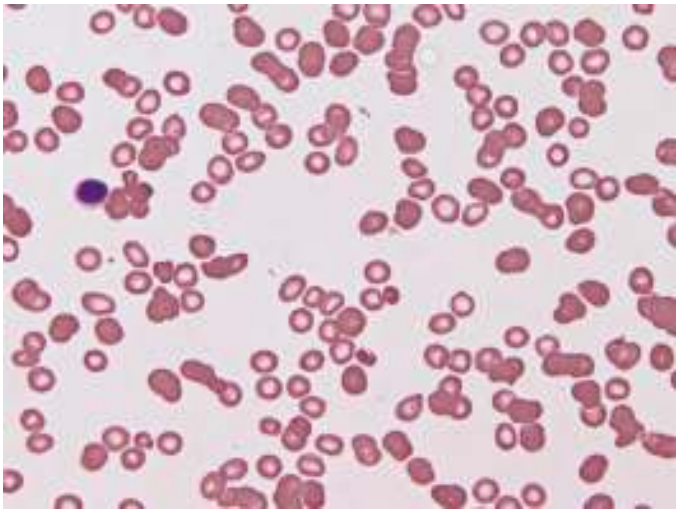


Fig. 9.5: Pancytopenia in Giemsa-stained peripheral blood smear. Pancytopenia is defined as deficiency of all three cellular components of the red blood cells, white blood cells and platelets (1000X).

Neutropenia (Agranulocytosis)

Neutropenia is defined as decrease in the neutrophil count $<1.5 \times 10^9$/L in adults and $<2.5 \times 10^9$/L in children. Neutropenia occurs due to suppression of myelopoiesis, destruction of neutrophils in the peripheral blood or pooling of neutrophil in the peripheral regions. Patient with neutropenia presents with sore throat, recurrent infections and delayed wound healing.

- Most bacterial infections cause neutrophilia with increased band forms exceeding reference range.
  - Some bacterial infections do not cause neutrophilia but result in neutropenia. Typhoid fever causes leukopenia or neutropenia or both.
  - Other causes of neutropenia include *Mycobacterium tuberculosis*, leishmaniasis, Rickettsia, brucellosis, tularemia, ehrlichiosis and *Staphylococcus aureus*.
  - Infants and preterm infants have small storage pools of neutrophils in the bone marrow. Therefore, neutropenia develops in severe or chronic infections in these infants because the demand of neutrophils is greater than the supply.
- After chemotherapy, radiation, bone marrow transplant, the absolute neutrophil count is usually depressed and then slowly rises, reflecting the fact the bone marrow is recovering and new blood cells are beginning to produce and undergoing maturation. Causes of neutropenia are given in Table 9.7.

Table 9.7 Causes of neutropenia

Bacterial Infections	
▪ Typhoid fever	▪ Endotoxic shock (gram-negative bacteria)
▪ Tuberculosis	
Viral Infections	
▪ Human immune deficiency virus	▪ Epstein-Barr virus (EBV)
▪ Parvovirus B19	▪ Hepatitis B virus (HBV)
Drug-induced Neutropenia	
▪ Anti-inflammatory drugs (phenylbutazone, ibuprofen)	
▪ Antithyroid drugs (thiouracil, carbimazole)	
▪ Antibacterial drugs (penicillin, gentamicin, doxycycline, ciprofloxacin, cephalosporins, cotrimoxazole)	
▪ Antiepileptic drugs (phenytoin, trimethadione, valproic acid)	
Autoimmune Disorders	
▪ Systemic lupus erythematosus (SLE)	▪ Blood transfusion reactions
	▪ Felty syndrome
Congenital Disorders	
▪ Severe congenital neutropenia	▪ Cyclic neutropenia
▪ Chronic benign neutropenia	
Hematological Disorders	
▪ Hypersplenism	▪ Myelodysplastic syndrome
▪ Splenomegaly	▪ Aplastic anemia
▪ Megaloblastic anemia	▪ X-ray irradiation induced neutropenia
▪ Chronic autoimmune neutropenia	

Lymphocytopenia

Lymphocytopenia is defined as decrease in the lymphocyte count less than 1,000/cumm ($<1 \times 10^9$/L) in adults and less than 2000/cumm ($<2 \times 10^9$/L) in the children in peripheral blood. It is observed in patients following therapeutic administration of corticosteroid and cytotoxic drugs; radiation and viral infections. Causes of lymphocytopenia in pathological state are given in Table 9.8.

Table 9.8 Causes of lymphocytopenia in pathological state

Bacterial Infections	
▪ Miliary tuberculosis	
Inherited Disorder	
▪ Ataxia-telangiectasia	
Inherit Drug-induced Lymphocyte Destruction ed Disorder	
▪ Cytotoxic drugs	▪ Antithymocyte globin administration
▪ Corticosteroid therapy	
Autoimmune Disorders	
▪ Systemic lupus erythematosus (SLE)	▪ Sarcoidosis
▪ Rheumatoid arthritis	▪ Myasthenia gravis
Immunodeficiency Disorders	
▪ Acquired immune deficiency syndrome	▪ Severe combined immune deficiency
Miscellaneous Disorders	
▪ Radiation therapy	▪ Malnutrition

Monocytopenia

Monocytopenia may be observed in patients receiving corticosteroid therapy or on hemodialysis, Epstein-Barr viral infection, aplastic anemia, and sepsis with splenomegaly. Profound monocytopenia is associated with hairy cell leukemia (HCL) and considered to be a diagnostic hallmark of disease.

QUALITATIVE VARIATIONS IN LEUKOCYTES

Qualitative inherited disorders of leukocytes include Alder-Reilly anomaly, Chédiak-Higashi anomaly, May-Hegglin anomaly (MHA), Pelger-Hüet anomaly, chronic granulomatous disease (CGD), myeloperoxidase deficiency and leukocyte adhesion deficiency (LAD).

Qualitative acquired defects of leukocytes include toxic granules in neutrophils, hypersegmented neutrophils, hyposegmented neutrophils, Auer rods, myeloid shift to left, smudge cells, variant lymphocytes with increased size in peripheral blood, lupus erythematosus (LE) cell phenomenon, Tart cells and leukemic cells (e.g. myeloblast, lymphoblast, monoblast, promyelocyte). Leukocyte inherited and acquired abnormalities and associated disorders are given in Table 9.9.

Table 9.9 Leukocyte inherited and acquired abnormalities and associated disorders

Disorder	Morphologic and Functional Defects	Clinical Features and Associated Disorder
Inherited leukocyte abnormality		
Alder-Reilly anomaly	▪ Defect involves protein–carbohydrate complexes within lysosomes ▪ Large, purplish cytoplasmic granules present in all mature leukocytes ▪ Leukocytes function normally	Associated with hereditary mucopolysaccharidosis such as Hurler syndrome
Chédiak-Higashi anomaly	▪ Autosomal recessive disorder due to LYST gene mutation ▪ Giant fused granules in neutrophils and lymphocytes ▪ Neutrophils phagocytose but do not kill microorganisms	▪ Serious, often fatal disorder with recurrent pyogenic infections ▪ Partial albinism ▪ Patchy gray hair ▪ Chédiak-Higashi anomaly accelerated phase associated with poor outcome due to multiorgan failure (85%)
May-Hegglin anomaly (MHA)	▪ Blue, Dohle-like cytoplasmic inclusions (RNA) in all leukocytes with giant platelets ▪ Leukocytes function normally	Bleeding tendency from associated thrombocytopenia and giant platelets
Pelger-Hüet anomaly	▪ Inherited disorder occurs due to lamin B receptor (LBR) gene mutation ▪ Hyposegmented neutrophils with hyposegmented bilobed nucleus, but normal granulation of neutrophils and eosinophils; coarse chromatin and pink cytoplasm; however, the neutrophil functions are normal ▪ Lamin B receptor (LBR) gene mutation, inherited disorder with hyposegmented but normal granulation of neutrophils and eosinophils	Hereditary myelogenous leukemia
Chronic granulomatous disease (CGD)	Defective respiratory burst	Recurrent infections, especially in childhood
Myeloperoxidase deficiency	Low or absent myeloperoxidase (MPO) enzyme in neutrophils with normal cell morphology	Usually benign disorder; recurrent infections occur due to decreased or absent bactericidal activity of neutrophils
Leukocyte adhesion deficiency (LAD)	Absence of cell-surface adhesion proteins affecting multiple cell functions but normal cell morphology	Serious disorder with recurrent infections and high mortality
Acquired leukocyte anomalies		
Toxic granules in neutrophils	Neutrophils contain black or purple granules in cytoplasm	Increased susceptibility to bacterial infections occurs due to inflammatory disease, burns and patient on chemotherapy
Hypersegmented neutrophils	Neutrophils contain >5 distinct lobes	Megaloblastic anemia due to vitamin B_{12} and folate deficiency; and hereditary constitutional hypersegmented of neutrophils
Hyposegmented neutrophils	Neutrophils with bilobed nuclei due to lamin B receptor (LBR) gene mutation	Pelger-Hüet anomaly, and pseudo-Pelger-Hüet anomaly in AML and AIDS

Contd...

Table 9.9 Leukocyte inherited and acquired abnormalities and associated disorders (*Contd...*)

Disorder	Morphologic and Functional Defects	Clinical Features and Associated Disorder
Auer rods	Rod-like long, red purple, refractile inclusions in neutrophils resulting from an abnormal fusion of azurophilic granules	Acute myelogenous leukemia (AML) or acute myelomonocytic leukemia (AMML)
Myeloid shift to left	Presence of band form neutrophils, myelocytes, metamyelocytes or promyelocytes in peripheral blood	Infections, tissue necrosis, chronic myelogenous leukemia, myeloproliferative syndrome and hyposplenism
Smudge cells	Disintegrated nucleus of a ruptured lymphocytes	Chronic lymphocytic leukemia
Variant lymphocytes with increased size in peripheral blood	Epstein-Barr viral infection causes cervical lymphadenopathy	Infectious mononucleosis and other viruses
Lupus erythematosus (LE) cell phenomenon	Neutrophil with a homogenous red-purple inclusion that distends the cytoplasm of cell	Systemic lupus erythematosus, other collagen disorders, chronic hepatitis, drug reactions and mechanical trauma *in vitro*
Tart cells	Neutrophil with phagocytosed nucleus of a granulocyte that retains some nuclear structure	Drug reactions (e.g. penicillin, procainamide) or actual phagocytosis
Leukemic cells (e.g. myeloblast, lymphoblast, monoblast, promyelocyte)	Presence of myeloblasts or lymphoblast or monoblasts and promyelocytes in peripheral blood	Acute leukemia, leukemoid reactions, severe infections, inflammatory disorders, malignancies, and recovery from bone marrow suppression

ALDER-REILLY ANOMALY

Alder-Reilly anomaly is an autosomal recessive disorder characterized by presence of large azurophilic granules in the cytoplasm of neutrophils, lymphocytes, and monocytes.

- Granulocytes show metachromatic and darkly staining inclusions (Alder-Reilly bodies) containing partially digested mucopolysaccharides because of deficient lysosomal enzymes.
- Alder-Reilly bodies, that resemble toxic granules can be demonstrated in the bone marrow and peripheral blood smear stained with Giemsa stain and toluidine stain.
- Leukocyte function is not impaired in Alder-Reilly anomaly. Alder-Reilly anomaly is associated with syndromes such as Hunter syndrome, Hurler syndrome, Tay-Sachs disease and Maroteaux-Lamy polydystrophic dwarfism. These clinical entities result in different clinical manifestations.
- Physical findings associated with Alder-Reilly anomaly include gargoylism (deformities of head, trunk, and limbs; mental retardation and hepatosplenomegaly) and dwarfism.

CHÉDIAK-HIGASHI SYNDROME

Chédiak-Higashi syndrome is an autosomal recessive disorder due to mutation in LYST gene (lysosomal trafficking regulator gene).

- LYST gene located on chromosome 1q42 encodes protein essential for assembly of microtubules in the cytoplasm. LYST gene mutation leads to defect in assembly of microtubules in neutrophils.
- Chédiak-Higashi syndrome is characterized by defective degranulation of neutrophils, impaired microbial killing, and recurrent bacterial infections due to *Staphylococcus aureus* forming soft tissue abscess. Neutrophils contain giant granules due to aberrant organelles. In Chédiak-Higashi syndrome, there are severe immunologic defects including recurrent bacterial infections, impaired chemotaxis, and abnormal natural killer function with fatal outcome.
- Basic defect in Chédiak-Higashi syndrome is in the formation of giant phagosomes within all white blood cells and defective granulation. Neutrophils phagocytose but do not kill microorganisms. Patient presents with partial albinism, patchy gray hair, increased susceptibility to pyogenic infections due to defective degranulation of neutrophils. Accelerated phase of Chédiak-Higashi syndrome is associated with fatal outcome due to multiorgan failure in 85% of cases. Neutrophils containing giant granules due to aberrant organelles in Chédiak-Higashi syndrome are shown in Fig. 9.6.

MAY-HEGGLIN ANOMALY

May-Hegglin anomaly (MHA) is an autosomal dominant disorder due to mutation in MYH9 gene. Disorder is characterized by basophilic cytoplasmic inclusions resembling Döhle body (RNA) within neutrophils, anemia, bleeding manifestations, thrombocytopenia, and giant platelets.

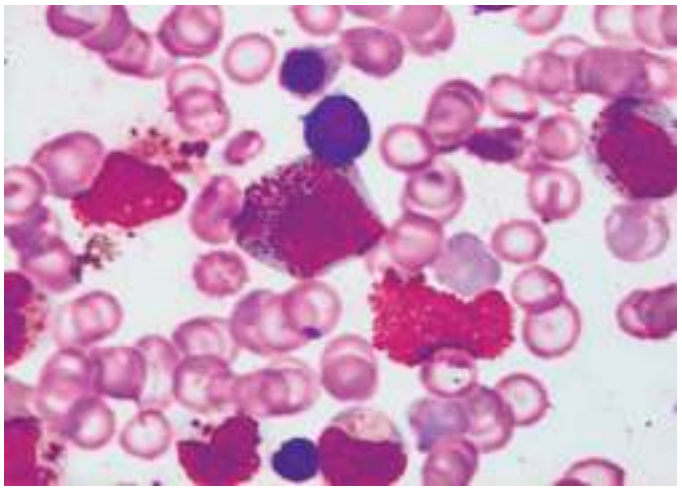


Fig. 9.6: Chédiak-Higashi syndrome. Neutrophils contain giant granules due to aberrant organelles in peripheral blood in Chédiak-Higashi syndrome. Chédiak-Higashi syndrome is characterized by oculocutaneous albinism, which causes abnormally light coloring (pigmentation) of the skin and eyes, vision problems such as reduced sharpness, rapid involuntary eye movements (nystagmus) and increased sensitivity to light (photophobia). Affected persons have fair skin and light-colored hair often with a metallic sheen (polished metal) (1000X).

PELGER-HÜET ANOMALY

Pelger-Hüet anomaly is an autosomal dominant disorder due to lamin B receptor (LBR) gene mutation. Disorder is characterized by hyposegmented nuclei with coarse chromatin and normal granulation in >70% of neutrophils and eosinophils. Neutrophils have normal functions. Two lobes of the nuclei of neutrophils are joined by thin chromatin bridge. Pelger-Hüet anomaly is associated with hereditary myelogenous leukemia.

CHRONIC GRANULOMATOUS DISEASE OF THE CHILDHOOD

Chronic granulomatous disease of the childhood is X-linked disorder characterized by defective respiratory burst due to defect in NADPH oxidase activity in neutrophils.

- Phagocytic cells (neutrophils) phagocytose microorganisms but fail to kill them resulting in recurrent infections, especially in childhood.
- Nitroblue tetrazolium (NBT) slide test is used for screening defects in NADPH oxidase. Positive NBT slide test means neutrophils turn blue, while negative NBT slide test means neutrophils color remains yellow.
  - The number of neutrophils with dark cytoplasmic granules of reaction product is counted.
  - Normally >95% of the neutrophils containing NADPH oxidase are positive for nitroblue tetrazolium. In chronic granulomatous disease, nitroblue tetrazolium test is negative due to the lack of NADPH oxidase activity in neutrophils.

MYELOPEROXIDASE DEFICIENCY

Myeloperoxidase deficiency is a benign disorder due to low or absent myeloperoxidase (MPO) enzyme in neutrophils. Cell morphology of neutrophils is normal.

- Myeloperoxidase deficiency is associated with recurrent infections due to decreased or absent bactericidal activity of neutrophils.
- Persons with myeloperoxidase deficiency have a respiratory burst with a normal nitroblue tetrazolium (NBT) test because they still have NADPH oxidase activity, but do not form HClO (bleach) due to lack of myeloperoxidase activity in neutrophils.

LEUKOCYTE ADHESION DEFICIENCY DISORDERS

Leukocyte adhesion deficiency (LAD) is a primary immunodeficiency autosomal recessive disorder that involves phagocytic cell defects due to absence of cell-surface adhesion proteins resulting in dysfunctional granulocytes and both T cells and B cells. The clinical picture of LAD deficiency is characterized by marked leukocytosis and bacterial recurrent infections in soft tissue and high mortality. Cell morphology of the leukocytes is normal.

Leukocyte Adhesion Deficiency Type 1 Disorder

Leukocyte adhesion deficiency type 1 (LAD-1) disorder is characterized by delay in detachment of the umbilical cord stump after birth. In normal infants, the umbilical cord stump falls off within the first two weeks of life, but in infants with LAD 1 disorder, separation of umbilical cord stump usually occurs at three weeks or later. Delayed separation of umbilical cord stump may also be observed in patients with IL-1 receptor-associated kinase 4 (IRAK4) deficiency or neutropenia.

- Bacterial and fungal infections most commonly occur in skin and mucous membranes lining nose and mouth.
- Life expectancy in persons with leukocytes adhesion deficiency type 1 is most often severely shortened. Due to recurrent infections, affected individuals may not survive past infancy.

Leukocyte Adhesion Deficiency Type 2 Disorder

Leukocyte adhesion deficiency type 2 (LAD-2) is a rare inherited disorder characterized by recurrent infections, persistent leukocytosis, and severe mental retardation.

- In LAD-2, neutrophils are deficient in expression of selectin ligand activity.
- In LAD-2, patients exhibit a deficiency in the expression of cell surface fucosylated glycan structures that

include the Hh and Lewis blood group determinants and the Sialyl Lewis X epitope.
- The molecular defect in LAD-2 has been localized to the pathway that synthesizes GDP-fucose from GDP-mannose.

INFECTIOUS MONONUCLEOSIS

Epstein-Barr virus (EBV), also known as human herpesvirus 4, is a member of the herpes family. It is one of the most common human viruses. EB virus spreads most commonly through body fluids, primarily saliva. EB virus can cause infectious mononucleosis (also called kissing disease and glandular fever) and other illnesses. Onset of infectious mononucleosis disease is abrupt, which most often occurs between 5 and 30 years of age. Salient features of infectious mononucleosis are given in Table 9.10.

MODE OF TRANSMISSION

Epstein-Barr virus (EB virus) spreads most commonly through body fluids, especially through oropharyngeal secretions (saliva). However, EB virus can also be transmitted through blood and semen during sexual contact, blood transfusions, and organ transplantations. EB virus can spread by using toothbrush or drinking glass that an infected person has recently used. Incubation period of EB virus infection is 1–2 months.

CLINICAL FEATURES

Infectious mononucleosis has been recognized as a clinical syndrome in teenagers/young adults consisting of fever, pharyngitis (sore throat), cervical lymphadenopathy in the first week, skin rash and splenomegaly. On clinical examination, enlarged lymph nodes are discrete and slightly tender. The enlarged lymph nodes gradually subside in the second week. Peripheral blood smear examination shows transformed atypical lymphocytes (CD8+ cytotoxic T cells).

PATHOPHYSIOLOGY

Epstein-Barr virus is transmitted via intimate contact with primarily oropharyngeal secretions. EB virus may also be shed from the uterine cervix, implicating the role of genital transmission in some cases. EB virus can be contracted via blood transfusion in occasional case. EB virus infects B cells in the oropharyngeal lining epithelium.
- Circulating B cells spread the infection throughout the reticuloendothelial cells in peripheral lymph nodes, spleen and liver.
- Epstein-Barr virus (EBV) infection of the B cells induces humoral and cellular response. The humoral response directed against EB virus structural proteins is the basis for technique used to diagnose infectious mononucleosis.
- However, T cell cellular response is essential in the control of EBV infection by CD8+ cytotoxic T cells and natural killer cells. CD8+ cytotoxic T cells control proliferating B cells infected by EB virus. T cell cellular response is critical in determining the clinical expression of EB virus infection.
- T cell cellular response determines clinical expression of EB virus infection. Efficient and quick T cell cellular response control primary EB virus infection and lifelong suppression of EB virus infection.
- Ineffective T cell response can lead to excessive and uncontrolled B cell proliferation resulting in B cell lymphoma.
- Cytokine release consequent to B cell immune response results in fever in EB virus infection.
- Proliferation of EB virus infected B cells leads to lymphocytosis.
  - Pharyngitis observed in EB virus-induced infectious mononucleosis occurs due to proliferation of EB virus-infected B cells in the oropharyngeal tissue.
  - EB virus transforms resting human B cells into indefinitely proliferating lymphoblastoid cell lines.

Table 9.10 Salient features of infectious mononucleosis

Parameters	Comments
Etiology	Epstein-Barr virus
Clinical features	Fever (38.3–38.9°C), tonsillitis/ severe pharyngitis (exudative or nonexudative), cervical lymphadenopathy (anterior, posterior, axillary, epitrochlear, mediastinal and mesenteric regions), fatigue, generalized nonpruritic skin rashes, petechiae on hard and soft palate (30%), bilateral palpebral edema, splenomegaly (50%), hepatomegaly (10%)
Diagnostic findings	Positive heterophile antibody test (monospot test) but false negative in 25% of cases during first week of illness, atypical lymphocytosis, transient hepatitis, increased alanine transaminase (ALT) and aspartate transaminase (AST)
Complications	Autoimmune hemolytic anemia and thrombocytopenia due to cross-reactivity of EB virus induced antibodies against red blood cells and platelets. Splenic rupture can occur due to trauma within three weeks of symptoms appear
Management	Avoid contact sports for ≥3 weeks due to risk of splenic rupture

COMPLICATIONS

In some cases, Epstein-Barr virus infection can lead to mild to serious complications such as immune hemolytic anemia, immune-mediated thrombocytopenia, hemophagocytic syndrome, secondary bacterial sore throat, hepatitis, myocarditis, conditions affecting central nervous system (meningoencephalitis, Guillain-Barré syndrome), and splenic rupture in rare cases. EB virus associated disorders are Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, body cavity lymphoma (HIV/AIDS patients) and X-linked lymphoproliferative disease (Duncan's syndrome).

Pathology Pearls: Epstein-Barr Virus-induced Disorders

- Immune hemolytic anemia
- Immune-mediated thrombocytopenia
- Hemophagocytic syndrome
- Secondary bacterial sore throat and hepatitis
- Viral myocarditis
- Meningoencephalitis
- Guillain-Barré syndrome
- Burkitt's lymphoma
- Nasopharyngeal carcinoma
- Hodgkin's disease
- Body cavity lymphoma (HIV/AIDS patients)
- X-linked lymphoproliferative disease (Duncan's syndrome)

LABORATORY DIAGNOSIS

Diagnosing Epstein-Barr virus (EB virus)-induced infectious mononucleosis is challenging because clinical manifestations are like other illnesses.

- Lymph nodes should not be biopsied as diagnosis is established by clinical features and detection of antibodies by serologic tests.
- About 90% of adults have antibodies that demonstrate that they have a current or past EB virus infection.
- Potential EB virus infections are often diagnosed without any blood testing. However, blood tests can detect the presence of antibodies associated with EB virus infection.

Paul and Bunnell Test

Paul and Bunnell originally described that heterophilic antibodies are present in 90–95% of adults in recent or past EB virus infections in the second to third weeks of infection.

- However, heterophilic antibody responses are not most often detected in infants and children under the age of four years.
- The heterophilic antibodies agglutinate horse red blood cells. Heterophilic antibodies are IgM antibodies, which agglutinate erythrocytes from sheep, goat, horse and camel.
- Epstein-Barr virus-induced heterophilic antibodies have no reactivity against guinea pig kidney cells in contrast to natural occurring antibodies (Forssman antibodies) or antibodies present in patients with serum sickness and other conditions. Heterophilic antibodies may be present in serum in response to microbial infections in patients.

Monospot Test

Several laboratory tests have been developed to aid in the diagnosis of infectious mononucleosis. Currently, serum testing for EB virus infection-specific antibodies is considered the gold standard for diagnosis, but rapid results are most often obtainable. Therefore, monospot test has been developed for rapid diagnosis.

- Principle of the monospot test is erythrocytes agglutination, much like Paul-Bunnell test which was developed prior to the monospot test.
- Monospot test is a latex agglutination test which utilizes equine erythrocytes as the primary substrate used to detect specific heterophilic antibodies produced by the human immune system in response to EB virus infection. When specific heterophilic antibodies are present in the patient's blood sample, exposure to equine erythrocytes will result in clumping of erythrocytes thus indicating a positive agglutination reaction, which is considered a positive test and therefore a diagnostic confirmation of the clinically suspected infectious mononucleosis especially in adult patients.
- The monospot test is **not indicated** for patients under the age of four years old children as the false-negative test results are unacceptably high.

Epstein-Barr Virus Specific Antibodies Assay

In addition to the monospot test, there are other blood tests to demonstrate more specific antibodies to EB virus infection: (a) viral capsid antigen (VCA), (b) early membrane antigen and (c) EB virus nuclear antigen.

- Antibodies to viral capsid antigen (VCA) appear early in the acute EB virus infection. One type of antibodies (anti-IgM) disappears after several weeks while another (anti-IgG) persists throughout life.
- Antibodies to early membrane antigen appear during an active EB virus infection. These antibodies are not detectable after several months, although these antibodies may persist for longer in some cases.
- Antibodies to EB virus nuclear antigen appear slowly in months following EB virus infection and can be detected throughout person's life.

Solid-phase Enzyme-linked Immunosorbent Assay

Solid-phase enzyme-linked immunosorbent assay is a reliable method to diagnose infectious mononucleosis by demonstration of IgM and IgG by complement fixation test and the indirect immunofluorescence test.

Molecular Techniques

Molecular techniques have become an important tool in Epstein-Barr virus in diagnostics.

- In the recent years, novel real-time polymerase chain reaction (RT-PCR) and *in situ* hybridization techniques have been developed to analyze EB virus DNA in specimens such as tissues, body fluids and peripheral blood, that offer increased time efficiency, reduced cross-contamination, high reproducibility, high sensitivity and allow determination of EB virus viral loads.
- Currently, monitoring of Epstein-Barr virus viral loads in different tissue compartments is used to assess the treatment response or prognosis in patients with oncological disorders or immunosuppression.

Laboratory Diagnosis of Infectious Mononucleosis

Routine Hematologic Findings

Total leukocyte count (TLC) is increased >15–30 × 10^9/L with absolute lymphocytosis. Morphology of transformed lymphocytes in infectious mononucleosis is given in Table 9.11.

Peripheral Blood Smear Examination

- Peripheral blood smear shows >10% transformed polyclonal T cells (CD8+ cytotoxic T cells). The indentation of cytoplasm of the lymphocyte by red blood cells gives rise to classic 'Dutch skirt' appearance of the T cell border.
- Differential white cell count shows lymphocytosis (>50%). Platelet count is within normal range.
- Morphologic variations of lymphocytes in infectious mononucleosis. Infectious mononucleosis in Giemsa-stained peripheral blood smear is shown in Fig. 9.7.

Bone Marrow Examination

- Bone marrow is performed to rule out leukemia.
- Bone marrow shows infiltration by transformed lymphocytes.

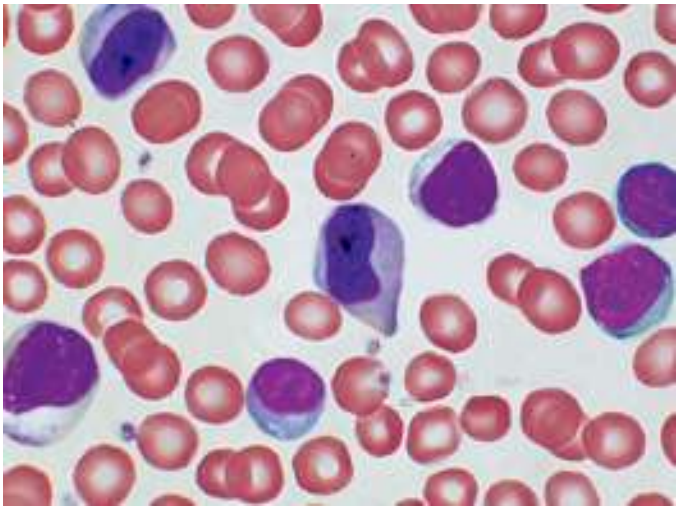


Fig. 9.7: Morphologic variations of lymphocytes in infectious mononucleosis in Giemsa-stained peripheral blood smear. It shows morphological variations in lymphocytes. Lymphocytes are large and irregular typical lymphocytes with indentation of the cytoplasm by red blood cells giving rise to the classic 'Dutch skirt' appearance of the border of lymphocytes (1000X).

Table 9.11 Morphology of transformed lymphocytes in infectious mononucleosis

Parameters	Plasmacytoid Cells (Type 1)	Monocytoid Cells (Type 2)	Blastoid Cells (Type 3)
Nuclear chromatin	Condensed chromatin giving cartwheel appearance with basophilic cytoplasm	Kidney-shaped nucleus with open chromatin	Open chromatin with thin cytoplasm

LEUKEMIAS

LEUKEMIAS: OVERVIEW

Hematopoietic stem cells (HSCs) and their progenitors maintain normal hematopoiesis during life-time of an individual. HSCs may differentiate into myeloid and lymphoid cell lineages.

- Leukemia is progressive disease due to clonal proliferation of HSCs either lymphoid or myeloid cell lineages resulting in accumulation of immature and nonfunctional HSCs in the bone marrow and blood circulation. Peripheral blood smear examination shows leukemic blasts with large nuclei, prominent nucleoli and few other distinguishing features.
- The leukemia cells show increased proliferation and/or decreased programmed cell death (apoptosis).
- Failure of normal production of red blood cells, leukocytes and platelets results in anemia, recurrent infection and thrombocytopenia. The net result is expansion of the leukemic clones and decrease in the normal HSCs in the bone marrow.

CLASSIFICATION OF LEUKEMIAS

Leukemias have been classified according to French-American-British classification and revised 2024 WHO classification. Acute leukemias consist of predominantly immature cells; chronic leukemias are composed of more mature cells.

- Four broad subtypes of leukemias most likely to be encountered by physicians are acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL) and chronic myelogenous leukemia (CML).
- Acute lymphoblastic leukemia (ALL) occurs more often in children, whereas other subtypes of leukemias affect adults. Risk factors for leukemias include a genetic predisposition and environmental factors, such as ionization radiation.
- Patient presents with nonspecific symptoms such as fever, fatigue, weight loss, bone pain, bleeding, and bruising.
- The two major categories of acute leukemias are classified according to origin of the cell with the primary defect: acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL).
  - If the defect primarily affects the maturation and differentiation and common myeloid progenitor cell, the leukemia is classified as AML.
  - If the defect primarily affects the common lymphoid progenitor cell, the leukemia is classified as acute lymphoblastic leukemia. Leukemic cells infiltrate liver, spleen, lymph nodes and other organs. In acute lymphoblastic leukemia, leukemic cells may cross blood–brain barrier and involve central nervous system.
- In subleukemic leukemia, blast cells are fewer in peripheral blood, and ≥20% in bone marrow. Aleukemic leukemia refers to low total leukocytes count ($<4 \times 10^9$/L) with absence of blasts in the peripheral blood smear. However, bone marrow shows ≥20% blasts.
- Hematopoietic stem cell-derived hematopoietic and lymphoid neoplasms found in the bone marrow, peripheral blood, lymph nodes and spleen are shown in Fig. 9.8. Origin of AML, ALL, CLL, B cell prolymphocytic leukemia (B-PLL), hairy cell leukemia (HCL), multiple myeloma (MM), Waldenström's macroglobulinemia is shown in Fig. 9.9.
- Characteristics of major subtypes of leukemia are given in Table 9.12. Immunophenotype markers for myeloblasts, B and T lymphoblasts are given in Table 9.13.

French-American-British (FAB) Classification of Leukemias

Leukemias were originally termed acute or chronic based on the life expectancy, but now are classified according to cell maturity. Leukemias are classified into acute myelogenous leukemia (AML-M0 to AML-M7), acute lymphoblastic leukemia (ALL-L1, ALL-L2, ALL-L3), chronic myelogenous leukemia (CML), and chronic lymphocytic leukemia (CLL). Other leukemias include hairy cell leukemia, prolymphocytic leukemia and T cell leukemia/lymphoma.

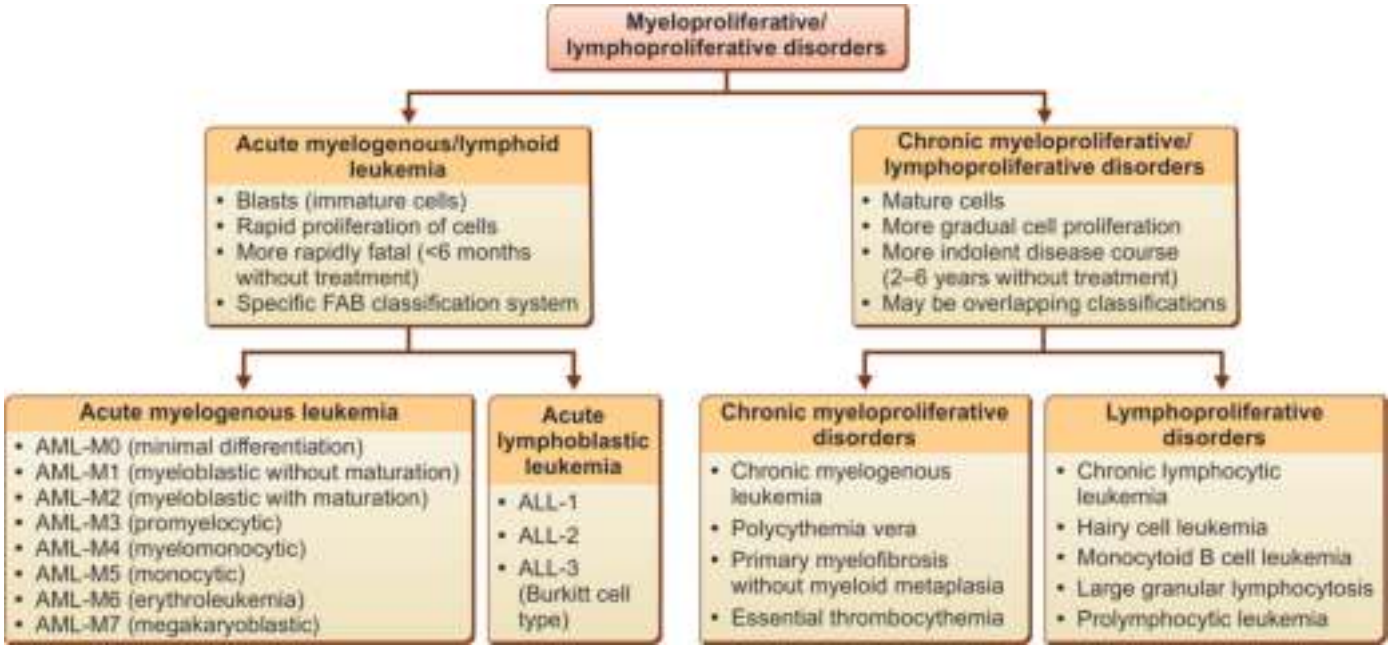


Fig. 9.8: Schematic representation of hematopoietic stem cell (HSC)-derived myeloid and lymphoid neoplasms. A neoplasm arising from hematopoietic stem cells are found in the bone marrow, peripheral blood, lymph nodes and spleen. HSC-derived neoplasms can also involve central nervous system and gastrointestinal tract either by metastasis, direct tumor infiltration or neoplastic transformation of extranodal lymphoid tissues.

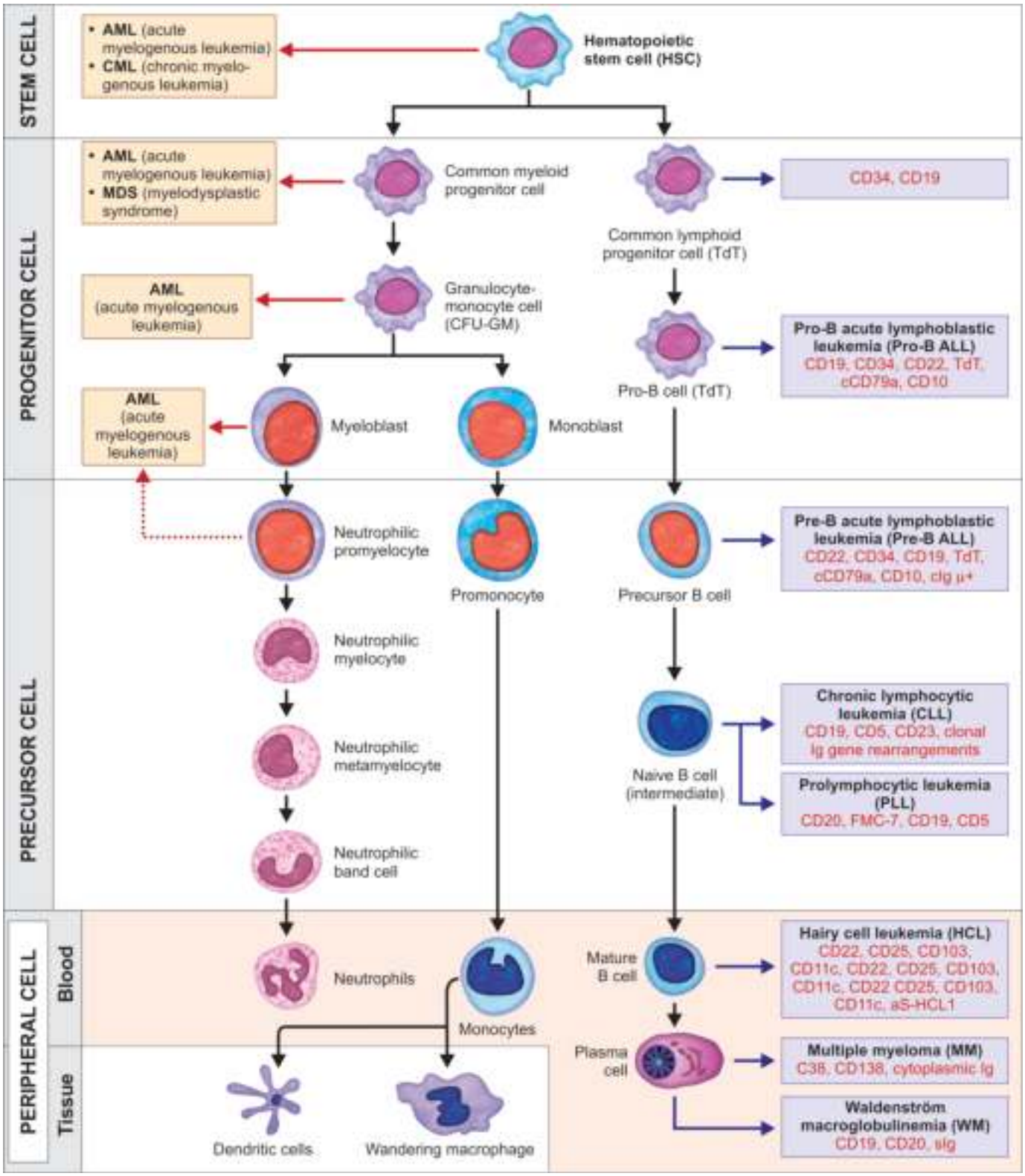


Fig. 9.9: Schematic representation of origin of acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HCL), multiple myeloma (MM), Waldenström heavy chain disease. AML and ALL may originate from any of the hematopoietic cells that fall within the pathways of the downward arrows. Importantly, the AML cell of origin acquires the capacity for self-renewal and maturation arrest.

Table 9.12 Characteristics of major subtypes of leukemia

Leukemia Subtype	Description	Age Group Affected	Clinical Features
Acute lymphoblastic leukemia (ALL)	Lymphoblasts on peripheral blood smear or bone marrow aspirate	Children 2–5 years and young adults in persons <20 years	Patient presents with fever, weakness, bleeding, musculoskeletal pain, or dysfunction. Clinical examination reveals hepatosplenomegaly and lymphadenopathy
Acute myelogenous leukemia (AML)	▪ Myeloblasts on peripheral blood smear or bone marrow aspirate ▪ Auer rods in myeloblasts on peripheral blood	Adults	Patient presents with fever, weakness, weight loss, bleeding, or bruising. Clinical examination reveals hepatosplenomegaly. Lymphadenopathy may occur in rare case
Chronic lymphocytic leukemia (CLL)	Clonal expansion of at least 5000 B lymphocytes per μl (5×10^9/μl) in the peripheral blood	Elderly adults >65 years	About 50% of patients remain asymptomatic
Chronic myelogenous leukemia (CML), BCR-ABL1 positive	Marked leukocytosis with myelocyte bulge and Philadelphia chromosome (BCR-ABL1 fusion transcript)	Adults	About 50% of patients remain asymptomatic. Clinical examination reveals massive splenomegaly, hepatomegaly and lymphadenopathy

Table 9.13 Immunophenotype markers for myeloblasts, B and T lymphoblasts

Lineage-specific Immunophenotype Markers	Expression
Myeloblast	
CD13, CD15, CD33, CD11b	Positive
CD34, c-KIT, HLA-DR	Positive/negative
Pro-B lymphoblast	
CD19	Positive
CD34	Positive
CD22	Positive
TdT (terminal deoxynucleotidyl transferase)	Positive
Cytoplasmic CD79a	Positive
CD10	Positive
Pre-B lymphoblast	
CD22	Positive
CD19	Positive
TdT (terminal deoxynucleotidyl transferase)	Positive
Cytoplasmic CD79a	Positive
CD10	Positive
Cytoplasmic mu	Positive
CD20, CD34, CD10, HLA-DR	Positive/negative
T lymphoblast	
CD3	Positive
TdT (terminal deoxynucleotidyl transferase)	Positive
CD5	Positive
CD7	Positive
CD1a	Positive
CD34, CD10	Positive/negative
HLA-DR, c-KIT (CD117)	Negative/positive (rarely)

- According to French-American-British (FAB) classification, in acute leukemias (AML or ALL) bone marrow and peripheral blood contain ≥30% blasts. FAB classification of ALL is based on cell size, cytoplasm, nuclear chromatin, nuclear shape, nucleoli, color and vacuolation of cytoplasm.
- Acute lymphoblastic leukemia (ALL) has three types: ALL-L1, ALL-L2 and ALL-L3. AML most often affects young adults and ALL is a disorder of children. Both ALL and AML more commonly affect males than females. ALL is most often seen in White population.
- Revised 2024 WHO classification of acute myelogenous and lymphoblastic leukemias and their features is given in Table 9.14. Revised 2024 WHO classification of acute myeloid and lymphoid leukemias and their features is given in Table 9.15.

Table 9.14 Revised 2024 WHO classification of acute myelogenous and lymphoblastic leukemias

Acute Myelogenous Leukemias	
▪ AML-M0:	Acute myelogenous leukemia with minimal evidence of myeloid differentiation
▪ AML-M1:	Acute myelogenous leukemia without maturation
▪ AML-M2:	Acute myelogenous leukemia with maturation
▪ AML-M3:	Acute promyelocytic leukemia
▪ AML-M4:	Acute myelomonocytic leukemia
▪ AML-M5:	Acute monocytic/monoblastic leukemia
▪ AML-M6:	Acute erythroleukemia
▪ AML-M7:	Acute myelogenous leukemia
Acute Lymphoblastic Leukemias	
▪ ALL-L1:	Acute lymphoblastic leukemia L1
▪ ALL-L2:	Acute lymphoblastic leukemia L2
▪ ALL-L3:	Acute lymphoblastic leukemia L3

Revised 2024 WHO Classification of Acute Leukemias

According to revised 2024 WHO classification of acute leukemias (acute myelogenous/acute lymphoblastic leukemia), diagnostic criteria are the presence of ≥20% blast cells as the cut off percentage in bone marrow and peripheral blood.

Table 9.15 Revised 2024 WHO classification of acute myeloid and lymphoid leukemias and their features

Leukemia Subtype	Description
Acute myelogenous leukemias	
AML-M0	▪ AML-M0 with minimally differentiation expressing myeloid lineage accounts for 2–3% ▪ AML-M0 is associated with poor prognosis
AML-M1	▪ AML-M1 without maturation demonstrates <10% promyelocytes or more mature myeloid cells ▪ Prognosis is favorable in AML-M1
AML-M2	▪ AML with maturation and differentiation into neutrophilic lineage ▪ AML-M2 is associated with favorable prognosis
AML-M3	▪ AML with promyelocytic differentiation (APL) in most cases demonstrate promyelocytes with heavy granulation and bilobed folded (cottage-loaf) nuclei (commonly associated with disseminated intravascular coagulation (DIC) due to release of thromboplastin-like substance); rarely microgranular variant with inconspicuous granules, accounts for 5–10% of leukemias ▪ Acute promyelocytic leukemia now represents most curable leukemia treated by administration of nontoxic cytotoxic chemotherapeutic drugs such as all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO)
AML-M4	▪ Acute myelomonocytic leukemia (AMML) shows myelocytic and monocytic differentiation (>20% monocytoid cells involving gums, skin or CNS) ▪ AML-M4E0 variant contains >5% abnormal eosinophils; associated with the inv(16) cytogenetic abnormality associated with favorable prognosis
AML-M5A	▪ Acute monoblastic/monocytic leukemia (AML-M5A) shows monoblasts without differentiation, extramedullary disease involving gums, skin, lymph node and central nervous system ▪ AML-M5A is associated with extramedullary disease, abnormalities of chromosome 11q23 and poor prognosis.
AML-M5B	▪ Acute monoblastic/monocytic leukemia (AML-M5B) showing more than 80% monoblasts with differentiated monocytes, or promonocytes ▪ Extramedullary AML-5B disease has poor prognosis
AML-M6A	▪ In acute erythroid leukemia (AML-M6A), bone marrow shows dysplastic nucleated erythroblasts (>50%) and myeloblasts (>20%) ▪ AML-M6A is associated with poor prognosis
AML-M6B	▪ In acute erythroid leukemia (pure erythroid leukemia) (AML-M6B), bone marrow shows dysplastic nucleated erythroblasts (>80%) ▪ AML-M6B is associated with poor prognosis
AML-M7	▪ Acute megakaryoblastic leukemia (AML-M7), bone marrow shows micromegakaryoblasts (common in Down's syndrome and myelofibrosis) ▪ AML-M7 accounts for <1% of leukemias ▪ AML-M7 is associated with poor prognosis
Acute lymphoblastic leukemias	
ALL-L1	▪ In acute lymphoblastic leukemia L1 (ALL-L1), lymphoblasts are uniform, homogenous with scant cytoplasm with regular nuclei and inconspicuous nucleoli ▪ ALL-L1 accounts for >80% of leukemias ▪ Children are affected associated with good prognosis
ALL-L2	▪ Acute lymphoblastic leukemia L2 (ALL-L2) demonstrates lymphoblasts, which are large, heterogenous with round to oval nuclei showing indentation or clefts with prominent nucleoli ▪ ALL-L2 accounts for 10–50% of leukemias ▪ Adults are affected associated with poor prognosis
ALL-L3	▪ Acute lymphoblastic leukemia L3 (ALL-L3) demonstrates lymphoblasts, which are large homogenous with round to oval nuclei, finely stippled chromatin, prominent nucleoli and abundant basophilic vacuolated cytoplasm ▪ ALL-L3 accounts for 3–4% of leukemias ▪ Adults are affected associated with poor prognosis

CD: Cluster of differentiation.

- Revised 2024 WHO classification of leukemias represents presence of ≥20% blast cells in bone marrow and peripheral blood. Presence of ≥30% blast cells were considered as a cut off percentage of diagnostic criteria of leukemia in bone marrow and peripheral blood as per original guidelines of French-American-British (FAB) classification of leukemias.
- PML-RARA molecular abnormality is the diagnostic feature of AML-M3. Revised 2024 WHO classification of acute leukemias and related neoplasm is discussed under AML and ALL entities.

PATHOGENESIS

Various predisposing factors are believed to participate in the pathogenesis of acute leukemias. Inherited genetic predisposition, ionizing radiation, chemotherapeutic agents (e.g. cyclophosphamide, chlorambucil, nitrosoureas, β-propiolactone, dimethyl sulfate, and dexproxibutene), tobacco smoke and benzene, pre-existing hematologic disorders (myelodysplastic dysplastic syndrome, paroxysmal nocturnal hemoglobinuria, familial aplastic anemia and acquired somatic mutations) associated with aging process play an important role in the development of acute myelogenous leukemia. Certain disorders predispose to acute lymphoblastic leukemia, most notably trisomy 21 (Down syndrome), in which the relative risk of ALL is increased 10- to 30-fold.

Chemical Agents

Benzene is widely used to prepare plastics, resins, synthetic fibers, dyes, detergents, drugs and pesticides. Natural source of benzene includes emissions from fires. Benzene is also a component of crude oil, gasoline and cigarette smoke.

- Prolonged exposure to benzene can lead to hematotoxicity and genomic toxicity that contribute to leukemogenic process leading to acute myelogenous leukemia.
- Benzene metabolites such as 1,4-benzoquinone (1,4-BQ) and hydroquinone (1,4-HQ) cause oxidative DNA damage through H_2O_2 generation in cells, preceding internucleosomal DNA damage leading to apoptosis or mutation.
- The outcome of the transformed cells to undergo apoptosis or mutation might be dependent on the intensity of DNA damage and ability to repair DNA.

Chemotherapeutic Agents

Chemotherapeutic agents such as cyclophosphamide, chlorambucil, nitrosoureas, β-propiolactone, dimethyl sulfate, and dexproxibutene, administered in patients with Hodgkin disease, NHL and ovarian carcinoma may cause somatic and cytogenetic mutations (monosomy 5, monosomy 7, 5p and 11q23) and evoke second form of cancer such as acute myelogenous leukemia (AML).

Ionizing Radiation

Ionizing radiation poses a potential worldwide threat in this nuclear age. Low doses of ionizing radiation can cause DNA mutations and chromosomal abnormalities leading to acute leukemias. Large doses of radiation can inhibit cell division. Survivors of atomic blasts in Hiroshima and Nagasaki develop acute myelogenous leukemia (AML) or chronic myelogenous leukemia (CML), but not ALL and CLL. Radiologists may develop acute myelogenous leukemia.

Human T-Lymphotropic Virus 1 (HTLV-1)

Human T-lymphotropic virus 1 (HTLV-1) can cause adult T cell leukemia or non-Hodgkin lymphoma (NHL) in Japan. HTLV-1 activates TAX gene that causes proliferation of T cells by neutralizing growth inhibitory signals by TP53 and CDKN2A genes. Additional gene mutations result in development of T-adult leukemia and non-Hodgkin lymphoma (NHL).

Bone Marrow Failure Syndrome

Bone marrow failure syndrome increases the risk for developing leukemia in patients with Fanconi syndrome, Diamond-Blackfan, ataxia-telangiectasia, familial aplastic anemia, and Bloom syndrome. Germline TP53 gene mutations, a cause of cancer-predisposing Li-Fraumeni syndrome, are present in 40% of acute lymphoblastic leukemia (ALL) patients with low hypodiploid chromosomes (32–39 chromosomes).

Genetic Disorders

Down syndrome, Bloom syndrome, Fanconi syndrome, dyskeratosis congenita, ataxia-telangiectasia, Li-Fraumeni syndrome, Kostmann syndrome, Klinefelter syndrome, osteogenesis imperfecta, Wiskott-Aldrich syndrome, leukemia in siblings and Diamond-Blackfan syndrome, increase the risk for developing acute myelogenous leukemia (AML).

Molecular Genetic Alterations

Molecular analysis has increased our understanding the role of Philadelphia chromosome in the pathogenesis of chronic myelogenous leukemia (CML).

- Philadelphia chromosome (Ph) is an acquired chromosomal translocation that results in a fusion gene called BCR-ABL1. Once the Philadelphia chromosome is detected, its rise and fall reflects the tumor burden; and hence can be used as a measure of the disease progression, remission and relapse. All the three cell lineages of the hematopoietic stem cells are involved in CML, the original transformed cell is pluripotent hematopoietic stem cell (HSC).

Table 9.16 Hereditary and acquired disorders associated with increased risk for acute leukemia/lymphoma

Predisposing Factors	Disorders and Causes	
Genetic susceptibility	▪ Down's syndrome (↑ risk of AML or ALL) ▪ Fanconi syndrome (↑ risk of AML) ▪ Ataxia-telangiectasia (↑ risk of ALL or NHL) ▪ Bloom syndrome ▪ Klinefelter's syndrome	▪ Osteogenesis imperfecta ▪ Leukemia in siblings ▪ Diamond-Blackfan syndrome ▪ Li-Fraumeni syndrome ▪ Xeroderma pigmentosum
Predisposing hemato-logical disorders	▪ Myeloproliferative syndrome (↑ risk of AML) ▪ Chronic myeloproliferative disorders (↑ risk of AML) ▪ Aplastic anemia	▪ Paroxysmal nocturnal hemoglobinuria (↑ risk of AML or rarely ALL)
Somatic mutations	▪ Chronic exposure to benzene ▪ Alkylating agents (chlorambucil, melphalan) ▪ Prolonged exposure to radiation	▪ Chronic exposure to benzene ▪ Alkylating agents (chlorambucil, melphalan)
Viral infection	▪ HTLV1 (↑ risk of adult T cell leukemia/ lymphoma)	▪ HIV infection
Immunologic disorders	▪ Wiskott-Aldrich syndrome ▪ Bruton's type X-linked agammaglobulinemia	▪ Immunosuppressive therapy
Chemical agents	▪ Prolonged benzene exposure	▪ Alkylating agents (chlorambucil, melphalan)

- On the other hand, chronic lymphocytic leukemia (CLL) is a disease of clonal B cells that have low proliferative rate and defect in the apoptosis of leukemic cells. CLL leukemic B cells appear mature but are actually arrested during early development resulting in less capable of differentiating into antibody-synthesizing plasma cells.
- Hereditary and acquired disorders associated with increased risk for acute leukemia/lymphoma are given in Table 9.16. Schematic representation of pathogenesis of acute leukemia is shown in Fig. 9.10. Schematic representation of pathogenesis of acute myelogenous leukemia showing class I and class II mutations is shown in Fig. 9.11.

LABORATORY DIAGNOSIS

A complete hemogram examination usually reveals leukocytosis and abnormally elevated or depressed cell lineages in leukemias.

- Coagulation studies reveal elevated prothrombin time–INR, decreased fibrinogen level and the presence of fibrin degradation products in acute promyelocytic leukemia also known as AML-M3 associated with disseminated intravascular coagulation (DIC).
- The diagnosis is confirmed by further examination of peripheral blood smear, bone marrow aspirate and bone marrow trephine biopsy.
- Cytogenetic analysis, molecular testing and immunophenotyping are performed to establish diagnosis of leukemias. Common laboratory tests for the evaluation of leukemias are given in Table 9.17.

Peripheral Blood Smear Examination

Examination of Romanowsky stained peripheral blood smear is an inexpensive but powerful reliable and diagnostic tool of hematologic disorders in both children and adults.

- In leukemias, malignant transformation and uncontrolled proliferation of an abnormally differentiated, long-lived myeloid progenitor cell results in high number of immature blood cells and replacement of normal bone marrow by leukemic cells.
- Changes in the number and the appearance of different types of blood cells such as anemia and thrombocytopenia of variable degrees are helpful in diagnosing leukemias.

Bone Marrow Smear Examination

Bone marrow smear examination is performed to obtain greater concentration of hematopoietic stem cells for identification of blast cells in acute myelogenous leukemia and acute lymphoblastic leukemia.

- Extent of bone marrow involvement is correlated with prognosis in chronic lymphocytic leukemia. It is essential to diagnose chronic myelogenous leukemia, BCR-ABL1 positive in its chronic, accelerated and blast phase.
- Bone marrow aspiration smears should be stained with May-Grunwald-Giemsa or Wright-Giemsa stain for optimal visualization of cytoplasmic granules and nuclear chromatin. It is recommended to count at least 500 nucleated cells on bone marrow cellular

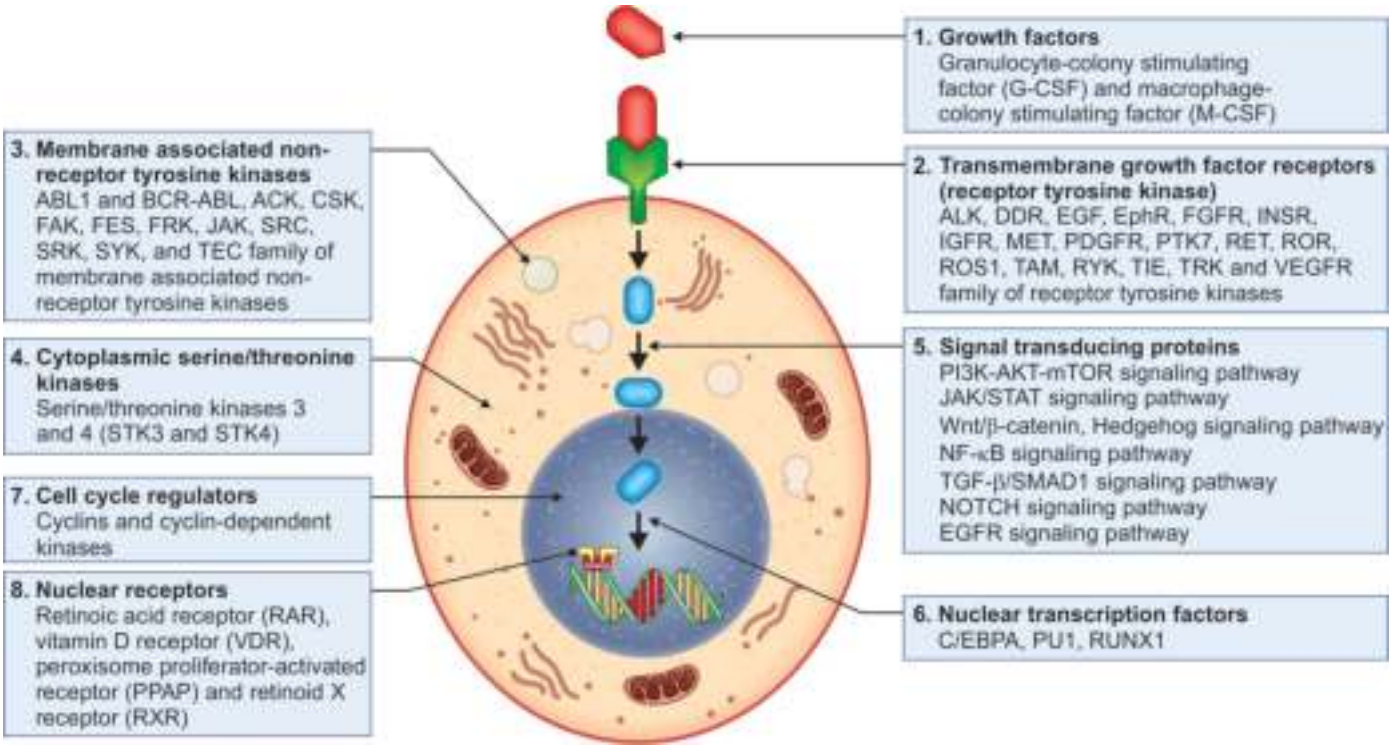


Fig. 9.10: Schematic representation of pathogenesis of acute myelogenous leukemia. It depicts mutations in growth factors, growth factor receptors, signaling transduction pathways leading to development of acute myelogenous leukemia as a consequence of a series of genetic changes in the hematopoietic precursor cell. These changes alter normal hematopoietic cell growth and differentiation resulting in an accumulation of large number of abnormal, immature myeloid cells in the bone marrow and peripheral blood.

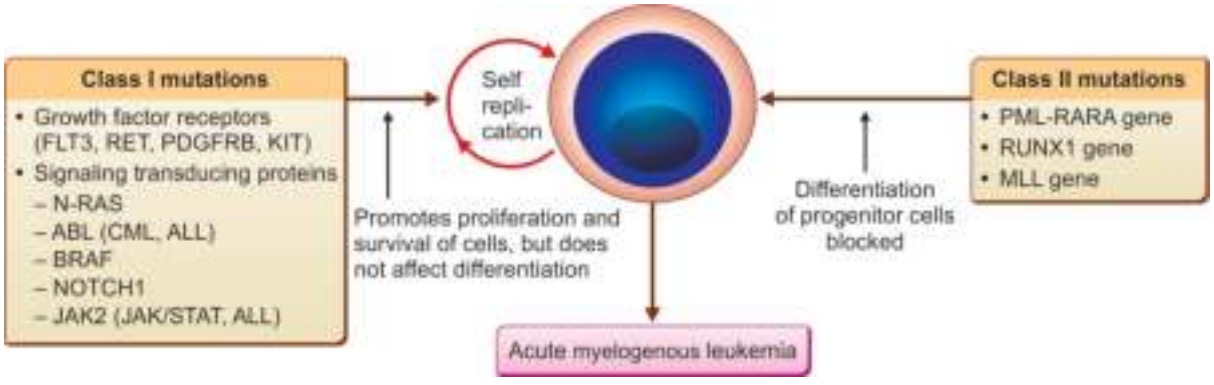


Fig. 9.11: Schematic representation of pathogenesis of acute myelogenous leukemia showing class I and II mutations. Class I mutations of growth factors and signaling transducing proteins act on hematopoietic stem cell that replicates. Class II mutations block the differentiation of progenitor cells. PML-RARA molecular abnormality is the diagnostic feature of AML-M3.

aspirate smears as close to the particle and undiluted with blood as far as possible.

- The cells to be counted in bone marrow aspirate smears include blasts, promyelocytes, myelocytes, metamyelocytes, band neutrophils, segmented neutrophils, eosinophils, basophils, lymphocytes, plasma cells, erythroid precursors, megakaryocytes, and mast cells. Comparison of acute and chronic leukemias is given in Table 9.18.

Bone Marrow Trephine Biopsy Examination

Bone marrow trephine biopsy examination is performed to obtain greater concentration of hematopoietic stem cells for identification of blast cells in acute myelogenous leukemia and acute lymphoblastic leukemia.

- Bone marrow trephine biopsies of suspected myeloid neoplasms should be ≥1.5 cm in length and obtained at right angles to the cortical bone,

Table 9.17 Common laboratory tests for the evaluation of leukemias

Laboratory Test	Characteristics	Clinical Application
Peripheral blood smear	Examination of peripheral blood smear under light microscope	Identification of Auer rods in myeloblasts in acute myelogenous leukemia
Bone marrow aspirate and/or bone marrow trephine biopsy examination	Examination of greater concentration of hematopoietic cells	▪ Identification of blast cells in acute myelogenous leukemia and acute lymphoblastic leukemia ▪ Extent of bone marrow involvement correlated with prognosis in chronic lymphocytic leukemia
Cytogenetic analysis	Examination of whole chromosomes through karyotyping or fluorescence *in situ* hybridization (FISH)	▪ Detection of the Philadelphia chromosome (BCR-ABL1 fusion gene) for the diagnosis of chronic myelogenous leukemia ▪ Identification of chromosomal abnormalities to diagnose leukemia subtype ▪ Cytogenetic analysis can be used to guide treatment and determine prognosis
Flow cytometry with immunophenotyping	Sorting and counting cell by specific markers, samples obtained from blood and/or bone marrow	▪ Counting cloned cells of lymphoid lineage for the diagnosis of chronic lymphocytic leukemia ▪ Identification of certain cell surface markers to diagnose leukemia subtypes
Molecular analysis	Analysis of specific mutations at the DNA level through polymerase chain reaction testing	▪ Detection of the Philadelphia chromosome (BCR-ABL1 fusion gene) for the diagnosis of chronic myelogenous leukemia ▪ Diagnosis of leukemia subtypes, monitoring response to therapy and prognosis

Table 9.18 Comparison of acute and chronic leukemias

Features	Acute Leukemia	Chronic Leukemia
Age group	All age groups	Adults
Clinical onset	Sudden onset	Insidious onset
Clinical course of disease (untreated cases)	Weeks to months	Months to years
Predominant cells	Blasts, some mature forms	Mature forms
Anemia	Mild to severe	Mild
Thrombocytopenia	Mild to severe	Mild
WBCs count	Variable	Increased

which provide information regarding overall cellularity, cytomorphologic details, proportion and maturation of hematopoietic stem cells, evaluation of bone marrow stroma and cancellous bone structure. Bone marrow trephine biopsy provides material for immunohistochemical studies that may have diagnostic and prognostic significance.

- Bone marrow trephine biopsy is essential whenever, one suspect myelofibrosis. Bone marrow trephine biopsy should be well fixed. Tissue sections should be 3–4 μm and stained with hematoxylin and eosin.
- Gomori silver methenamine stain is used for evaluation of collagen and reticulin fibers in myelofibrosis. Periodic acid–Schiff stain (PAS) may be used to detect megakaryocytes.

Cytochemistry

Cytochemistry is commonly performed to diagnose leukemias. In principle, it identifies differences in the cellular content of different cytoplasmic enzymes such as myeloperoxidase (MPO) stain, nonspecific esterase (NSE) stain, periodic acid–Schiff (PAS) stain, Sudan black stain and acid phosphatase (AP) stain.

- Cytochemical stains are sometimes difficult to interpret even in the most experienced hands, especially when leukemic blasts are undifferentiated enough to make a morphologic diagnosis difficult.
- Cytochemical staining techniques in hematology are given in Table 9.19. Cytochemistry in acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (AML) is given in Table 9.20.

Table 9.19 Cytochemical staining techniques in hematology

Cytochemical Stain	Element Stained	Positive Staining Reaction	Negative Staining Reaction	Diagnostic Utility
Myeloperoxidase (MPO) stain	MPO present in primary granules of granulocytic cells	▪ Myeloblasts and promyelocytes (strong staining) ▪ Monocytes (weak staining)	Lymphoblasts	▪ Differentiating AML versus ALL ▪ AML positive ▪ ALL negative
Sudan black B (SBB) stain	Lipids present in primary, secondary granules and lysosomes of monocytes	▪ Myeloblasts and promyelocytes (strong staining) ▪ Monocytes (weak staining)	Lymphoblasts	▪ Differentiating AML versus ALL ▪ AML positive ▪ ALL negative
Leukocyte esterase AS-D chloroacetate esterase stain	Specific esterase enzyme present in neutrophils	Myeloblasts	Lymphoid cells	Demonstration of cells of myeloid origin
Leukocyte esterase (alpha-naphthyl esterase) stain	Nonspecific esterase enzyme present in monocytes	▪ Monoblasts ▪ Monocytes	▪ Granulocytes ▪ Lymphoid cells	Differentiating myeloid versus monocytic origin
Tartrate-resistant acid phosphatase (TRAP) stain	Isoenzymes of acid phosphatase	Hairy cell lymphocytes (isoenzyme 5 of acid phosphatase is not inhibited with the addition of tartrate, thus positive reaction)	All cells except hairy cell lymphocytes	Positive staining and diagnosis of hairy cell leukemia
Periodic acid–Schiff (PAS) stain	▪ Glycogen ▪ Mucoproteins ▪ High molecular weight carbohydrates	Multiple cell types	Normal erythroblasts	▪ AML of erythroid (coarse granular staining) or megakaryocytic origin ▪ ALL (some cases)
Leukocyte alkaline phosphatase (LAP)	LAP present in the secondary granules of mature neutrophils	Neutrophils (activity of LAP is demonstrated in mature band cells and segmented neutrophils and scoring done on a 0–4 rating scale)	Not applicable	▪ Differentiation of CML versus leukemoid reaction ▪ CML (low activity) ▪ Leukemoid reaction (high activity)
Terminal deoxynucleotidyl transferase (TdT)	DNA polymerase in cell nuclei	Lymphoblasts	▪ Myeloblasts ▪ Monoblasts	Demonstration of lymphoblasts

AML: Acute myelogenous leukemia, ALL: Acute lymphoblastic leukemia, CML: Chronic myelogenous leukemia.

Table 9.20 Cytochemistry in acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL)

Cytochemical Stain	Acute Myelogenous Leukemia (AML)	Acute Lymphoblastic Leukemia (ALL)
Myeloperoxidase	Positive	Negative
Sudan black B	Positive	Negative
Nonspecific esterase	Positive (AML-M4, AML-M5)	Negative
PAS	Positive diffuse staining (AML-M6)	Positive
Acid phosphatase	Negative	Positive (T cell ALL)

Pathology Pearls: Cytochemical Stains used Diagnosing Leukemias

Leukocyte Myeloperoxidase Stain

- Leukocyte myeloperoxidase is present in the myeloblasts, that plays a role in killing bacteria. Myeloblasts show positivity for myeloperoxidase.
- Myeloperoxidase (MPO) is positive in myeloblast of acute myelogenous leukemia and negative in lymphoblast of acute lymphoblastic leukemia.
- Myeloperoxidase enzyme stain negativity in lymphoblasts in acute lymphoblastic leukemia 2 (ALL-L2) is shown in Fig. 9.12. Myeloperoxidase enzyme stain positivity in myeloblasts in a case of acute myelogenous leukemia M2 (AML-M2) is shown in Fig. 9.13.

Leukocyte Esterase Stain

- Leukocyte esterases are found in monocytes and neutrophils in different concentrations.
- α-Naphthyl esterase is strongly positive in monocytes and weakly positive in neutrophils.
- Reverse expression is true of naphthol AS-D chloroacetate esterase, i.e. weakly positive in macrophages and strongly positive in neutrophils.
- Leukocyte esterases are useful to differentiate the monocytic from granulocytic precursors in acute myelogenous leukemia.
- Nonspecific esterase stain (α-naphthyl-acetate-esterase) positivity in a case of T cell lineage acute lymphoblastic leukemia (T-ALL) is shown in Fig. 9.14. Nonspecific esterase stain positivity in a case of acute myelogenous leukemia M3 (AML-M3) is shown in Fig. 9.15.

Periodic Acid–Schiff Stain

- Periodic acid–Schiff (PAS) stain demonstrates the presence of polysaccharides. Neutrophilic granules stain with PAS stain technique.
- Lymphocytes may have PAS positive granules.
- PAS stain is negative in acute myelogenous leukemia blasts, but acute lymphoblastic leukemia lymphoblasts may demonstrate variable positivity.
- Periodic acid–Schiff (PAS) stain positivity in blasts in a case of acute lymphoblastic leukemia L2 (ALL-L2) is shown in Fig. 9.16.

Acid Phosphatase Stain

- Acid phosphatase is present in macrophages and osteoblasts.
- T cell acute lymphoblastic leukemia (T-ALL) and hairy cell leukemia (HCL) are also positive for acid phosphatase.
- Acid phosphatase is tartrate resistant in hairy cell leukemia.
- Acid phosphatase staining on bone marrow aspirate smear is shown in Fig. 9.17.

Leukocyte Alkaline Phosphatase Stain

- Leukocyte alkaline phosphatase is present in the more mature cells of the myeloid lineage, band form and neutrophils.
- Leukocyte alkaline phosphatase is useful for the differential diagnosis between chronic myelogenous leukemia (CML), where alkaline phosphatase is low from leukemoid reactions, where alkaline phosphatase is normal.

Sudan Black Stain

- Sudan black is a lipid stain positive in neutrophilic granules of precursors and mature granulocytes.
- Sudan black is also present in myeloblast of acute myelogenous leukemia but not in lymphoblast of acute lymphoblastic leukemia.

Terminal Deoxynucleotidyl Transferase

- Terminal deoxynucleotidyl transferase (TdT) is present in thymocytes and thymocyte precursors. TdT is positive in most patients with acute lymphoblastic leukemia except the rare B cell acute lymphoblastic leukemia.
- TdT is absent in acute myelogenous leukemia (AML).

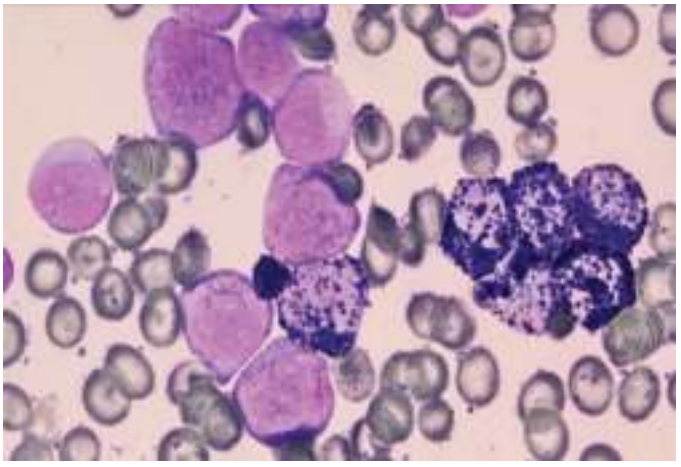


Fig. 9.12: Myeloperoxidase (MPO) enzyme stain in acute lymphoblastic leukemia 2 (ALL-L2). Blasts are showing negative results with myeloperoxidase stain and the neutrophils and eosinophils are showing positivity in the form of dense greenish blue granules in the cytoplasm (1000X).

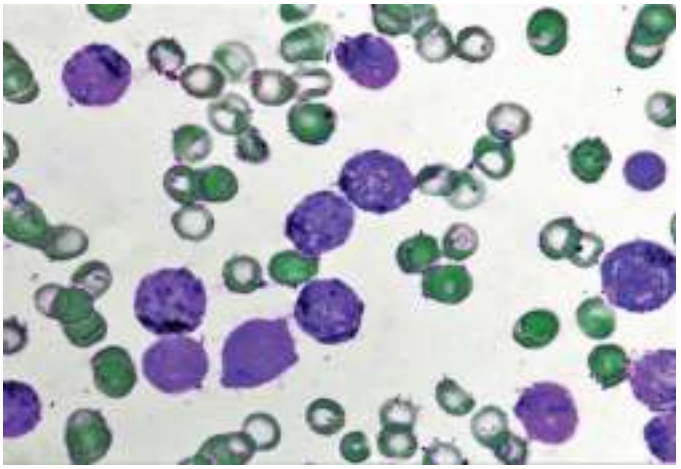


Fig. 9.13: Myeloperoxidase (MPO) enzyme stain positivity in myeloblasts in a case of acute myelogenous leukemia M2 (AML-M2). Myeloperoxidase is the hallmark enzyme of the myeloid lineage. It can be detected by cytochemistry, flow cytochemistry or immunohistochemistry. The diagnosis of acute myeloblastic leukemia (AML) is easy if more than 3% of myeloblast cells are confirmed to be cytochemically myeloperoxidase positive (1000X).

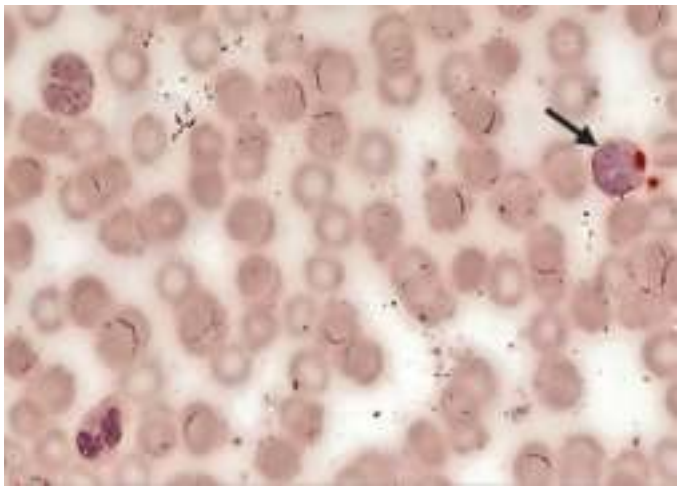


Fig. 9.14: Nonspecific esterase stain (α-naphthyl-acetate-esterase) positivity in a case of T cell lineage acute lymphoblastic leukemia (T-ALL). Bone marrow trephine biopsy section is showing reddish brown focal positivity in the blast (marked by arrow). On immunophenotyping this case turned out to be of T-lineage acute lymphoblastic leukemia (1000X).

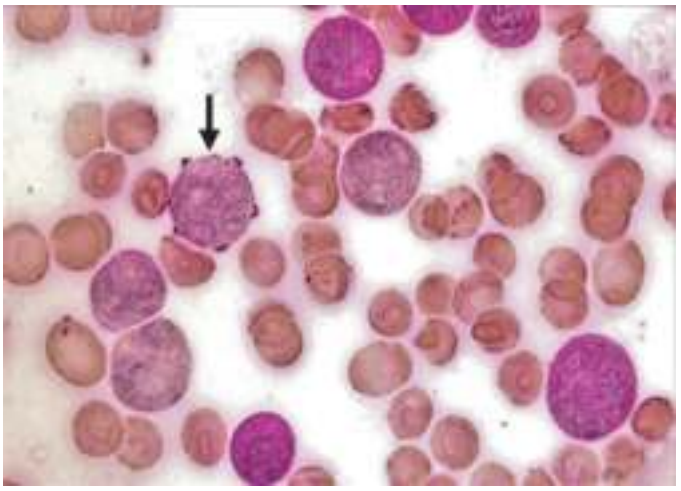


Fig. 9.16: Periodic acid–Schiff (PAS) stain positivity in blasts in a case of acute lymphoblastic leukemia L2 (ALL L2). Periodic acid–Schiff (PAS) stain of the same case as above showing granular magenta positivity in the blast cell (marked by arrow) and neutrophils are showing dense confluent positivity (acting as positive control) (1000X).

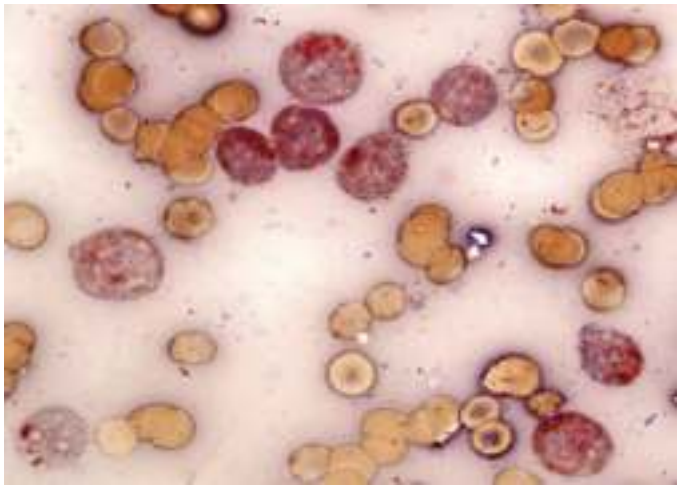


Fig. 9.15: Nonspecific esterase stain positivity in a case of acute myelogenous leukemia M3 (AML-M3) (1000X).

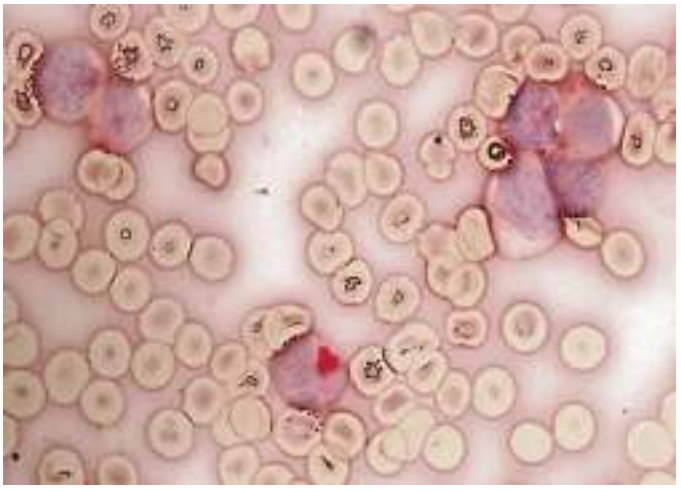


Fig. 9.17: Acid phosphatase stain on bone marrow aspirate smear shows polar magenta colored positivity in the blast cells. This case was of T-ALL on immunophenotyping (1000X).

Immunophenotyping

Flow cytometry is performed for immunophenotyping for sorting and counting cell by specific surface markers to diagnose leukemias on samples obtained from blood and/or bone marrow.

- Monoclonal antibodies are useful to differentiate acute myelogenous leukemia (AML) from acute lymphoblastic leukemia (ALL) in cases inconclusive on cytochemistry.
- Patients with AML express HLA-DR, cytoplasmic anti-MPO, CD13, CD33 and CD65. HLA-DR, CD10, CD19, CD20 are positive in acute lymphoblastic leukemia (ALL). B-ALL and T-ALL cases are positive with CD2, CD3, CD5 and CD7.
- Monocyte is positive with CD14. AML-M6 is positive with glycophorin A. AML-M7 is positive for CD33, CD61, vWF, GpIIb/IIIa and glycophorin A.
- Immunophenotyping of AML and B-ALL, T-ALL, chronic lymphocytic leukemia, B-prolymphocytic leukemia and hairy cell leukemia is given in Table 9.21. Expression of markers for diagnosis of AML and mixed phenotype acute leukemia (MPAL) is shown in Fig. 9.18.

Cytogenetic Analysis

Cytogenetic analysis is study of whole chromosomes through karyotyping or fluorescence *in situ* hybridization. Cytogenetic analysis is done to detect the Philadelphia

Table 9.21 Immunophenotyping of acute myelogenous leukemia (AML) and B cell acute lymphoblastic leukemia (B-ALL), T cell acute lymphoid leukemia (T-ALL), chronic lymphocytic leukemia, B-prolymphocytic leukemia and hairy cell leukemia

Leukemia	Expression
Acute myelogenous leukemia (AML)	HLA-DR+, CD13+, CD33+, CD65+, cAnti-MPO+
B-lineage acute lymphoblastic leukemia	CD19+, CD210+, CD22+, CD24+, CD79a+
Pro-B acute lymphoblastic leukemia	CD19+, CD34+, CD22+, TdT+, cytoplasmic CD79a+, CD10+
Pre-B acute lymphoblastic leukemia	CD22+, CD34+, CD34+, CD19+, TdT+, cytoplasmic CD79a+, CD10+, cytoplasmic Ig mu+
Cortical/thymic T cell acute lymphoblastic leukemia (T cell-ALL)	TdT+, CD10+, CD1a+, CD2+. CD3+. CD5+. CD7+
Chronic lymphocytic leukemia (CLL)	CD19+, CD5+, CD23+ clonal Ig gene rearrangements+
B cell-prolymphocytic leukemia (B-PLL)	CD20+, FMC-7+, CD19+, CD5+
Hairy cell leukemia (HCL)	CD19+, CD20+, CD200+, monoclonal surface immunoglobulin-aS-HCL1+, CD22+ CD25+, CD103+, CD11c+

CD1 is specific marker for Langerhans cells; TdT: Terminal deoxynucleotidyl transferase.

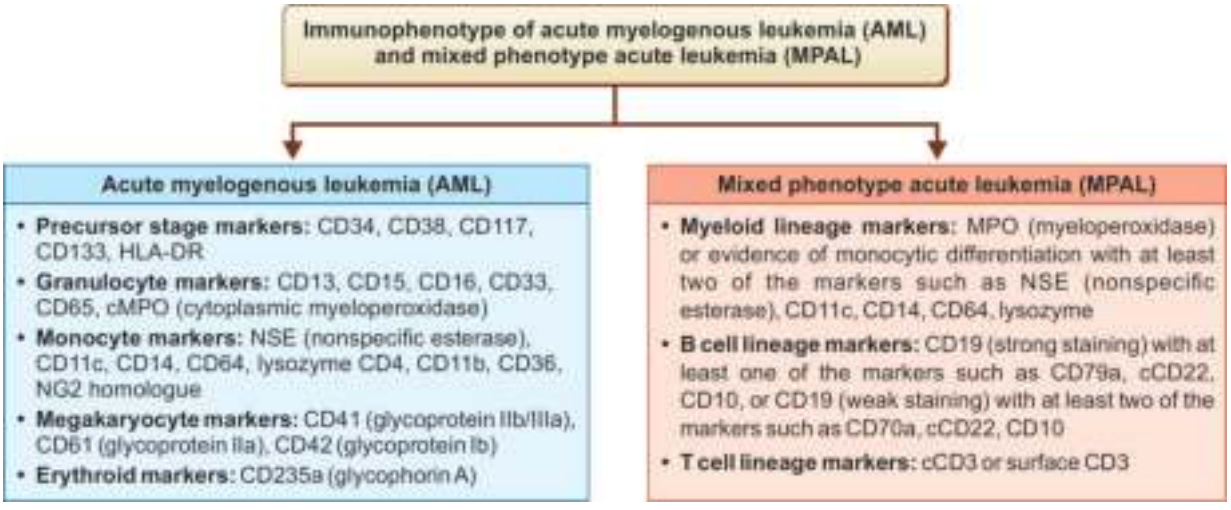


Fig. 9.18: Schematic representation of immunophenotype of acute myelogenous leukemia (AML) and mixed phenotype acute leukemia (MPAL).

chromosome (BCR-ABL1 fusion gene) for the diagnosis of chronic myelogenous leukemia. It can be used to guide treatment and determine prognosis. Cytogenetic abnormalities in acute and chronic leukemias are given in Table 9.22.

Molecular Genetic Analysis

Molecular genetic analysis is performed to identify specific mutations at the DNA level through polymerase chain reaction to detect the Philadelphia chromosome (BCR-ABL1 fusion gene) for the diagnosis of chronic myelogenous leukemia. Molecular genetic analysis also aids in the diagnosis of leukemia subtypes, can also be used to guide treatment and determine prognosis.

TREATMENT

Treatment of leukemias are treated by targeted therapy, chemotherapy, radiotherapy, monoclonal antibodies, or hematopoietic stem cell transplantation.

- Complications of leukemias treatment include tumor lysis syndrome and serious infections from immunosuppression in leukemic patients.
- Leukemic patients should be monitored closely for development of secondary malignancies, cardiac complications, and endocrinal disturbances such as metabolic syndrome, hypothyroidism and hypogonadism.

PROGNOSIS

Five-year survival rates are highest in the younger patients and patients with chronic lymphocytic leukemia (CLL) and chronic myelogenous leukemia (CML).

- Prognosis in patients with acute myelogenous leukemia (AML) ranges from mortality within a few days of initiating treatment.
- Acute lymphoblastic leukemia (ALL) is the second most common in children and adults. In adults, 75% of ALL cases develop from B cell lineage with

Table 9.22 Cytogenetic abnormalities in acute and chronic leukemias

Tumor	Chromosomal Abnormalities	Fusion Transcript, Involved Genes
Acute myelogenous leukemia (AML)		
AML-M1 (CD13+, CD15+, CD64+)	t(9;22)	BCR-ABL
AML-M2	t(8;21) favorable prognosis	CBa-ETO
AML-M3 (acute promyelocytic leukemia)	▪ t(15;17) (q22;q21) ▪ t(11;17) (q22;q21) ▪ t(5;17) (q35;q21) ▪ t(11;17) (q13;q21)	▪ RARA/PML (transcriptional factor in 95%) ▪ PLZF-RARA (<5%) ▪ NPM-RARA (<1%) ▪ NuMA-RARA (<1%)
AML-M4 eosinophil	inv(16) (p13'q22) favorable prognosis	CBFb/MYH1
Acute lymphoblastic leukemia (ALL)		
ALL-B cell lineage (good prognosis)	t(12;21)	CBFa-ETV6
ALL pre-B cell lineage (cytoplasmic Ig positive) with poor prognosis	t(1;19)	PBX1-E2A (transcription factor)
Chronic myelogenous leukemia (CML)		
Chronic myelogenous leukemia (CML), BCR-ABL1 positive	t(9,22) (q34,q11)	BCR-ABL
Chronic lymphocytic leukemia (CLL)		
Chronic lymphocytic leukemia (CD19+, CD20+, CD23+, CD5+)	▪ Trisomy 12 ▪ Deletions of 11q,13q and 17p	Not applicable

the remainder ALL cases develop from clonal T cell lineage.

- Despite advances in management, the backbone of therapy in leukemias remains multi-agent chemotherapy with vincristine, corticosteroids and an anthracycline with allogeneic hematopoietic stem cell transplantation for eligible candidates.

Clinical Pearls: Uses of Hematopoietic Growth Factors in Oncology Practice

- **Hematopoietic growth factors:** These belong to family of regulatory molecules that play important roles in the growth, and differentiation of blood progenitor cells, as well as functional activation of mature cells.
- **Recombinant growth factors:** The commercial availability of recombinant hematopoietic growth factors/cytokines (G-CSF, GM-CSF, EPO, IL-11, darbepoetin-α) has led to their wide clinical application in oncology clinical practice for prophylactic and therapeutic purpose.
  - **Prophylactic use:** It refers to administration of hematopoietic growth factor to prevent febrile neutropenia in patients receiving chemotherapy or subsequent cycles of chemotherapy.
  - **Therapeutic use:** It refers to administration of a hematopoietic growth factor at the time, when neutropenia or neutropenic fever is documented in a patient who had not been receiving colony-stimulating factors (CSF) previously.

Leukemia Disease Burden Impact

Leukemia disease burden refers to the number of leukemic cells, the size of the tumor or amount of cancer in the body. Tumor mutation burden (TMB) is defined as the total number of nonsynchronous mutations per coding are of tumor genome.

- TMB has been determined using whole exome sequencing, but due to the high costs, targeted panel sequencing is currently being explored to measure tumor mutation burden.
- Leukemic disease burden of 10^{12} leukemic clones is believed to be sufficient for recognizable signs and symptoms.
- Lethal levels of leukemic disease burden occur, when there is presence of 10^{13-14} leukemic clones. The classic triad of anemia, recurrent infections and bleeding manifestations in cases of acute leukemias occur as a result of replacement of normal hematopoietic stem cells by leukemic cells.

ACUTE LYMPHOBLASTIC LEUKEMIA

ACUTE LYMPHOBLASTIC LEUKEMIA: OVERVIEW

Acute lymphoblastic leukemia (ALL) represents the most common pediatric malignancy, accounting for approximately 25% of childhood cancer. ALL is derived from lymphoid precursor B cell (80%), lymphoid precursor T cell (20%) and mature B cells in the bone marrow.

- According to revised 2024 WHO classification of acute myelogenous leukemia (AML) or acute lymphoblastic leukemias diagnostic criteria is the presence of **≥20% blast cells** as the cut off percentage in bone marrow and peripheral blood. It represents a change from original guidelines as per French-American-British (FAB) classification of leukemias, where **≥30%** blast cells were considered as the cut off percentage diagnostic criteria in bone marrow and peripheral blood.
- Clonal lymphoblasts continuously multiply in the bone marrow and cause damage to normal progenitor cells of different cell-lineages (i.e. erythroid, myeloid and megakaryocytes) resulting in anemia, neutropenia (recurrent infection) and thrombocytopenia (bleeding tendencies).
- Acute lymphoblastic leukemia involves bone marrow (100%), peripheral blood and distant organs such as anterior mediastinal mass (10%), central nervous system (5%), testes (5%) and other sites such as eye, skin, pericardium, pleura, kidney, breast, ovary, penis (priapism, and gastrointestinal tract intussusception) in <5% of cases. Disease is most common during childhood with a peak at 4–5 years of age (85%) and adulthood (50%) with peak in old age.
- Acute lymphoblastic leukemia (ALL) patients are more responsive to therapy associated with long-term disease-free survival.
  - Rapid progression of the disease can be fatal in weeks to months, if left untreated.
  - Patients present with hepatosplenomegaly, fever, fatigue, cervical lymphadenopathy, bone pain, bleeding manifestations, anorexia and abdominal pain.
- Revised 2024 WHO classification of acute lymphoblastic leukemia/lymphoma is given in Table 9.23. French-American-British classification of acute lymphoblastic leukemias and their features is given in Table 9.24.

Table 9.23 Revised 2024 WHO classification of acute lymphoblastic leukemia/lymphoma

B Cell Derived Acute Lymphoblastic Leukemia/Lymphoma
B-lymphoblastic leukemia/lymphoma, not otherwise specified (NOS)
B-lymphoblastic leukemia/lymphoma with t(9;22) (q34.1;q11.2); BCR-ABL1
B-lymphoblastic leukemia/lymphoma with t(v;11q23.3); KMT2A-rearrangement
B-lymphoblastic leukemia/lymphoma with t(12;21) (p13.2;q22.1); ETV6-RUNX1
B-lymphoblastic leukemia/lymphoma with hyperdiploidy
B-lymphoblastic leukemia/lymphoma with hypodiploidy
B-lymphoblastic leukemia/lymphoma with t(5;14) (q31.1; q32.1);IGH/IL3
B-lymphoblastic leukemia/lymphoma with t(1;19) (q23;p13.3); TCF3-PBX1
Provisional entity: B cell acute lymphoblastic leukemia/lymphoma with BCR-ABL1 like
Provisional entity: B cell acute lymphoblastic leukemia/lymphoma with iAMP21 (intrachromosomal amplification of chromosome 21)
T Cell Derived Acute Lymphoblastic Leukemia/Lymphoma
T cell lymphoblastic leukemia/lymphoma
Early T cell precursor lymphoblastic leukemia/lymphoma
NK Cell Derived Acute Lymphoblastic Leukemia/Lymphoma
Adapted from revised 2024 WHO classification of acute lymphoblastic leukemia

Table 9.24 French-American-British (FAB) classification of acute lymphoblastic leukemias and their features

Leukemia Subtype	Description
ALL-L1	▪ ALL-L1 accounts for >80% of leukemias and affects children ▪ In ALL-L1, lymphoblasts are uniform, homogenous with scant cytoplasm with regular nuclei and inconspicuous nucleoli ▪ ALL-L1 is associated with good prognosis
ALL-L2	▪ ALL-L2 accounts for 10–50% and affects adults ▪ In ALL-L2, lymphoblasts are large, heterogenous with round to oval nuclei showing indentation or clefts with prominent nucleoli ▪ ALL-L1 is associated with poor prognosis
ALL-L3	▪ ALL-L3 accounts for 3–4% of leukemias and affects adults ▪ In ALL-L3, lymphoblasts are large homogenous with round to oval nuclei, finely stippled chromatin, prominent nucleoli and abundant basophilic vacuolated cytoplasm ▪ ALL-L3 is associated with poor prognosis

CD: Cluster of differentiation.

PREDISPOSING FACTORS

Acute lymphoblastic leukemia (ALL) accounts for 80% of all childhood leukemia. ALL is most common in children 2–8 years of age (85%), and another peak at 50 years of age.

- Only a small proportion of patients with childhood ALL have an underlying genetic abnormality in less than 5% of cases.
- Acquired chromosomal abnormalities such as aneuploidy (hyperploidy) and/or translocations are demonstrated in >90% of ALL cases. Such chromosomal abnormalities may be prenatal in origin in some cases.
- The genes involved in leukemogenesis are frequently transcription factors expressed in the hematopoietic tissues in bone marrow.
- Genetic disorders and environmental factors associated with acute lymphoblastic leukemia (ALL) are given in Table 9.25.

Molecular Genetic Alterations

Recent technical advances in next generation sequencing have shed light on genetic abnormalities in the hematopoietic stem cells and progenitor cells as the prerequisite to molecular genesis of acute lymphoblastic leukemia. Hyperdiploid ALL also occurs before birth, but these cases require postnatal events for full malignant transformation. Polymorphism at six genomic loci is associated with increased risk of ALL in genetic alterations: ARID5B, IKZF1, CEBPE, GATA3, CDKN2A, CDKN2B, BMI-PIP4K2A, TP53. The IKZF1 deletion is associated with poor prognosis in ALL patients. Loss of CDKN2A or CDKN2B increases the risk of ALL.

Congenital Disorders

Certain disorders predispose to acute lymphoblastic leukemia (ALL), most notably trisomy 21 (Down syndrome), in which the relative risk is increased 10- to 30-fold. Other predisposing factors for development of ALL include Fanconi anemia, Bloom syndrome and ataxia-telangiectasia.

Congenital Immunodeficiencies

There is no definite evidence of an association between leukemia and congenital immunodeficiencies such as X-linked agammaglobulinemia (Bruton agammaglobulinemia) and severe combined immunodeficiency (SCID) syndrome.

Germline TP53 Gene Mutations

Germline TP53 gene mutations, a cause of cancer-predisposing Li-Fraumeni syndrome, are present in 40% of ALL patients with low hypodiploid (32–39 chromosomes).

Fraternal (Dizygotic) Twins and Siblings

There is increased risk for development of ALL during the first decade of life in affected fraternal twins and siblings.

- Fraternal (dizygotic) twins produced derived from two separate fertilized eggs, which develop two separate amniotic sacs and placentae.
- In the case of identical twins, when leukemia affects a twin, there is increased risk for development of leukemia in another twin owing to ALL transfer *in utero* via the shared placental circulation before the age of one year.
  - In identical twins with t(4;11)/MIL-AF4, the concordance rate is nearly 100% within a week to few months period.
  - In identical twins with ETV6-RUNX1 fusion gene or T cell phenotype is lower in postnatal latency period. These cases require additional genetic alterations for development of acute lymphoblastic leukemia.

Environmental Factors

Acute lymphoblastic leukemia may be induced by environmental factors such as prolonged exposure to ionization radiation and chemical agents (benzene) in some cases.

Table 9.25 Genetic disorders and environmental factors associated with acute lymphoblastic leukemia (ALL)

Categories	Disorders
Genetic disorders	Down syndrome, Fanconi anemia, Bloom syndrome, ataxia-telangiectasia
Germline mutations in TP53 gene	Li-Fraumeni syndrome: ALL (40% cases) with low hypodiploid (32–39 chromosomes)
Fraternal twins and siblings	Increased risk of ALL during first decade
Identical twins	Increased risk of ALL in identical twins t(4;11)/MIL-AF4 and identical twins with ETV6-RUNX1 fusion
Loss of CDKN2A or CDKN2B	Increased risk of ALL
Environmental factors	Ionization radiation and chemical agent (benzene)

- Patients treated for other cancers with ionizing radiation and chemotherapy often develop leukemias. But clear etiological factors for ALL cannot be demonstrated in a majority of cases.
- *In utero* exposed to diagnostic X-rays increases the risk for developing ALL. Human T cell leukemia virus 1 (HTLV-1) endemic in Japan, which is the etiological agent for developing an aggressive adult T cell leukemia/lymphoma.

MOLECULAR PATHOGENESIS

Recent studies revealed genomic landscape of evolution of childhood acute lymphoblastic leukemia (ALL) prior to diagnosis, except for mixed-lineage leukemia (MLL)-rearranged involving fusions of 11q23 with one of more than 80% different genes in infant ALL, in which a single genetic mutation is likely to be initiating event in pathogenesis of ALL.

- All other subtypes of childhood ALL occur due to series of genetic events within cell that have acquired an initiating gene mutation during intrauterine life.
- A recurring leukemia associated chromosomal abnormality is demonstrated by karyotyping, fluorescence *in situ* hybridization (FISH) or molecular techniques in about 80% cases in children.
- High hyperdiploidy and ETV6-RUNX1-carrying cells are demonstrated at low levels in 1% of cord blood samples from normal neonates. Out of these, only 1% of neonates develop ALL after several years, thus these rearrangements are not sufficient to cause ALL.
- The initiating mutations drive transcriptional and epigenetic dysregulation and aberrant self-renewal and disrupt lymphoid development resulting in maturation arrest. Additional genetic mutations disrupt multiple molecular pathways such as cell cycle regulation and apoptosis.
- Normally hematopoietic stem cell (HSC) transforming to pre-B cell/pro-B cell and then mature B cell are regulated by various genes (e.g. ARID5B, IKZF1, CEBPE, GATA3, CDKN2A, CDKN2B, BMI-PIP4K2A and TP53). Predisposition of hematopoietic stem cell to pre-/pro-B cell occurs due to mutations of genes.
  - Initial gene mutations and ETV6/RUNX1 transform hematopoietic stem cell to pre-/pro-B cell pre-leukemic clone.
  - Further mutations in B cell transcriptional factor PAX2 gene leads to developmental arrest of B cell development.
- Now the pre-B cell/pro-B cell leukemic clone with the cooperation of mutations in CRLF2, JAK2, IZKF1, PDGFRB result in increased proliferation of clonal cells and their decreased apoptosis. Heterologous clones are demonstrated at ALL diagnosis. Basic aim of treatment of ALL to eradicate clones. Failure to treatment results in relapse.
- Schematic representation of molecular pathogenesis of acute lymphoblastic leukemia (ALL) is shown in Fig. 9.19. Schematic representation of immunophenotype of B cell and T cell lineage acute lymphoblastic leukemias is shown in Fig. 9.20.

CLINICAL FEATURES

Presenting signs and symptoms in acute lymphoblastic leukemia (ALL) are always caused by lymphoblast infiltration in the bone marrow with resultant involvement of peripheral blood and distant organs such as lymph nodes (75%), spleen, liver, anterior mediastinal mass (10%), central nervous system (5%), testes (5%), eye, skin, pericardium, pleura, kidney, breast, ovary, penis, priapism, and gastrointestinal tract intussusception in <5% of cases. The lymph nodes that are immobile and painless are commonly malignant. Painful lymphadenopathy is usually inflammatory in nature.

- Clonal lymphoblasts continuously multiply in the bone marrow and cause damage to normal progenitor cells of different cell lineages in bone marrow (erythroid, myeloid and megakaryocytes) resulting in anemia, neutropenia (recurrent infections) and thrombocytopenia (bleeding tendencies).
- Patients present with cervical lymphadenopathy (75%), hepatosplenomegaly (70%), and anterior mediastinal mass in the decreasing frequency. On clinical examination, enlarged lymph nodes are discrete and firm in consistency.
- Generalized lymphadenopathy is associated with high white blood cell count and fatal outcome. T cell ALL usually presents with bulky lymphadenopathy, mediastinal mass, pleural effusion, and/or hyperleukocytosis. Patient presenting with mediastinal mass has poor prognosis.
- Painless enlargement of scrotum may be due to a testicular infiltration by leukemic cells or hydrocele resulting from lymphatic obstruction in 1–2% of cases.
- Ocular involvement is more common during ALL relapse involving the retina, ocular nerve, orbit, cornea, or anterior chamber with hypopyon.
- Central nervous system involvement is usually leptomeningeal rather than parenchymal and may present with cranial nerve palsies in 5% of cases.
- Involvement of organs/tissues in acute lymphoblastic leukemia (ALL) and common presenting signs and symptoms are given in Table 9.26.

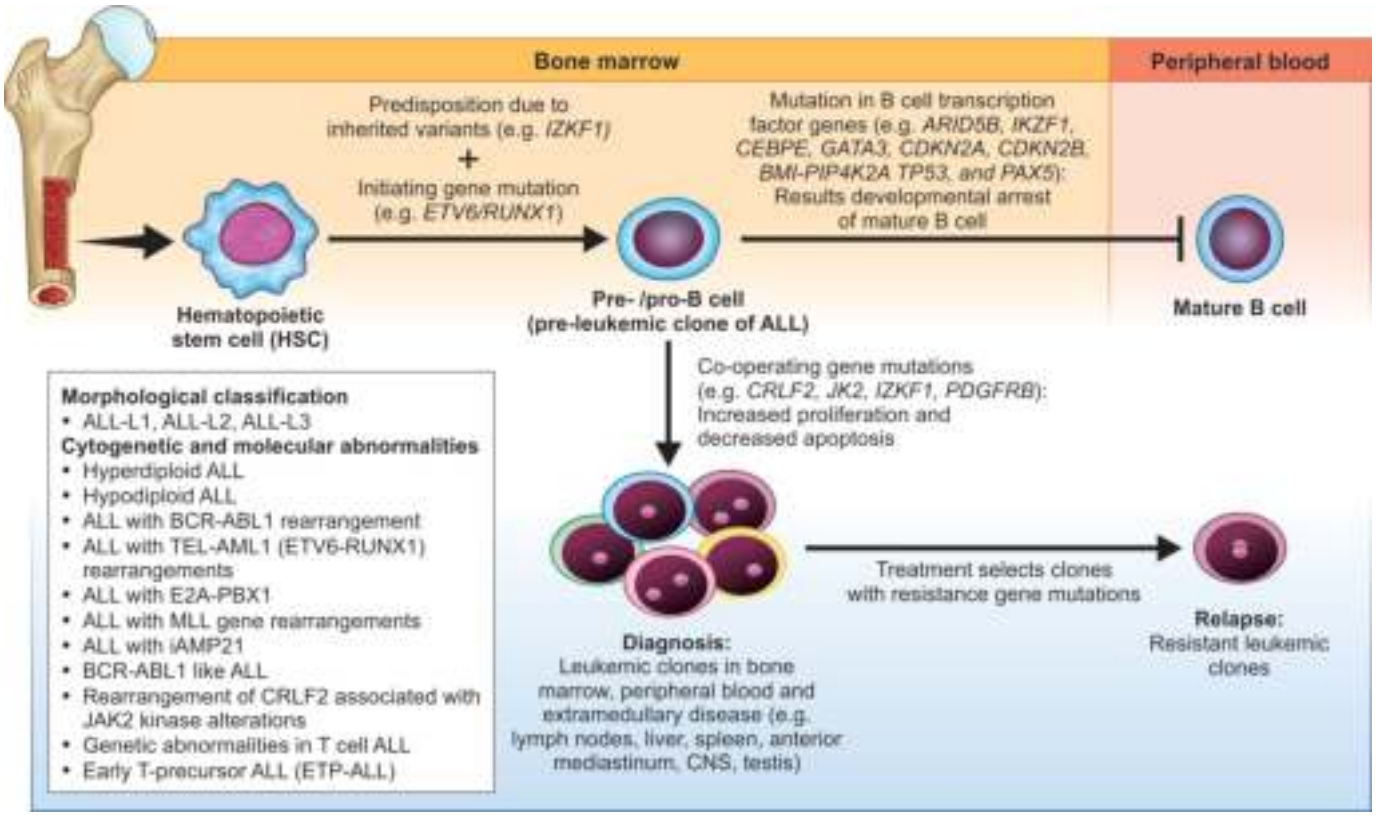


Fig. 9.19: Schematic representation of molecular pathogenesis of acute lymphoblastic leukemia (ALL). It shows morphological classification and cytogenetic and molecular abnormalities; and clinical course. Molecular pathogenesis of acute lymphoblastic leukemia involves a number of abnormal gene expression including TEL-AML1, BCR-ABL1, RAS and PI3K leading to dysregulated cell cycle. Risk factors such as parvovirus B19 infection, environmental toxins, high birth weight can induce abnormal DNA methylation and DNA damage leading to development B-ALL.

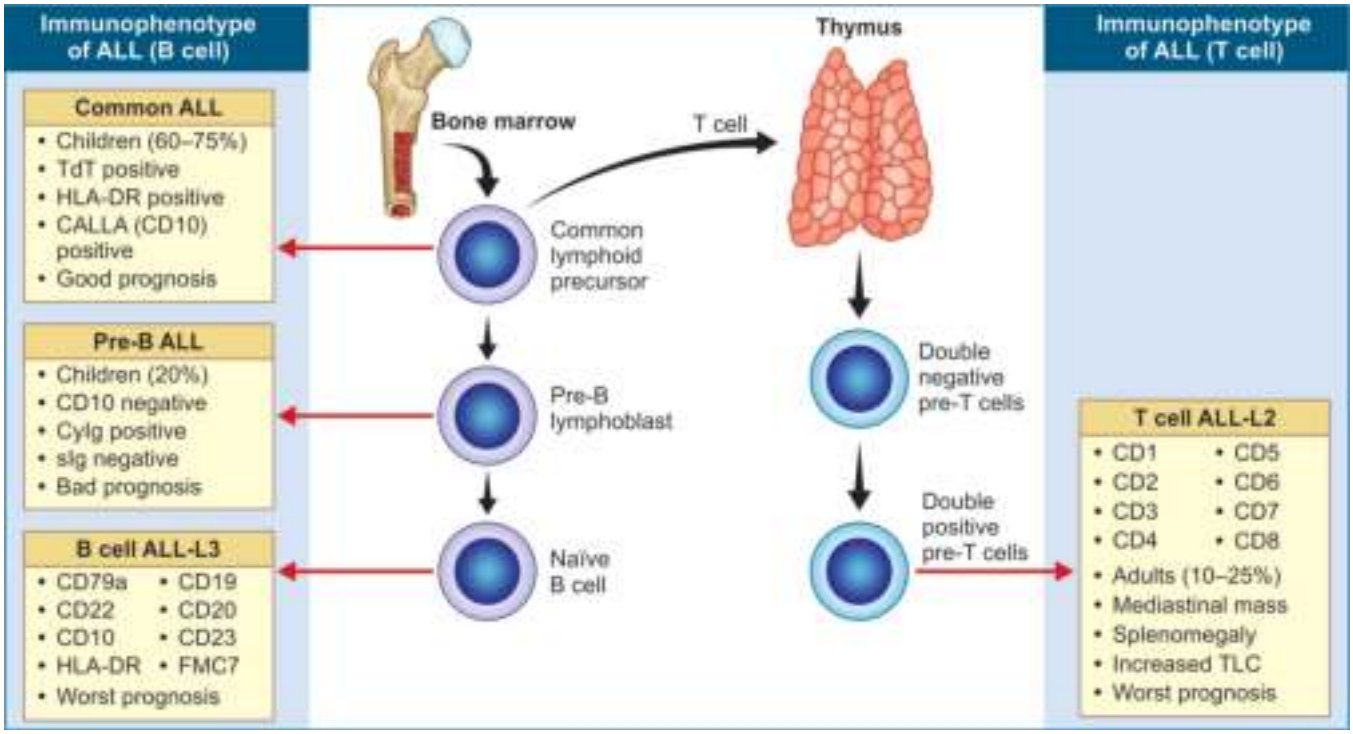


Fig. 9.20: Schematic representation of immunophenotype of B cell and T cell lineage acute lymphoblastic leukemias.

Table 9.26 Involvement of organs/tissues in acute lymphoblastic leukemia (ALL) and common presenting signs and symptoms

Organs/Tissues Involvement
▪ Bone marrow (100%)
▪ Cervical lymphadenopathy (75%)
▪ Mediastinal mass in anterior compartment (10%)
▪ Central nervous system (5%)
▪ Painless involvement of testes (1–2%)
▪ Involvement of organs in <5% of cases (eye, skin, pericardium, pleura, kidney, breast, ovary, priapism, intussusception)
Common Presenting Signs and Symptoms
▪ Hepatosplenomegaly (70%)
▪ Fever (50%)
▪ Fatigue (50%)
▪ Generalized lymphadenopathy (50%)
▪ Bleeding manifestations (40%)
▪ Bone or joint pains (40%)
▪ Anorexia (20%)
▪ Abdominal pain (10%)

- Petechial hemorrhages on the abdomen of a child in acute lymphoblastic leukemia (ALL) are shown in Fig. 9.21. Mediastinal lymph nodes in acute lymphoblastic leukemia (ALL) on CT scan are shown in Fig. 9.22. Abdominal lymph nodes in acute lymphoblastic leukemia (ALL) patient on CT scan are shown in Fig. 9.23.

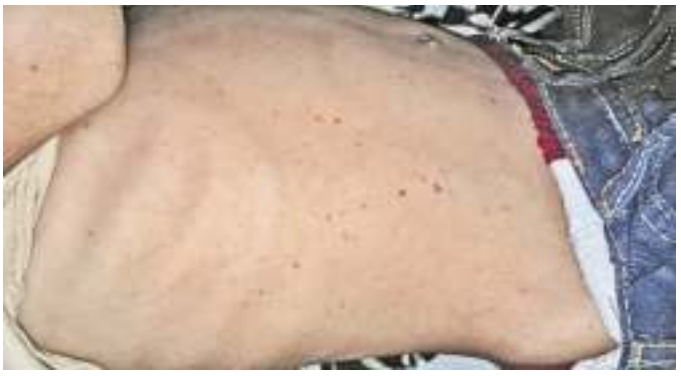


Fig. 9.21: Petechial hemorrhages on the abdomen of a child in acute lymphoblastic leukemia (ALL).

LIFE-THREATENING COMPLICATIONS OF ALL AND MANAGEMENT

There are several life-threatening emergent clinical complications of ALL, that require emergent intervention.

- Hyperleukocytosis is treated by cytoreduction, leukapheresis and low-dose radiotherapy.
- Broad spectrum antibiotics should be administered in neutropenia with fever manifestations.
- Thrombocytopenia is treated by platelet transfusion.
- Disseminated intravascular coagulation (DIC) is treated by fresh frozen plasma or cryoprecipitate. Tumor lysis syndrome is treated by supportive measures.
- Airway obstruction, superior vena cava syndrome, central nervous system manifestations and spinal cord compression are treated by corticosteroids and/or radiation.

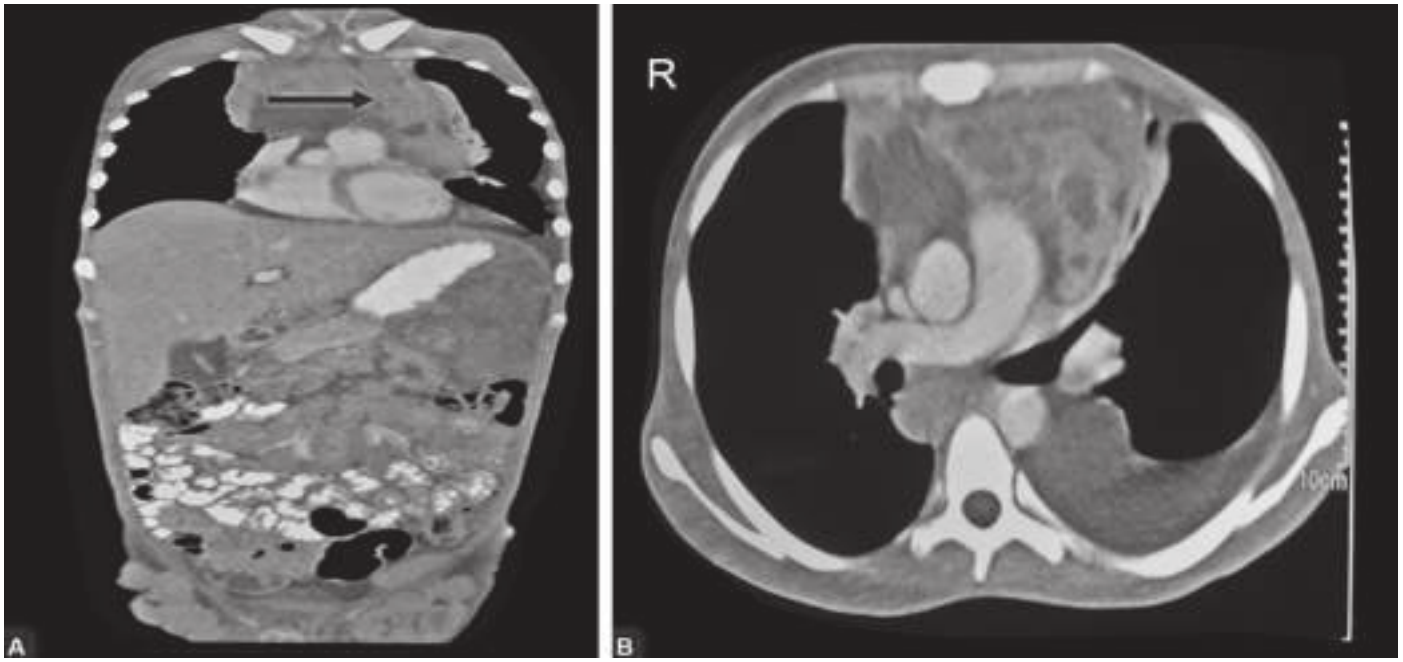


Fig. 9.22: Mediastinal lymph nodes in acute lymphoblastic leukemia (ALL) on CT scan. Coronal image (A) and transverse image (B) on CT scan shows mediastinal mass in a case of T-ALL.

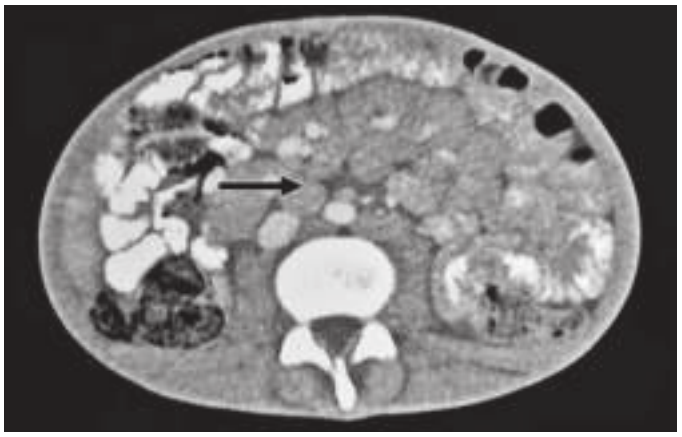


Fig. 9.23: Abdominal lymph nodes in acute lymphoblastic leukemia (ALL) patient on CT scan. Transverse image on CT scan is showing lymph nodes (shown by arrow) in the abdomen.

- Ocular involvement is treated by radiation. Pericardial tamponade is treated by pericardiocentesis and corticosteroids.
- Emergency clinical presentations and management of acute lymphoblastic leukemia (ALL) are given in Table 9.27.

LABORATORY DIAGNOSIS

Diagnosis of acute lymphoblastic leukemia is confirmed by the demonstration of lymphoblasts in the blood and/or bone marrow. Routine hematological analysis, immunophenotype, flow cytometry, cytogenetics, and molecular studies are performed to define ALL subtypes and further identify the prognostic factors.

- Approximately 80% of ALL cases are derived from pro-B cell (pre-B) with immunophenotype (CD19+, CD34+, CD22+, TdT+, cytoplasmic CD79a+, CD10+). precursor B cell (pre-B) with immunophenotype (CD22+, CD34+, CD19+, TdT+, cytoplasmic CD79a+, CD10+, cytoplasmic mu+), and 10–20% is of T cell origin with immunophenotype (TdT+, CD10+, CD1a+, CD2+, CD3+, CD5+, CD7+).
- Certain cytogenetic abnormalities are not apparent on routine karyotyping, thus molecular testing should be performed to demonstrate t(12;21) in 25% of ALL cases in children.
- Lumbar puncture is performed to evaluate the possibility of meningeal leukemia. Central nervous system involvement by ALL leads to cranial nerve palsies.
- According to revised 2024 WHO classification of acute leukemias (lymphoblastic/myelogenous leukemia), diagnostic criteria is the presence of ≥20% blast cells as the cut off percentage in bone marrow and peripheral blood, which represents a change from original guidelines where ≥30% blast cells were considered as the cut off percentage of diagnostic criteria in bone marrow and peripheral blood as per French-American-British (FAB) classification of leukemias.
- Morphology subtyping of various forms of ALL have been used according to French-American-British (FAB) classification: ALL-L1 (small uniform lymphoblasts), ALL-L2 (variable size lymphoblasts) and ALL-L3 (large lymphoblasts with vacuoles—bubble-like appearance as seen in Burkitt lymphoma).
- The revised 2024 WHO international panel recommends that FAB classification be abandoned, since the morphologic classification has no clinical or prognostic relevance. Revised 2024 WHO international panel instead advocates the use of the immunophenotypic classification. Each ALL subtype is then further classified by determining the surface markers of the leukemic lymphoblasts, called immunotyping. There are two main immunologic types of ALL:

Table 9.27 Emergency clinical presentations and management of acute lymphoblastic leukemia (ALL)

Emergent Clinical Presentation	Intervention/Management
Hyperleukocytosis (high leukocyte count)	Cytoreduction by leukapheresis; low dose of chemotherapy
Airway obstruction	Oxygen, corticosteroids administration, and/or radiation therapy
Superior vena cava syndrome	Corticosteroids and/or radiation therapy
Pericardial tamponade	Pericardiocentesis, corticosteroids administration
Neutropenia with fever and superadded infections	Broad spectrum antibiotics
Thrombocytopenia	Platelet transfusion
Disseminated intravascular coagulation (DIC)	Fresh frozen plasma, cryoprecipitate administration
Tumor lysis syndrome	Intravenous hydration, allopurinol and supportive measures, clinically indicated hemodialysis
Central nervous system manifestations such as increased intracranial pressure and nerve palsies	Corticosteroids and/or radiation therapy
Spinal cord compression	Corticosteroids and/or radiation therapy
Ocular involvement	Radiation

pre-B cell lineage ALL and pre-T cell lineage ALL. The mature B cell ALL-L3 is now classified as Burkitt leukemia/lymphoma. Subtyping helps to determine the prognosis and most appropriate therapy in ALL patients.

Laboratory Diagnosis of Acute Lymphoblastic Leukemias

- ALL-L1 usually affects children and young adults with peak incidence during 4–5 years of age and constitutes 75–80% of ALL cases.
- ALL-L2 most often affects children and young adults with peak incidence during 4–5 years of age constitutes 20–30% of ALL cases.
- ALL-L3 is now classified as Burkitt leukemia/lymphoma type. It constitutes 1–2% of ALL cases.

Biochemical Investigations

- Elevated uric acid and phosphorus levels may be demonstrated due to rapid tumor proliferation in patients with high tumor burden in acute lymphoblastic leukemia (ALL).
- Raised liver enzymes are detected due to infiltration of liver by leukemic cells in 10–20% of patients.
- A minority of patients develop mild cholestatic jaundice due to lymph nodes compression at the portal hepatic or portal tract infiltration by leukemic cells.

Peripheral Blood Smear Examination

White blood cells

- In acute lymphoblastic leukemia (ALL), initial total leukocyte count is markedly elevated ranging between 20×10^9/L and 200×10^9/L with ≥20% lymphoblasts and mean 40–95%. Higher the white blood cell count, there is decreased chance of remission of disease.
- Low white blood cell count is associated with good prognosis. In subleukemic/ aleukemic leukemia, peripheral blood smear demonstrates a few lymphoblasts.
- Reactive hypereosinophilia with pulmonary infiltration and cardiomyopathy is a feature of B cell lineage ALL subtype.
- ALL with t(5;14)(q3;q32), which results in activation of IL-3 gene on chromosome 5 by the enhancer of the Ig gene on chromosome 14.
  - **ALL-L1 (B cell or T cell):** Lymphoblasts are small with high nucleocytoplasmic ratio and containing large nucleus with homogenous chromatin, regular nucleoli, and scanty cytoplasm. Lymphoblasts are positivity for PAS stain. Prognosis is good in these patients. Acute lymphoblastic leukemia L1 (ALL-L1) in Giemsa-stained peripheral blood smear is shown in Figs 9.24 and 9.25.
  - **ALL-L2 (mostly T cell):** Lymphoblasts are of variable size with high nucleocytoplasmic ratio and containing irregular indented nuclei with slightly opened chromatin, prominent nucleoli and mild to moderate cytoplasm. Lymphoblasts are negative for PAS stain. ALL-L2 is associated with poor prognosis. Acute lymphoblastic leukemia L2 (ALL-L2) in Giemsa-stained peripheral blood smear is shown in Fig. 9.26.
  - **ALL-L3 (mature B cell):** Lymphoblasts are of large size containing large nucleus with homogenous chromatin with prominent nuclei and vacuolated basophilic cytoplasm giving bubble-like appearance. ALL-L3 lymphoblasts are like those seen in Burkitt's lymphoma. ALL-L3 lymphoblasts are positive for Sudan black B stain. Acute lymphoblastic leukemia L3 (ALL-L3) in Giemsa-stained peripheral blood smear is shown in Fig. 9.27.

Red blood cells

Peripheral blood smear examination shows normocytic normochromic picture.

Platelets

Platelet count is lower in acute lymphoblastic leukemia (<30,000/μl) than normal range (1,50,000–4,00.000/μl).

Bone Marrow Smear Examination

- Bone marrow is highly cellular with nearly absence of adipocytes. Lymphoblasts range between **≥20%,** which replace or suppress normal hematopoiesis in acute lymphoblastic leukemia in 100% of cases.
- In aleukemic leukemia, lymphocytes are absent in peripheral blood, but bone marrow shows ≥20% lymphoblasts.
- In subleukemic leukemia, a few lymphoblasts are present in peripheral blood and bone marrow shows ≥20% lymphoblasts. Erythroid and megakaryocytic series are suppressed.
- Morphologic features in acute lymphoblastic leukemia are described below:
  - **ALL-L1 (B cell or T cell):** Lymphoblasts are small with high nucleocytoplasmic ratio and containing large nucleus with homogenous chromatin, regular nucleoli, and scanty cytoplasm. Lymphoblasts are positive for PAS stain. Prognosis is good in these patients. Acute lymphoblastic leukemia L1 (ALL-L1) in Giemsa-stained in bone marrow aspirate smear is shown in Fig. 9.28.
  - **ALL-L2 (mostly T cell):** Lymphoblasts are of variable size with high nucleocytoplasmic ratio and containing irregular indented nuclei with slightly opened chromatin, prominent nucleoli and mild to moderate cytoplasm. Lymphoblasts are negative for PAS stain. ALL-L2 is associated with poor prognosis.
  - **ALL-L3 (mature B cell):** Lymphoblasts are of large size containing large nucleus with homogenous chromatin with prominent nuclei and vacuolated basophilic cytoplasm giving bubble-like appearance. These lymphoblasts are like those seen in Burkitt's lymphoma. Lymphoblasts are positive for Sudan black B cytochemical stain. Acute lymphoblastic leukemia L3 (ALL-L3) in Giemsa-stained bone marrow aspirate smear is shown in Fig. 9.29.

Cytochemistry

- Lymphoblasts in ALL-L1 and ALL-L2 subtypes are positivity for PAS stain.
- Whereas lymphoblasts in ALL-L3 subtype are positivity for Sudan black or Oil red O stains.

Bone Marrow Trephine Biopsy Examination

Acute lymphoblastic leukemia L1 (ALL-L1) in hematoxylin and eosin-stained section is shown in Fig. 9.30. Acute lymphoblastic leukemia L3 (ALL-L3) in hematoxylin and eosin-stained section is shown in Fig. 9.31. Bone marrow fibrosis in childhood acute lymphoblastic leukemia in hematoxylin and eosin-stained bone marrow trephine biopsy section is shown in Fig. 9.32. Bone marrow fibrosis in adult acute lymphoblastic leukemia patient in silver-stained bone marrow trephine biopsy is shown in Fig. 9.33.

Immunophenotyping

- B cell lineage ALL markers are CD19, CD20, CD22, CD24, CD79a, PAX5, BOB1, OCT2, PU1, HLA-DR and terminal deoxynucleotidyl transferase (TdT).
- T cell lineage ALL markers are cCD1, CD2, cCD3, CD4, CD5, CD7 and TdT. CD10 positivity is demonstrated in all subgroups except for some B cell lineage ALL.
- CD10 is expressed in childhood B cell ALL (80%) and T cell ALL (20%). ALL with CD10 positivity is amenable to chemotherapy. Thus, CD34 positivity in ALL is associated with better prognosis.
- Flow cytometry or immunohistochemistry can be used to demonstrate terminal deoxynucleotidyl transferase (TdT) in lymphoblasts in ALL.
- TdT was thought to be a lymphoid specific marker. But TdT is also demonstrated on more immature hematopoietic cells, sometimes including those of myeloid lineage.
- Therefore, TdT cannot be the sole determinant of lymphoid lineage.

Markers	Expression
B cell markers in ALL	
CD10	Positive (80%)
CD19	Positive
CD20	Positive
CD22	Positive
CD24	Positive
CD79a	Positive
PAX5	Positive
BOB1	Positive
OCT2	Positive
PU1	Positive
HLA-DR	Positive
TdT	Positive
T cell markers in ALL	
cCD1	Positive
CD2	Positive
cCD3	Positive
CD4	Positive
CD5	Positive
CD7	Positive
TdT	Positive

Acute lymphoblastic leukemia with CD10 positivity is amenable to chemotherapy. Thus, CD10 positivity is a favorable prognostic marker.

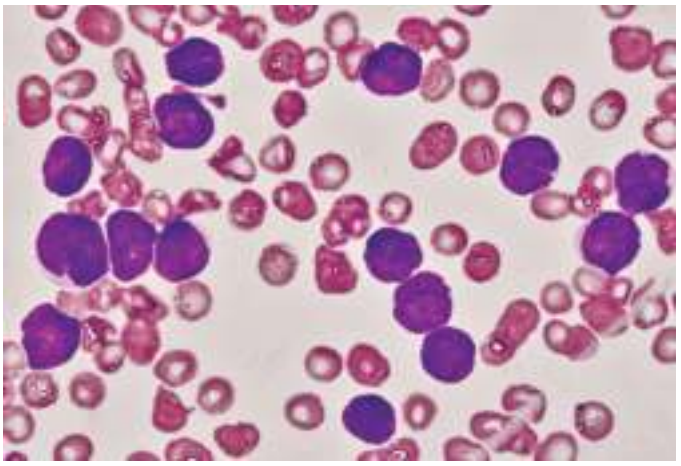


Fig. 9.24: Acute lymphoblastic leukemia L1 (ALL-L1) in Giemsa-stained peripheral blood smear. Peripheral blood smear examination shows small, uniform lymphoblasts with high nucleocytoplasmic ratio, coarse chromatin, inconspicuous nucleoli and scant cytoplasm (1000X).

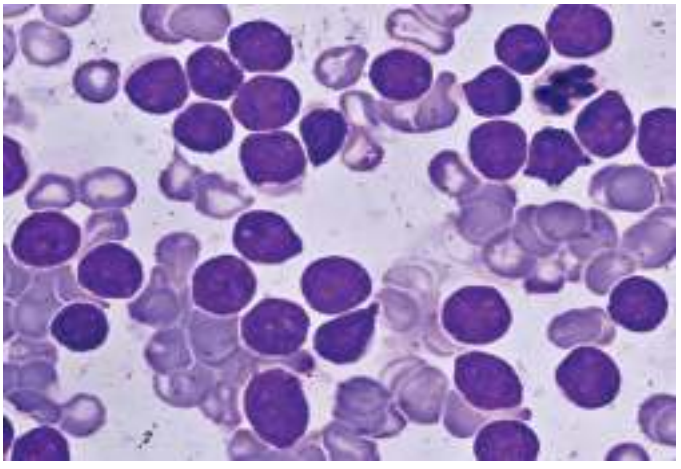


Fig. 9.25: Acute lymphoblastic leukemia L1 (ALL-L1) in Giemsa-stained peripheral blood smear. Peripheral blood smear is showing uniform population of lymphoblasts with high nucleocytoplasmic ratio, coarse chromatin, inconspicuous nucleoli and all of them showing nuclear notching. There is a minimal amount of cytoplasm in lymphoblasts (1000X).

Pathology Pearls: Terminal Deoxynucleotidyl Transferase

- Flow cytometry or immunohistochemistry can be used to demonstrate terminal deoxynucleotidyl transferase (TdT) in cells. TdT was thought to be a lymphoid specific marker.
- However, TdT is also demonstrated in more immature hematopoietic stem cells, sometimes including those of myeloid lineage.
- Therefore, TdT cannot be the sole determinant of lymphoid lineage.

Lymphoblast Morphology in ALL Subtypes

According to French-American-British (FAB) classification, three morphological subtypes of acute lymphoblastic leukemia have been defined: ALL-L1,

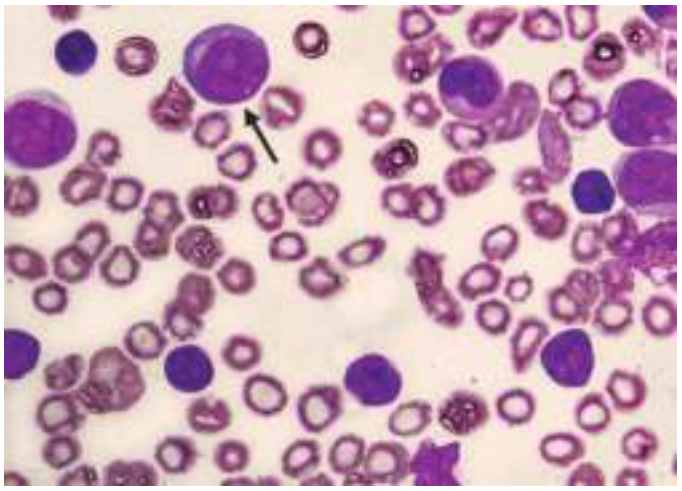


Fig. 9.26: Acute lymphoblastic leukemia L2 (ALL-L2) in Giemsa-stained peripheral blood smear. Peripheral blood smear examination shows variable sized lymphoblasts with high nucleocytoplasmic ratio, slightly opened up chromatin, prominent nucleoli and mild to moderate cytoplasm (arrow) (1000X).

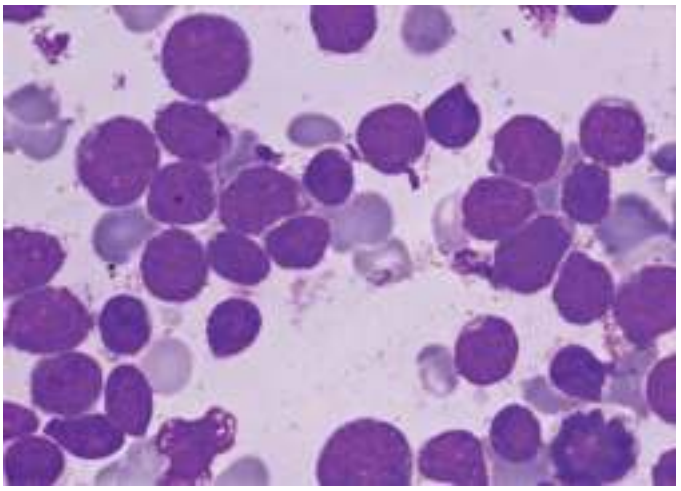


Fig. 9.28: Acute lymphoblastic leukemia L1 (ALL-L1) in Giemsa-stained in bone marrow aspirate smear. Bone marrow aspirate smear is showing ALL-L1 morphology lymphoblasts, with high N:C ratio, coarse chromatin, inconspicuous nucleoli, scant agranular cytoplasm and mild variation in the cell size, completely replacing the hematopoietic elements (1000X).

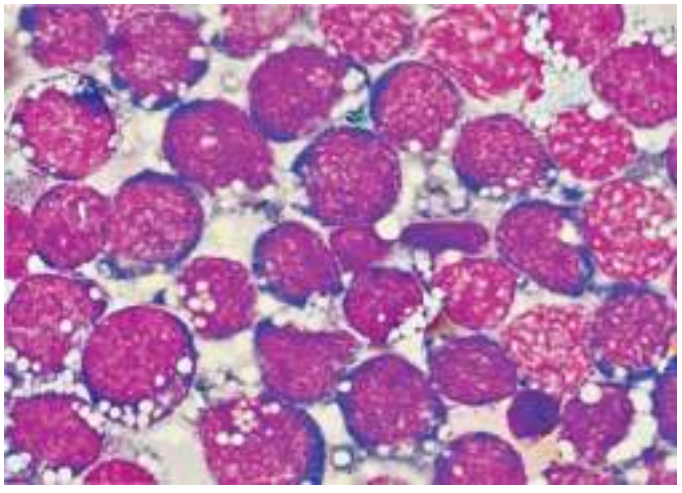


Fig. 9.27: Acute lymphoblastic leukemia L3 (ALL-L3) in Giemsa-stained peripheral blood smear. Peripheral blood smear examination shows large size lymphoblasts with large nucleus with homogenous chromatin with prominent nuclei and vacuolated basophilic cytoplasm giving bubble-like appearance. These lymphoblasts are similar to those seen in Burkitt's lymphoma (1000X).

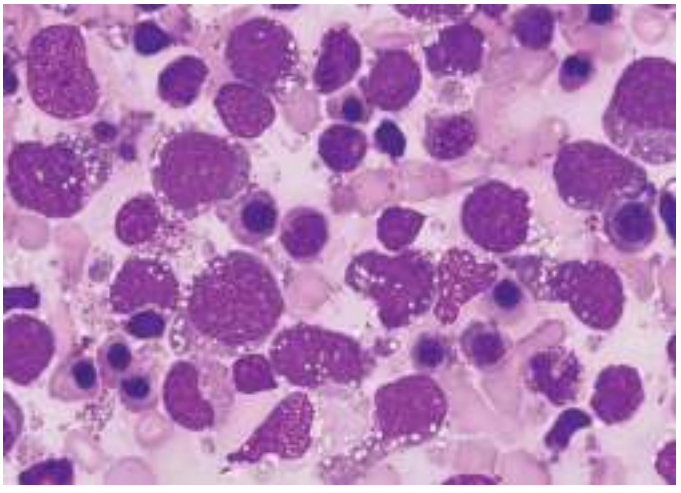


Fig. 9.29: Acute lymphoblastic leukemia L3 (ALL-L3) in Giemsa-stained bone marrow aspirate smear. Bone marrow examination shows large size lymphoblasts with basophilic vacuolated cytoplasm. These lymphoblasts are similar to those seen in Burkitt's lymphoma (1000X).

ALL-L2 and ALL-L3. Distinction between ALL-L1 and ALL-L2 morphology has no clinical significance.

- However, detection of ALL-L3 is of clinical and prognostic relevance. ALL-L3 subtype has been observed in ≤5% of adult patients, which is indicative of mature B cell neoplasm usually termed Burkitt's leukemia with different treatment options. ALL-L3 should be confirmed by demonstration of antigenic cell surface markers.
- Further French-American-British (FAB) classification and revised 2024 WHO classification of ALL/lymphomas categorized into number of subgroups based on morphological features, cytogenetic abnormalities, antigen cell surface markers and rearrangement of the immunoglobulin heavy chain or T cell receptor genes.
- Morphological subtypes of acute lymphoblastic leukemia according to French-American-British (FAB) classification is given in Table 9.28. Revised 2024 WHO classification of ALL/lymphomas is given in Table 9.29.

Immunophenotyping in ALL Subtypes

A series of monoclonal antibodies are employed to demonstrate antigens expressed on the surface of

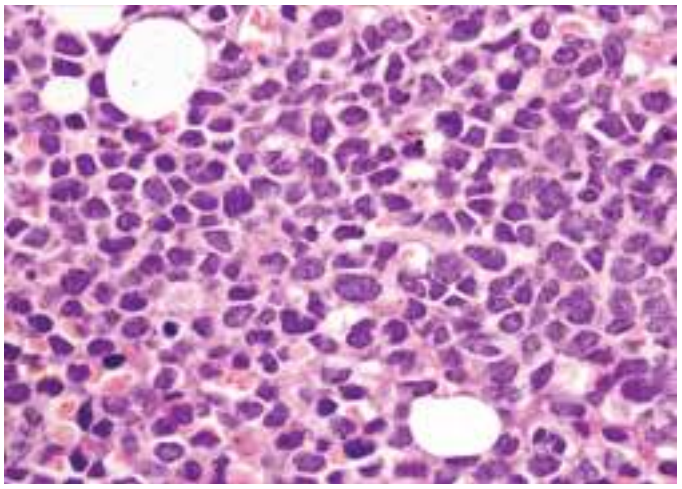


Fig. 9.30: Acute lymphoblastic leukemia L1 (ALL-L1) in hematoxylin and eosin-stained section. Bone marrow trephine biopsy section is showing a hypercellular marrow. The bone marrow is replaced by sheets of lymphoblasts having high N:C ratio, clumped chromatin, no nucleoli and minimal eosinophilic cytoplasm—ALL-L1 morphology (1000X).

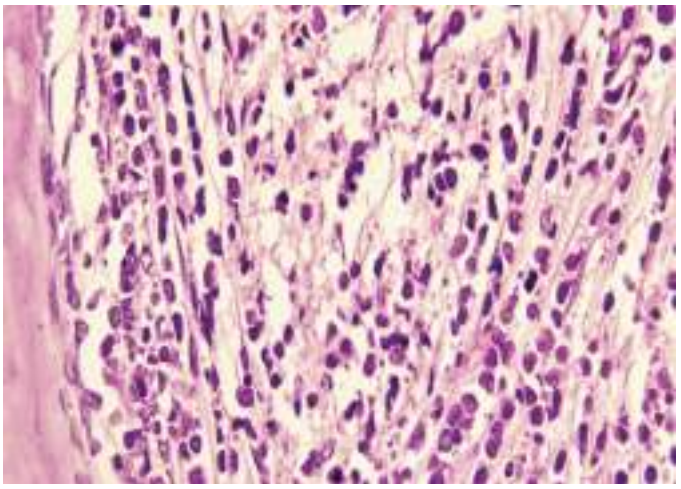


Fig. 9.32: Bone marrow fibrosis in childhood acute lymphoblastic leukemia in hematoxylin and eosin-stained bone marrow trephine biopsy section. It is showing bone marrow fibrosis with streaming of the lymphoblasts and absence of hematopoietic precursors (400X).

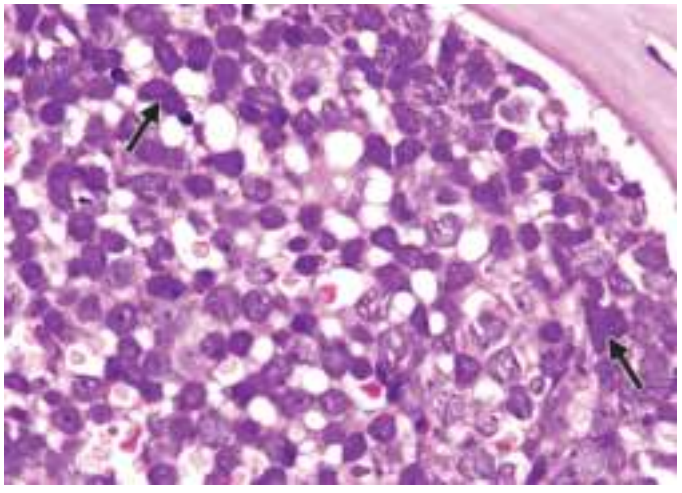


Fig. 9.31: Acute lymphoblastic leukemia L2 (ALL-L2) in hematoxylin and eosin-stained bone marrow trephine section. Bone marrow trephine biopsy section examination is hypercellular marrow, which is showing sheets of lymphoblast with high nucleocytoplasmic ratio and clumped chromatin, eosinophilic cytoplasm with indistinct margins. Some of the blasts are showing prominent nucleoli and occasional mitotic figure (marked by arrow)—ALL-L2 (1000X).

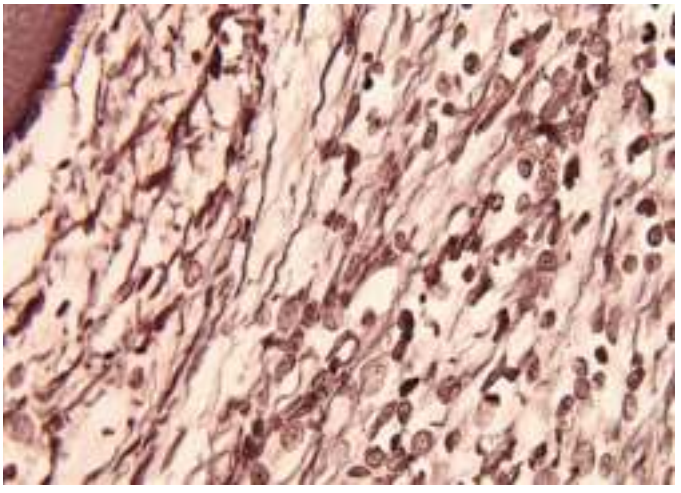


Fig. 9.33: Bone marrow fibrosis in adult acute lymphoblastic leukemia patient in silver-stained bone marrow trephine biopsy. It is showing replacement of bone marrow by dense reticulin fibers with streaming of the lymphoblasts and absence of hematopoietic precursors corresponding to grade 2 marrow fibrosis (400X).

normal lymphoid cells and leukemic lymphoblasts. The basic aim of the immunological classification is to subdivide acute lymphoblastic leukemias according to presence or absence of B cell or T cell markers, or B-phenotypic/hybrid acute leukemia. A marker is positive if ≥20% of leukemic lymphoblasts are stained with monoclonal antibodies.

- Terminal deoxynucleotidyl transferase is a specialized DNA polymerase expressed by immature, pre-B lineage lymphoid cells, pre-T lineage lymphoid cells and acute lymphoblastic leukemia/lymphoma cells in >95% of ALL cases.
- Demonstration of terminal deoxynucleotidyl transferase (TdT) activity suggests that a leukemic lymphoblast is derived from lymphoid lineage rather of myeloid lineage. Comparison of acute lymphoblastic leukemia derived from B cell and T cell lineage is given in Table 9.30.

Table 9.28 Morphological subtypes of acute lymphoblastic leukemia (ALL) according to French-American-British (FAB) classification

Features	ALL-L1	ALL-L2	ALL-L3
Epidemiology and immunophenotype class			
Frequency	70–75%	20–30%	1–2%
Age group	Children	Adults	Adults
Immunophenotype class	B-ALL, T-ALL	Adult T cell ALL	B cell ALL (equivalent to Burkitt's type ALL)
Prognosis	Good prognosis	Worse prognosis	Poor prognosis
Lymphoblast cell morphology			
Cell size	Small size lymphoblasts	Mixture of small and large lymphoblasts (heterogenous population)	Large size lymphoblasts
Nuclear shape	Regular shape with occasional clefting or indentation	Irregular shape with clefting or indentation common	Regular round to oval in shape
Nuclear chromatin	Homogenous chromatin	Heterogenous chromatin	Finely stippled and homogenous
Nucleoli	Nucleoli small or inconspicuous	Nucleoli (≥1) prominent	Nucleoli (≥1) prominent
Amount of cytoplasm	Scanty	Moderate	Moderate to abundant
Cytoplasmic basophilia	Slight or moderate cytoplasmic basophilia	Variable cytoplasmic basophilia	Very deep cytoplasmic basophilia
Cytoplasmic vacuolation	Absent or few cytoplasmic vacuoles	Variable cytoplasmic vacuolation	Prominent cytoplasmic vacuolation
Cytochemistry			
PAS stain	Positive	Negative	Negative
Acid phosphatase stain	Positive/negative	Positive/negative	Positive/negative
Oil red O stain	Negative	Negative	Positive

Table 9.29 Revised 2024 WHO classification of ALL/lymphomas

B Cell or T Cell ALL/Lymphoma	Recurrent Genetic Abnormalities
B cell ALL/lymphoma, not otherwise specified (NOS)	No recurrent genetic abnormalities
B cell ALL/lymphoma with recurrent genetic abnormalities	B cell ALL/lymphoma with recurrent genetic abnormalities ▪ t(9;22) (q34.1;q11.2);BCR-ABL1 ▪ t(v;11q23.3); KMT2 rearranged ▪ t(12;21) (p13.2;q22.1); ETV6-RUNX1 ▪ Hyperploidy >50 chromosomes ▪ Hypoploidy <45 chromosomes ▪ t(5;14) (q31.1;q32.3) IL3-IGH ▪ t(1;19) (q23;p13.3); TCF3-PBX1 ▪ Provisional entity B-ALL/lymphoma with BCR-ABL1-like
T-ALL/lymphoma	Provisional entity early T cell precursor lymphoblastic leukemia/lymphoma
Natural killer cell (NK cell) lymphoblastic leukemia/ lymphoma	Provisional entity natural killer cell lymphoblastic leukemia/lymphoma

Hematology Pearls: Immunophenotyping of Acute Lymphoblastic Leukemia

- Terminal deoxynucleotidyl transferase (TdT) is demonstrated in all subgroups of acute lymphoblastic leukemia (ALL).
- CD10 positivity is demonstrated in all subgroups except some B cell lineage ALL. HLA-DR is demonstrated in all subgroups of ALL.
- Common ALL (frequency 70–75% in children associated with good prognosis) express TdT, HLA-DR and CD10+ (CALLA).
- Pre-B cell-derived ALL (frequency 20% in children associated with poor prognosis) expresses cytoplasmic Ig, but negative for surface Ig and CD10.

B Cell and T Cell Lineage and Biphenotypic Acute Lymphoblastic Leukemias

More than 70% of adults ALLs are derived from B cell, i.e. precursor B cell (80%), and mature B cell in the bone marrow. B cell lineage ALLs demonstrate immunoglobulin gene rearrangement.

- Terminal deoxynucleotidyl transferase (TdT) is a specialized DNA polymerase expressed only by pre-B and pre-T lymphoblasts in >95% of ALL cases.
- Demonstration of TdT activity suggests that a leukemic blast is of lymphoid rather than myeloid lineage.

Table 9.30 Comparison of acute lymphoblastic leukemia (ALL) derived from B cell and T cell lineage

Parameters	B Cell Lineage ALL	T Cell Lineage ALL
Age group	2–10 years	13–17 years
Frequency in Indian population	80%	20%
Tumor load	Low tumor load	High tumor load
Clinical features	Extensive involvement of bone marrow and peripheral blood	Mediastinal lymphadenopathy, splenomegaly, marked leukocytosis
Molecular genetic alterations	▪ t(9;22)(q34;q11.2); BCR-ABL1 (Philadelphia chromosome) ▪ t(v;11q23.3); translocation between MLL (also called KMT2A), i.e. MLL rearrangement ▪ t(12;21)(p13;q22); TEL-AML1 ▪ Hyperploidy >50 chromosomes ▪ Hypoploidy <45 chromosomes ▪ t(5;14) (q31;q32); IL3-IGH ▪ t(1;19)(q23p13.3); E2A-PBX1	– ▪ t(v;11q23.3); translocation between MLL (also called KMT2A), i.e. MLL rearrangement – – ▪ Hypoploidy <45 chromosomes – –
Immunophenotype lineage markers	CD10(CALLA), CD19, CD20, CD22, CD24, CD79a, BOB1, OCT2, PU1, TdT, HLA-DR, monoclonal surface immunoglobulin, TCR gene rearrangement	cCD1, CD2, CD4, CD5, CD7, TdT
Treatment response	Responsive to therapy	Unresponsive to therapy
Prognosis	Good prognosis	Poor prognosis

T-lineage ALL shows TCR gene rearrangement and presents with mediastinal mass.

Common Acute Lymphoblastic Leukemia

Common ALL is characterized by the presence of ALL antigen, a glycoprotein (gp100/CD10). Common ALL lymphoblasts do not express markers of mature B cells such as cytoplasmic immunoglobulins or surface immunoglobulins.

Early Pre-B Cell Lineage Acute Lymphoblastic Leukemia

The lymphoblasts of early pre-B cell lineage ALL resemble B cell precursors in normal bone marrow, which show positivity for CD19, cCD22, CD79a, CD10 (90%), TdT (90%), CD34 (>75%) and CD2 (50%). These cells lack expression of surface and cytoplasmic immunoglobulins.

- ALL with rearrangement of the myeloproliferative leukemia gene (MPL gene) has immunophenotype of early pre-B cell ALL, which expresses CD15, CD65 and chondroitin proteoglycan sulfate, and lack expression of CD10 and CD22.
- Hyperploidy (chromosome number >50) is typically associated with weak or undetectable CD45 expression in ALL cases. It should be noted that myeloproliferative leukemia gene (MPL gene) encodes thrombopoietin receptor protein, which promotes cell growth and cell division of megakaryocytes, which produce platelets involved in blood clotting.

Pro-B Cell Lineage Acute Lymphoblastic Leukemia

Pro-B cell lineage ALL is also termed early B-precursor ALL, which constitutes about 11% of adult ALL. This variant of ALL was formerly termed non-B ALL, non-T ALL or null ALL; as neither of B cell nor T cell features could be demonstrated. The leukemic lymphoblasts express terminal deoxynucleotidyl transferase (TdT) and CD19.

Pre-B Cell Lineage Acute Lymphoblastic Leukemia

Pre-B cell lineage ALL immunophenotype is defined by the accumulation of cytoplasmic immunoglobulin μ heavy chains (cIgM) without detectable surface immunoglobulins (sIgM), that accounts for 25% of cases.

- Expression of both heavy chains cIgM and sIgM without kappa or lambda light chains in pre-B cell ALL is a rare finding, and such cases are designated transitional pre-B cell lineage ALL.
- Pre-B cell lineage ALL expresses CD19, CD22, CD79a, CD10 and TdT. CD34 is expressed in 66% of cases. CD34 is weakly expressed in some cases.

Mature B Cell Lineage Acute Lymphoblastic Leukemia

Mature B cell lineage ALL accounts for 3–4% of childhood ALL patients. The lymphoblasts express surface immunoglobulin μ (mu) heavy chains (sIgM) plus κ or λ light chains, which may express common ALL antigens and occasionally cytoplasmic immunoglobulin.

- Commonly, lymphoblasts have morphology according to the FAB classification. The lymphoblasts

express CD19, CD22, CD20 and CD10. The leukemic cells lack CD34 expression.

- The less common subtype of B cell lineage ALL demonstrates lymphoblasts with ALL-L1 and ALL-L2 morphology and express terminal deoxynucleotidyl transferase (TdT) and/or CD34.
- These patients should be treated as for stage IVB non-Hodgkin lymphoma (Burkitt type), if these cases have rearrangement of the c-Myc gene.
- B cell lineage acute lymphoblastic leukemia (B-ALL) in hematoxylin and eosin-stained bone marrow trephine biopsy section is shown in Fig. 9.34.

T Cell Lineage Acute Lymphoblastic Leukemia

T cell lineage acute lymphoblastic leukemia (T-ALL) usually affects 15% of children and young persons. Incidence is lower in children and higher with advancing age.

- In T cell lineage ALL, lymphoblasts express T cell antigen (gp40, CD7) and cytoplasmic or surface CD3. The leukemic lymphoblasts also express CD2, CD5 and TdT; CD1a, surface CD3, CD4, CD8, CD10 and/or CD21 in 45% of cases. HLA-DR expression is uncommon.
- T-lineage ALL has been divided into three stages of immunophenotypic differentiation: early, mid or common and late stages.
- T cell acute lymphoblastic leukemia (T-ALL) in Giemsa-stained peripheral blood smear is shown in Fig. 9.35. Expression of the immunohistochemical memory marker CD45 RO in cytoplasm of helper T cell lineage lymphoblast population in childhood acute lymphoblastic leukemia in bone marrow trephine biopsy is shown in Fig. 9.36. CD10 positivity in

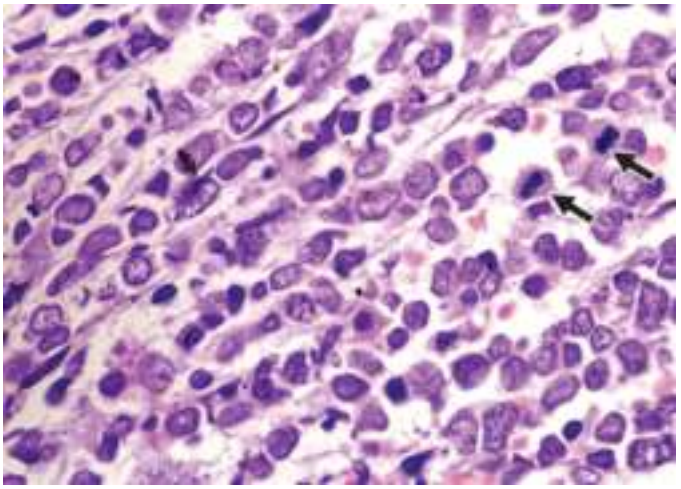


Fig. 9.34: B cell lineage acute lymphoblastic leukemia (B-ALL) in hematoxylin and eosin-stained bone marrow trephine biopsy section. Bone marrow trephine biopsy is showing sheets of blasts, with high mitosis (shown by arrows) and streaming of cells, replacing the hematopoietic elements in a case of B-ALL (1000X).

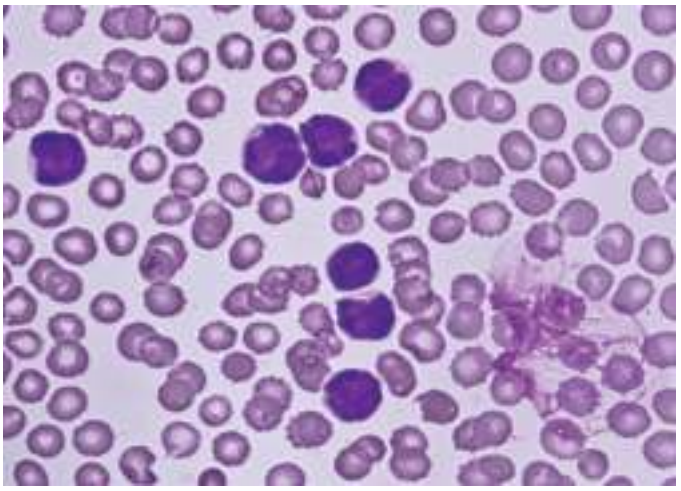


Fig. 9.35: T cell lineage acute lymphoblastic leukemia (T-ALL) in Giemsa-stained peripheral blood smear. T-ALL is a hematological malignancy characterized by the clonal proliferation of immature T cell precursors. In the acute variant, the leukemic cells are medium-sized to large lymphoid cells with irregular nuclei and basophilic cytoplasm. The characteristics of T cell ALL have been described as 'flower cells, with many nuclear convolutions and lobes. It is worth mentioning that T cell ALL in the chronic variant is generally small, with slight nuclear abnormalities such as notching and indentations (1000X).

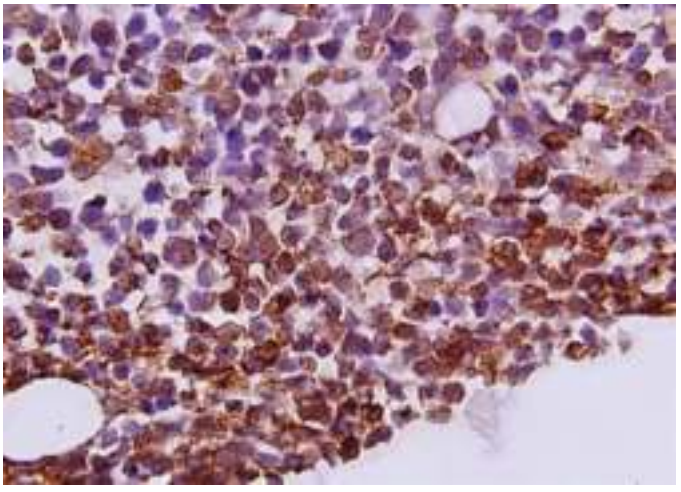


Fig. 9.36: Expression of the immunohistochemical memory marker CD45 RO in cytoplasm of T cell lineage lymphoblast population in childhood acute lymphoblastic leukemia in bone marrow trephine biopsy (400X).

T cell lineage acute lymphoblastic leukemia (T-ALL), also known as common acute lymphoblastic antigen (ALL antigen), is shown in Fig. 9.37. CD10 positive lymphoblasts from acute lymphoblastic leukemia (T-ALL) in bone marrow trephine biopsy section is shown in Fig. 9.38.

Biphenotypic Mixed Lineage Acute Leukemias

Biphenotypic mixed lineage leukemias are defined by the coexpression of markers of lymphoid and myeloid cell lineages on blast cells without typical phenotype

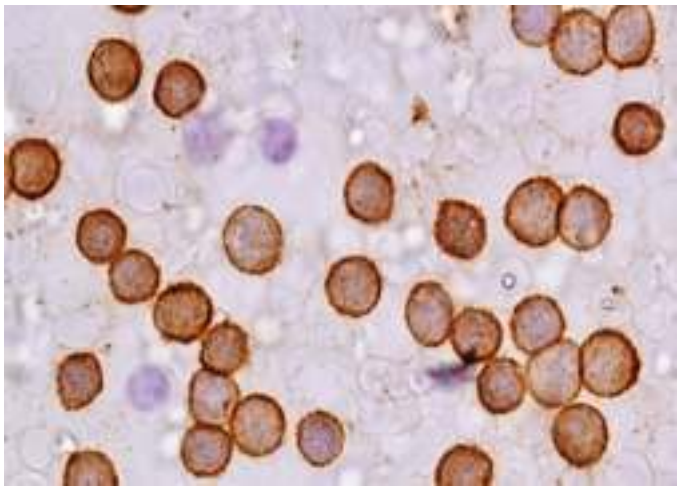


Fig. 9.37: CD10 positivity in T cell acute lymphoblastic leukemia (T-ALL), also known as common acute lymphoblastic antigen (ALL antigen). CD10 is a zinc metallopeptidase expressed in early lymphoid progenitors and normal germinal center cells. CD10 is almost expressed on the surface of precursor B cell lineage acute lymphoblastic leukemia (80%) and Burkitt lymphoma and much less frequently precursor of T cell lineage lymphoblastic leukemia (20%)/lymphoma. Acute lymphoblastic leukemia with CD10 positivity is amenable to chemotherapy. Thus, CD10 positivity is a favorable prognostic marker (1000X).

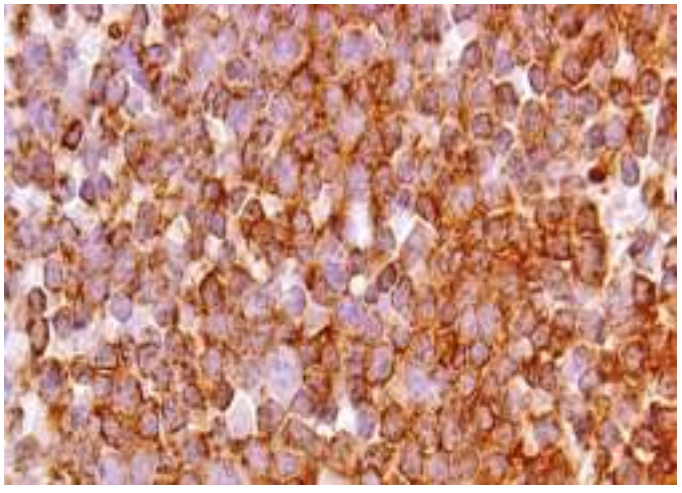


Fig. 9.38: CD10 positive lymphoblasts from acute lymphoblastic leukemia (T-ALL) in bone marrow trephine biopsy section. It shows CD10 positivity in lymphoblasts (400X).

of either acute lymphoblastic leukemia (ALL) or acute myelogenous leukemia (AML).

- Bilineage leukemias are those with two population of blast cells expressing either lymphoid or myeloid antigens.
- Bilineage leukemias should be differentiated from ALL by coexpression of myeloid markers and from AML by coexpression of lymphoid markers.
- Stages of immunophenotypic differentiation of T cell lineage acute lymphoblastic leukemia (ALL) are given in Table 9.31.

Table 9.31 Stages of immunophenotypic differentiation of T cell lineage acute lymphoblastic leukemia (ALL)

Stage of T Cell Lineage ALL	Immunophenotypic Differentiation	Expression
Early stage T cell lineage ALL	▪ CD7 ▪ cCD3 ▪ sCD3 ▪ CD4 ▪ CD8	▪ Positive ▪ Positive ▪ Negative ▪ Negative ▪ Negative
Mid or common stage T cell lineage ALL	▪ cCD3 ▪ CD4 ▪ CD8 ▪ CD1a ▪ sCD3	▪ Positive ▪ Positive ▪ Positive ▪ Positive ▪ Negative
Late stage T cell lineage ALL	▪ sCD3 ▪ CD4 or CD8 ▪ CD1a	▪ Positive ▪ Positive ▪ Negative

cCD3: cytoplasmic CD3; sCD3: surface CD3.

Molecular Genetic Alterations in Acute Lymphoblastic Leukemia

Both acute lymphoblastic leukemias/lymphoblastic lymphomas (ALL/lymphoma) have overlapping clinical presentations.

- Broadly ALL/lymphoma is divided into tumors of B cell and T cell lineage, and rarely NK cell lineage. Immunophenotyping is required to determine the lineage of ALL/lymphoma because different subtypes are morphologically indistinguishable.
- Most cases of ALL/lymphoma have cytogenetic and/or molecular abnormalities that are associated with distinctive phenotypes prognostic features and/or influence the choice of treatment.
- The World Health Organization (WHO) classification system uses immunophenotype and cytogenetic/molecular features to define specific categories of ALL/lymphoma.
- Cytogenetic analysis must be performed in all patients suffering from acute lymphoblastic leukemias. The demonstration of a specific karyotype is performed for the confirmation of the diagnosis.
- Demonstration of chromosomal abnormalities is important independent prognostic variables for disease-free survival or selection of patients to a specific targeted therapy.
- B cell lineage ALL/lymphoma with recurrent genetic abnormalities are given in Table 9.32. Salient features of B cell acute lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities are given in Table 9.33. Cytogenetic alterations in acute lymphoblastic leukemia (ALL) are given in Table 9.34.

Table 9.32 B cell lineage ALL/lymphoma with recurrent genetic abnormalities

▪ A translocation between 9 and 22, i.e. t(9; 22), known as Philadelphia chromosome occurs in 5% in pediatric cases and about 20% of adults in ALL. It is associated with poor prognosis
▪ A translocation between chromosomes 4 and 11 occurs in about 4% of cases in infants under 12 months of age
▪ Cytogenetic abnormality such as hyperdiploidy (>50 chromosomes) is associated with good prognosis
▪ Other cytogenetic abnormalities include t(12; 21), t(4;11), TEL-AML1, t(1;19), 11q23 rearrangements, polyploidy and hypodiploidy. These cytogenetic abnormalities sometimes predict poor prognosis

Prognostic significance of cytogenetic findings in acute lymphoblastic leukemia are given in Table 9.35.

- Acute lymphoblastic leukemia (ALL) is a neoplastic disorder characterized by clonal expansion of leukemic cells in the bone marrow, lymph nodes, spleen or thymus gland. Majority of the ALL—patients harbor acquired somatic mutations that contribute to the unrestricted cell proliferation, prolonged survival and/or impaired differentiation of the lymphoid hematopoietic progenitors. Cytogenetic/molecular genetic alterations in acute lymphoblastic leukemia are discussed below.

Hyperdiploid Acute Lymphoblastic Leukemia

Hyperdiploid acute lymphoblastic leukemia (51–65 chromosomes) is the most common genetic abnormality observed in 35% of children and 10% of adults; and associated with favorable outcome related to sensitivity to methotrexate. Some patients with hyperdiploidy and trisomies of chromosomes 4, 10 and 17 have a favorable prognosis.

Hypodiploid Acute Lymphoblastic Leukemia

Hypodiploid ALL (<45 chromosomes) is demonstrated in less than 2% of ALL and associated with unfavorable prognosis especially for patients with near haploid (<30 chromosomes) or low hypodiploid (30–39 chromosomes) karyotype.

Acute Lymphoblastic Leukemia with BCR-ABL1 Rearrangement

The translocation t(9;22) (q34;q11) encodes a chimeric gene (BCR-ABL fusion gene) consisting of 3′ portion of ABL1 on chromosome 9 joins to the 5′ portion of BCR gene on chromosome 22. In ALL, chromosomal breaks tend to occur in the minor breakpoint cluster regions forming a 190 kDa BCR-ABL fusion gene.

- Genomic analysis of BCR-ABL fusion gene in ALL demonstrates deletions of IKZF1 in 85% of cases. Moreover, deletion of IKZF1 appears to be acquired

Table 9.33 Salient features of B cell acute lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities

Genomic Abnormality	Age Group	Clinical Features	Immunophenotype (Positive Expression)	Aberrant Antigen Expression	Prognosis
t(9;22) (q34;q11.2); BCR-ABL1 (Philadelphia chromosome)	Adults	Organomegaly	CD10, CD19, TdT, CD25	CD13, CD33	Worst prognosis
t(v;11q23.3); translocation between MLL (also called KMT2A), i.e. MLL rearrangement	Infants <1 year	Marked leukocytosis and CNS involvement	CD19, CD15, CD10, neural/glial antigen 2 (NG2), melanoma associated chondroitin sulfate proteoglycans (MCSP)	Not applicable	Poor prognosis
t(12;21)(p13;q22); TEL-AML1	Children	Similar to other ALL features	CD19, CD10, CD34	CD13	Favorable prognosis
Hyperploidy >50	Children	Similar to other ALL features	CD19, CD10, CD34	Not applicable	Favorable prognosis
Hypoploidy <45 chromosomes	Children and adults	Similar to other ALL features	CD19, CD10	Not applicable	Poor prognosis
t(5;14)(q31;q32); IL3-IGH	Children and adults	Asymptomatic eosinophilia	CD19, CD10	Not applicable	Unpredictable prognosis
t(1;19)(q23p13.3); E2A-PBX1	Children	Similar to other ALL features	CD19, CD10, CD9, cytoplasmic μ (cμ) heavy chain	Not applicable	Better prognosis with intensive therapy

In hyperploidy state, each cell contains one or more extrachromosomes. In hypoploidy state, each cell contains one or more fewer chromosomes than normal.

Table 9.34 Cytogenetic alterations in acute lymphoblastic leukemia (ALL)

ALL Type	Cytogenic Findings	Frequency
ALL-B cell type	▪ t(2;8) ▪ t(8;22)	>90%
ALL pre-B cell (cytoplasmic Ig of mu, heavy chain)	▪ t(1;19) (q23;p13) ▪ t(9;22) in adults	25%
ALL-T cell	▪ t(14q11)	25%
Mixed lineage ALL	▪ t(11p23) ▪ t(14q32) ▪ t(9;22) (q34;q11)	Unknown

Table 9.35 Prognostic significance of cytogenetic findings in acute lymphoblastic leukemia

Cytogenetic Abnormalities	Prognosis
Hypodiploidy in 100% of chromosomes	Unfavorable prognosis
Hyperdiploidy in >50% of chromosomes	Favorable prognosis
Hyperdiploidy in 47–50% of chromosomes	Intermediate prognosis

lesion at the time of transformation from chronic myelogenous leukemia to lymphoid blast phase.

- BCR-ABL1 ALL is refractory to standard chemotherapy. These patients respond to first generation receptor tyrosine kinase inhibitor (RTKI) such as imatinib as well as second and third generation RTKIs such as nilotinib, dasatinib and bosutinib.

Acute Lymphoblastic Leukemia with ETV6-RUNX1 Rearrangements

The chromosomal translocation, t(12;21) (p13; q22) is the most prevalent translocation in pediatric ALL resulting in ETV6-RUNX1 chimeric fusion gene (formerly TEL-AML1). Children with ETV6-RUNX1 chimeric fusion protein have excellent prognosis with very low rates of relapses related to optimal asparaginase therapy.

Acute Lymphoblastic Leukemia with E2A-PBX1 Fusion Gene

About 20–25% of pre-B cell ALL cases demonstrate t(1;19) (q23;p13) chromosomal translocation abnormality that juxtaposes the E2A (TCF3 transcriptional factor 3) gene on chromosome 19 and the PBX1 gene on chromosome 1 resulting in E2A-PBX1 fusion transcription protein.

- Another fusion gene is created by t(17;19) (q22;p13) chromosomal translocation in which E2A is fused to the gene that encodes the transcription factor, i.e. hepatic leukemic factor (HLF).
- Chromosomal translocation t(1;19) (q23;p13) is demonstrated by conventional cytogenetics, fluorescence *in situ* hybridization (FISH) or reverse transcriptase polymerase chain reaction (RT-PCR).
- Patients usually presents with hypercalcemia and coagulopathy associated with poor prognosis.
- Acute lymphoblastic leukemia demonstrates MLL gene rearrangements. Structural cytogenetic alterations involving band 11q23 of chromosome 11 are frequently demonstrated in ALL affecting infants and children. The targeted therapy is administered in mixed-lineage leukemia (MLL) gene.
  - Chromosomal translocation t(4;11) (q21;q23) involving band 11q23 in ALL produces a chimeric protein that contains the N-terminal portion of MLL linked to the C-terminal portion of AFF1 (AF4/FMR2 family member) and other more than 50 genes.
  - MLL gene encodes DNA binding protein that regulates the expression of numerous genes including multiple HOX genes. Protein produced by normal MLL gene regulates hematopoiesis. In contrast MLL fusion protein transforms hematopoietic stem cells into leukemia-initiating cells. Prognosis of ALL in infants is poor.

Acute Lymphoblastic Leukemia with iAMP21 Alterations

Intrachromosomal amplification of a region of chromosome 21 (iAMP21); three or more extra copies of RUNX1 on an abnormal chromosome 21 is recently identified recurrent genomic abnormality associated with poor outcome in children with ALL. The patients have a common/pre-B cell immunophenotype of ALL.

BCR-ABL1-like Acute Lymphoblastic Leukemia

Out of B-lineage ALL, 10–20% of cases demonstrate a gene expression profile like that of BCR-ABL1; but lacks BCR-ABL1 rearrangement. The frequency of BCR-ABL1 like ALL is 10% in children and 25–30% in young adults.

- BCR-ABL1 like ALL cases usually demonstrate IKZF1 alteration, overexpression of CLRF2, and tyrosine kinase activating rearrangements involving ABL1, JAK2, PDGFRB and several others. These ALL patients are associated with worse outcome.
- About 50% of BCR-ABL1 like ALL cases demonstrate CRLF2 rearrangements and JAK2 mutations. Next-generation sequencing such as transcriptome and whole-genome sequencing has demonstrated that the remaining cases harbor a diverse range of rearrangements, deletions and sequence mutations that activate cytokine receptor and RTK signaling involving ABL1, JAK2, IL7R and PDGFR.
- BCR-ABL1 like ALL patients are treated by targeted therapy directed at the underlying genetic pattern with BCR-ABL inhibitors (e.g. dasatinib) or JAK2 inhibitors (e.g. ruxolitinib).

B Cell Lineage Acute Lymphoblastic Leukemia with CRLF2 Rearrangement and JAK2 Kinase Alterations

Approximately 8% of B-lineage ALL cases harbor a genomic rearrangement of CRLF2: a translocation of the immunoglobulin heavy chain gene on 14q32 to CRLF2 in the pseudoautosomal region 1 of Xp22.3/Yp11.3.

- CRLF2 encodes cytokine-like factor 2, the receptor for thymic stromal lymphopoietin (TSLP). CRLF2 rearrangement is commonly seen in ALL associated with Down syndrome.
- Approximately 50% of cases with CRLF2 rearrangement harbor concomitant activating mutations in Janus kinase genes JAK1 or JAK2 and associated with poor prognosis.

T Cell Lineage Acute Lymphoblastic Leukemia with Genetic Abnormalities

Cytogenetic alterations in T cell acute lymphoblastic leukemia include SCL (TAL1), LMO1 (TTG1), LMO2 (TTG2) and HOX11. An additional genetic alterations demonstrated in T cell lineage ALL is the deletion from chromosome 9p21 of the CDKN2A and CDKN2B genes, which encode $p16^{INK4a}$ and $p16^{INK4b}$ including cyclin-dependent kinase inhibitors. NOTCH1 mutation has been implicated in the pathogenesis of T cell lineage ALL.

Early T-Precursor Acute Lymphoblastic Leukemia

Early T-precursor ALL (ETP-ALL) is a recently described subtype of aggressive acute lymphoblastic leukemia of unknown genetic basis, which originates from early T cell precursors (ETPs), the subset of thymocytes that retain stem cell-like features. Early T-precursor ALL lacks CD1 and CD8 expression, and weak CD5 expression. Patients respond poorly to lymphoid cell-directed therapy.

- Genetic sequencing of ETP-ALL analysis reveals somatic mutations in genes regulating cytokine receptor and RAS signaling (K-RAS, N-RAS, FLT3, IL7R, JK3, JK1, SH2B3 and BRAF), inactivating lesions disrupting hematopoietic development (GATA3, ETV6, RUNX1, IKZF1 and EP300); and recurrent mutations of DNM2, ECT21 and RELN.
- Mutational spectrum of these cases is similar to myeloid tumors. These findings suggest that addition of myeloid-directed therapies might improve the poor outcome of ETP-ALL.

Hematology Pearls: Diagnostic Cytogenetic/Molecular Techniques for Acute Lymphoblastic Leukemia

- The diagnostic techniques for acute lymphoblastic leukemia (ALL) include standard cytogenetics, fluorescence *in situ* hybridization (FISH), and reverse transcriptase polymerase chain reaction (RT-PCR). These techniques allow the detection of Philadelphia chromosome positive acute lymphoblastic leukemia, with the chromosomal translocation t(9;22)(q34;q11) and demonstration of the corresponding BCR-ABL1 gene rearrangement.
- Further ALL entities that have been detected include t(5;14)(q31;q32)/IL3-IGH, t(1;19)(q23p13.3)/PBX-E2A, t(12;21)(p13;q22); TEL-AML1, t(4;11)(q21;q23)/MLL-AFA4, abnormalities 11q23/MLL, hyperploidy and hypoploidy.
- Gene expression profiling, single nucleotide polymorphism array analysis, array-comparative genomic hybridization. Next generation sequencing recognizes newly defined ALL entities with fatal outcome: Philadelphia chromosome like ALL and early T-precursor ALL.

PROGNOSIS

Age of the patient is a strong prognostic determinant in ALL. Prognosis is good in children and poor in infants and children. A higher initial total leukocyte count and presence of central nervous system involvement by leukemic cells also determine high-risk disease.

- Early response to post-induction therapy is measured by persistence or absence of minimal residual disease (MRD). Recently, genomic analysis has revealed molecular alterations and profiles have been associated favorable or unfavorable prognosis.
- Common B cell precursor subset of ALL cases expressing CD10, hyperdiploidy, and t(12;21)/TEL-AML1 rearrangement have been associated with good prognosis. The t(12;21)/TEL-AML1 generates TEL-AML1 (ETV6-RUNX1) fusion gene. Patient has poor prognosis showing chromosomal t(9; 22), t(1;19), 11q23 rearrangements and hypodiploidy. Prognostic factors in acute lymphoblastic leukemia (ALL) are given in Table 9.36.

TREATMENT

Approximately >90% cases of ALL achieve complete remission with treatment. Relapse is common in 75% of cases without prophylactic intrathecal chemotherapy. ALL in adults has worse prognosis and requires bone marrow transplantation as well as chemotherapy.

- Most infants under one year at diagnosis harbor rearrangements in the KMTT2A (formerly MLL) gene have a poor prognosis. These infants should be treated on age and weight specific protocols with certain agents to reduce the risk of severe toxicity.
- T-ALL patients are treated and are benefited by administration of high-dose methotrexate, dexamethasone and asparaginase.
- Philadelphia chromosome positive ALL patients are treated by receptor tyrosine kinase inhibitor. Patients with CNS involvement require CNS sterilization by administration of intrathecal chemotherapy

Table 9.36 Prognostic factors for acute lymphoblastic leukemia (ALL)

Features	Favorable Prognosis in ALL Patients	Unfavorable Prognosis in ALL Patients
Age group	2–10 years	<1 year or >10 years
Gender predilection	Girls	Boys
Racial predilection	White population	Black population
FAB classification	ALL-L1 subtype	ALL-L2, ALL-L3 subtypes
ALL derived from cell type	▪ ALL (early pre-B cell) ▪ Common acute lymphoblastic leukemia antigen (common ALL antigen)	▪ ALL (pre-B cell) ▪ ALL (B cell) ▪ ALL (pre-T cell)
Extramedullary involvement (testes and central nervous system)	Testis and CNS not involved	Testis and CNS involved
Total white blood cell count	Low white blood cell count (<30,000/ml)	High white blood cell count (>50,000/ml)
Hemoglobin	>10 g/dl	<7 g/dl
Platelet count	>100,000/ml	30,000/ml
Molecular genetics	▪ Hyperploidy involving >50% chromosomes (favorable) ▪ t(12;21) (p13;q22); TEL-AML1 ▪ t(1;19) (q23p13.3); E2A-PBX1	▪ Hypoploidy involving 100% chromosomes ▪ t(9;22) (q34;q11.2); BCR-ABL1 (Philadelphia chromosome) ▪ 11p23 ▪ t(14q;11) ▪ t(8;14) ▪ t(1;19) ▪ 11q23 rearrangement
Immunophenotype	CD10+ (amenable to therapy)	CD10 negative (not amenable to therapy)
Response to therapy	Rapid early responder	Slow early responder
Time to clear lymphoblasts from peripheral blood	<1 week	>1 week
Time of remission	<4 weeks	>4 weeks
Post-induction minimal residual disease (MRD)	Minimal residual disease negative after one month therapy in children and 3 months therapy in adults	Minimal residual disease persisting for 3–6 months after therapy

Hyperploidy involving 47–50% of chromosomes has intermediate prognosis. Translocation (9;22) in ALL is cytogenetically identical but distinct from demonstrated in CML/minor breakpoint region is more commonly involved in ALL. But in CML, major breakpoint region is typically involved in childhood ALL, a BCR/ABL fusion protein P190 is produced.

in combination with systemic chemotherapeutic agents such as dexamethasone and high-dose methotrexate.

- Radiation is most often reserved for those ALL cases with meningeal leukemia or CNS relapse. Testicular involvement resolves completely during the induction phase of therapy.
- Administration of molecular targeted agents has improved outcome in ALL patients. BCR-ABL receptor tyrosine kinase inhibitors (RTKIs) such as imatinib mesylate (Gleevec), dasatinib, and nilotinib have been successfully utilized in combination with chemotherapy to improve the survival in Philadelphia chromosome positive ALL patients.
- Monoclonal antibody-based therapies that target differentiation antigens expressed on the surface of lymphoblasts (e.g. CD19, CD20, CD22, CD52) have also been used in the setting of ALL. Rituximab combined with conventional chemotherapy has improved survival in adults with CD20 positive Philadelphia chromosome negative ALL.
- For adult patients with relapsed ALL, allogeneic stem cell transplantation with HLA-matched sibling donor has improved the survival of ALL patients.

MINIMAL RESIDUAL DISEASE

Minimal residual disease (MRD) is the detection of residual leukemic cells, not recognizable by light microscopy. Bone marrow of ALL patients in morphologic complete remission may contain up to 1010 residual leukemic cells.

- Molecular quantitation of MRD of ALL is analyzed by using real-time polymerase chain reaction (RT-PCR).
- Methods are based on the demonstration of leukemia-specific aberrant immunophenotypes by flow cytometry, analysis of leukemia-specific rearranged immunoglobulin or T cell receptor sequences by real-time quantitative polymerase chain reaction (RT-PCR), or the detection of fusion genes associated with chromosomal abnormalities (e.g. BCR-ABL, MLL-AF4).

ACUTE MYELOGENOUS LEUKEMIA

ACUTE MYELOGENOUS LEUKEMIA: OVERVIEW

Acute myelogenous leukemia (AML) is a hematopoietic stem cell (HSC) disorder characterized by unrestricted proliferation of myeloblasts in the bone marrow. It may originate from any of the cells that fall within the downward pathways.

- Importantly, AML cell of origin acquires capacity for self-renewal and maturation arrest, which results in accumulation of immature nonfunctional leukocytes in the bone marrow and blood circulation.
- Failure of normal production of red blood cells, leukocytes and platelets results in anemia, recurrent infections and thrombocytopenia.
- AML accounts for 15–20% in children and adolescents; and 85% in adults. The incidence of AML rises rapidly after the age of 60 years and the median age at diagnosis is 67 years.
- Clinical course of acute myelogenous leukemia (AML) is short and marked by anemia, neutropenia, recurrent respiratory tract infection, fever, and hemorrhage, and death occurs within 6–12 months. These patients are less responsive to therapeutic intervention.

PATHOGENESIS

Inherited genetic predisposition, environmental mutagens such as radiation, drugs (e.g. alkylating agents, topoisomerase II inhibitors:), tobacco smoke and benzene; preexisting hematological disorders; and acquired somatic mutations associated with aging process play a role in the development of AML.

- Genetic causes are suggested by the increased incidence of AML in identical twins as well as known association of AML with various congenital disorders.
- Genetic predisposition plays important role in the development of AML in children and young adults. Risk factors for development of acute myelogenous leukemia are given in Table 9.37.

CLINICAL FEATURES

Patients usually present with bone marrow failure that causes anemia, bleeding manifestations from

Table 9.37 Risk factors for development of acute myelogenous leukemia (AML)

Categories	Etiology and Disorders
Environmental factors	Benzene, ioniting radiation, tobacco smoking
Genetic disorders	Down syndrome, Bloom syndrome, Fanconi syndrome, dyskeratosis congenita, ataxia-telangiectasia, Li-Fraumeni syndrome, Kostmann syndrome, Klinefelter syndrome, osteogenesis imperfecta, Wiskott-Aldrich syndrome, leukemia in siblings, Diamond-Blackfan syndrome
Pre-existing hematological disorders	Myelodysplastic syndrome (MDS), myeloproliferative disorders, familial aplastic anemia, paroxysmal nocturnal hemoglobinuria (PNH)
Treatment-induced acute myelogenous leukemia (AML)	▪ **Alkylating agents (chlorambucil, melphalan):** Acute myelogenous leukemia (AML) usually arises from myelodysplastic syndrome (**MDS**), after 3–10-year latency period and is associated with deletions involving chromosomes 5 or 7 ▪ **Topoisomerase II inhibitors:** Acute myelogenous leukemia (AML) arises without preceding myelodysplastic syndrome. AML involves chromosome 11q23, chromosome 11q23, exhibits monocytic morphology ▪ **Radiotherapy alone or in combination chemotherapy:** Patients may develop acute myelogenous leukemia (AML)

thrombocytopenia and neutropenic recurrent infections of upper respiratory tract.

- Tissue infiltration with leukemic blasts involving gums, meninges, skin, liver, spleen, lymph nodes are most often associated with monocytic morphology.
- Striking bruising and life-threatening hemorrhage raise suspicion of disseminated intravascular coagulation (DIC) frequently seen in acute promyelocytic leukemia (AML-M3).
- Leukostasis and hyperviscosity syndrome cause organ dysfunction with blast cell counts and symptoms of decreased tissue perfusion.

Bone Marrow Failure

Symptoms and signs on presentation due to bone marrow failure include fatigue, breathlessness, fever, focal bacterial infections, petechiae, bruising and bleeding. Severe bleeding suspects acute promyelocytic leukemia (AML-M3).

Leukemic Blasts Infiltration in Multiple Organs

Leukemic blasts in patients with AML-M4 or AML- M5 or AML-M7 can infiltrate into gums, liver, spleen, lymph nodes, skin (diffuse erythematous skin rashes) and central nervous system (meninges, Vth and VIIth cranial nerves) and visual changes such as retinal involvement, intracranial hemorrhage, and papilledema. AML-M4, AML-M5 and AML-M7 result in pancytopenia and bone marrow fibrosis.

Leukostasis and Hyperviscosity Syndrome

Leukostasis and hyperviscosity syndrome cause organ dysfunction in AML cases with high blast cell counts more than 1,00,000 µl.

- Leukostasis is more common in acute myelogenous leukemia than in acute lymphoblastic leukemia, because the blast cells of AML have a larger cell volume than blast cells in ALL. Leukostasis is a medical emergency in patients with AML and in the CML blast phase in which blast cells are accumulated in small blood vessels.
- Skin rash with neutrophilic infiltrates in dermis and chloromas are rare findings in AML.
- Extramedullary AML disease pretends a poor prognosis.
- Bone marrow failure usually occurs in AML. Intracranial hemorrhage often associated with hyperviscosity is studied by CT scan. MRI demonstrates thickened nerve sheaths. Lung infiltrates are demonstrated by CT scan.

Chloroma

Chloroma is a rare malignant tumor solid mass formation in acute myelogenous leukemia composed of granulocyte precursor cells such as myeloblasts, promyelocytes and myelocytes. Children seem to more affected than adults. In many cases, chloroma does not cause major symptoms.

- About 50% of chloroma cases are diagnosed only during autopsy. The symptoms are caused by tumor mass itself located into the tissue/organ.
- Chloroma in the central nervous system is associated with symptoms such as cauda equina syndrome or radiculopathy.

Sweet Syndrome

Sweet syndrome, also called acute febrile dermatosis, is a rare finding in acute myelogenous leukemia, that typically presents with acute-onset tender plaques or nodules, fever, arthralgia, ophthalmic manifestations and headache. Diagnosis is established on clinical manifestations and histologic examination of plaque.

Hematology Pearls: French-American-British (FAB) and Revised 2024 WHO Classification of Acute Myelogenous Leukemia (AML)

- The classification of AML has evolved from the morphology-based French-American-British (FAB) to the more comprehensive revised 2024 WHO classification. AML has been categorized into eight subtypes (AML-M0–AML-M7) according to FAB classification based on morphology, cytochemical staining, and immunophenotype of the predominant cells.
- Revised 2024 WHO classification of acute myelogenous leukemia and acute lymphoblastic leukemia and their features is given in Table 9.38. Revised 2024 WHO classification of acute myelogenous leukemias is given in Table 9.39.
- Revised 2024 WHO classification of acute leukemias integrates new clinical, prognostic, morphologic, immunophenotyping and genetic data. Latest 2024 WHO classification also includes myeloid neoplasms with germline predisposition. Family history is essential to establish diagnosis of AML.
- According to revised 2024 WHO classification of acute leukemias (myelogenous/ lymphoblastic leukemia), diagnostic criterion is the presence of ≥20% lymphoblast cells as the cut off percentage in bone marrow and peripheral blood.
- According to French-American-British (FAB) classification of acute leukemias (myelogenous/lymphoblastic leukemia), original diagnostic criterion was the presence of ≥30% lymphoblast cells as the cut off percentage in bone marrow and peripheral blood.
- AML group with recurrent genetic abnormalities has prognostic significance. Most common ones such as t(8;21) and t(16;10) are considered acute myelogenous leukemia even if blast cell count in the bone marrow is ≤20%.

LABORATORY DIAGNOSIS

The most common laboratory findings in acute myelogenous leukemia are anemia, neutropenia and thrombocytopenia and myeloblasts in the bone marrow and peripheral blood.

Table 9.38 Revised 2024 WHO classification of acute myelogenous leukemia and acute lymphoblastic leukemia and their features

Leukemia Subtype	Description
Acute myelogenous leukemias	
AML-M0	▪ AML-M0 with minimally differentiation expressing myeloid lineage accounts for 2–3% ▪ Myeloperoxidase stain is positive in <3% of cases ▪ Two or more myeloid markers by flow cytometry: CD34+, HLA-DR+, CD33+/–; CD13+/– ▪ Frequent complex cytogenetic abnormalities such as 11q13 are present ▪ AML-M0 is associated with poor prognosis
AML-M1	▪ AML-M1 without maturation: less than 10% promyelocytes or more mature myeloid cells ▪ AML-M1 accounts for 20% of leukemias ▪ Myeloblast contains faint granules, distinct nucleoli and occasional Auer rod ▪ Myeloperoxidase stain positive (>3%) ▪ Two or more myeloid markers by flow cytometry: CD34+, HLA-DR +, CD33+/–; CD13+/– ▪ Frequent complex cytogenetic abnormalities: –6, –7, –17, del 3p, +21, +8 ▪ Prognosis is favorable
AML-M2	▪ AML with maturation and differentiation into neutrophilic lineage ▪ Myeloblast contains faint granules, distinct nucleoli and occasional Auer rods ▪ AML-M2 accounts for 30–40% of leukemias ▪ Myeloperoxidase stain positive (>10%) ▪ Subset of patients demonstrate cytogenetic abnormality: t(8;21) ▪ Two or more myeloid markers by flow cytometry: CD34–, HLA-DR+, CD33+, CD13+ ▪ AML-M2 is associated with favorable prognosis
AML-M3	▪ In AML-M3, AML with promyelocytic differentiation (APL), promyelocytes show heavy granulation and bilobed folded (cottage-loaf) nuclei (commonly associated with disseminated intravascular coagulation (DIC) due to release of thromboplastin like substance); rarely microgranular variant with inconspicuous granules in majority of cases ▪ AML-M3 accounts for 5–10% of leukemias ▪ Myeloperoxidase stain is positive ▪ Two myeloid markers by flow cytometry include: CD33+, CD13+, CD34–, HLA-DR– ▪ Most cases have t(15–17) chromosomal translocation associated with favorable prognosis ▪ Bruising and life-threatening hemorrhage raises suspicion of disseminated intravascular coagulation (DIC) associated with overall good prognosis ▪ AML-M3 is treated by targeted therapy directed against the leukemogenic event, t(15.17) PML-RARA fusion transcript, lead to improved outcome ▪ Acute promyelocytic leukemia now represents most curable leukemia treated by administration of nontoxic cytotoxic chemotherapeutic drugs such as all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO)
AML-M4	▪ AML-M4 (acute myelomonocytic leukemia—AMML) shows myelocytic and monocytic differentiation (>20% monocytoid cells involving gums, skin or central nervous system) ▪ AML-M4 accounts for 15–20% of leukemias ▪ Myeloperoxidase stain is positive in leukemic cells ▪ Nonspecific esterase stain is positive ▪ Complex cytogenetic abnormalities: inv(16), t(16;16), –5, –7, t(6;9) ▪ Two or more myeloid markers by flow cytometry: CD34–, HLA-DR+, CD33+, CD13+, CD14/CD64+ ▪ AML-M4E0 variant contains >5% abnormal eosinophils; associated with the inv(16) cytogenetic abnormality associated with favorable prognosis
AML-M5A	▪ AML-M5A (acute monoblastic/monocytic leukemia—AMoL) shows monoblasts without differentiation, extramedullary disease involving gums, skin, lymph node and central nervous system ▪ AML-M5A accounts for 5% of leukemias ▪ Nonspecific esterase (NSE) stain is positive ▪ AML-M5A is associated with extramedullary disease, abnormalities of chromosome 11q23 and poor prognosis
AML-M5B	▪ AML-M5B (acute monoblastic/monocytic leukemia—AMoL) shows more than 80% monoblasts with differentiated monocytes, or promonocytes ▪ AML-M5B accounts for 5% of leukemias ▪ Nonspecific esterase (NSE) stain is positive

Contd...

Table 9.38 Revised 2024 WHO classification of acute myelogenous leukemia and acute lymphoblastic leukemia and their features (*Contd...*)

Leukemia Subtype	Description
AML-M6A	▪ In AML-M6A (acute erythroid leukemia), bone marrow shows dysplastic nucleated erythroblasts (>50%) and myeloblasts (>20%) ▪ Erythroblasts are strongly positive for periodic acid–Schiff (PAS) stain and glycophorin. PAS highlights the abundant glycogen in the tumor cells, the staining will disappear after diastase treatment ▪ Complex cytogenetic abnormalities include: –5q, –7, –3, +8 ▪ AML-M6A is associated with poor prognosis
AML-M6B	▪ In AML-M6B (acute erythroid leukemia is known as pure erythroid leukemia), bone marrow shows dysplastic erythroblasts (>80%) ▪ Erythroblasts are strongly positive for periodic acid–Schiff (PAS) positive and glycophorin ▪ Complex cytogenetic abnormalities include: –5q, –7, –3, +8 ▪ AML-M6B is associated with poor prognosis
AML-M7	▪ In AML-M7 (acute megakaryoblastic leukemia), bone marrow shows micromegakaryoblasts (common in Down syndrome and myelofibrosis) ▪ AML-M7 accounts for <1% of leukemias ▪ Complex cytogenetic abnormalities: +8, +21, inv or del 3p are present ▪ Platelet peroxidase is positive ▪ Diagnosis is confirmed by immunophenotyping (CD41, CD61, platelet peroxidase) or electron microscopy ▪ AML-M7 is associated with poor prognosis
Acute lymphoblastic leukemias	
ALL-L1	▪ In ALL-L1, lymphoblasts are uniform, homogenous with scant cytoplasm with regular nuclei and inconspicuous nucleoli ▪ ALL-L1 accounts for >80% of leukemias ▪ Children are affected associated with good prognosis
ALL-L2	▪ In ALL-L2, lymphoblasts are large, heterogenous with round to oval nuclei showing indentation or clefts with prominent nucleoli ▪ ALL-L2 accounts for 10–50% of leukemias ▪ Adults are affected associated with poor prognosis
ALL-L3	▪ In ALL-L3, lymphoblasts are large homogenous with round to oval nuclei, finely stippled chromatin, prominent nucleoli and abundant basophilic vacuolated cytoplasm ▪ ALL-L3 accounts of 3–4% of leukemias ▪ Adults are affected associated with poor prognosis

CD: Cluster of differentiation.

- According to revised 2024 WHO classification of acute myelogenous leukemia and acute lymphoblastic leukemia, diagnostic criteria are the presence of ≥20% blast cells as the cut off percentage in bone marrow and peripheral blood, which represents a change from original guidelines where blasts ≥30% were considered as diagnostic criteria in bone marrow and peripheral blood as per French-American-British classification.
- The myeloblasts have distinct immunophenotype detected by flow cytometry. In aleukemic leukemia, myeloblasts are detected only in bone marrow.
- Coagulopathy is resulting from disseminated intravascular coagulation (DIC).
- Hyperuricemia is related to increased to high cell turnover and chemotherapy in AML. Rapidly rising serum concentrations of uric acid, potassium phosphate with decreased calcium level herald a tumor lysis syndrome that can cause acute renal failure.
- Muramidase released by leukemic myeloblasts can cause renal tubular dysfunction and electrolyte abnormalities. Lactic acidosis tends to occur due to anerobic glycolysis by leukemic cells.
- Blood biochemical investigations reveal hyperuricemia, elevated blood urea nitrogen and creatinine, high lactic dehydrogenase (LDH), hypokalemia (tubular dysfunction), spurious hypoglycemia and

Table 9.39 Revised 2024 WHO classification of acute myelogenous leukemias (AML) and related precursor neoplasms

AML and Recurrent Genetic Abnormalities	
▪ AML with t(8,21) (q22;q22.1); RUNX1-RUNX1T1	▪ AML with megakaryoblastic leukemia and t(1;22) (p13.3;q13.3); RBM15-MKL1
▪ AML with inv (16) (p13.1q22) or t(16;16) (p13.1;q22); CBFB-MYH11	▪ AML with BCR-ABL1 (provisional entity)
▪ Acute promyelocytic leukemia (APL) with PML-RARA	▪ AML with mutated NPM1
▪ AML with t(9;11) (p21.3;q23.3); KMT2A-MLLT3	▪ AML with biallelic mutations of CEBPA
▪ AML with t(6;9) (p23;q34.1); DEK-NUP214	▪ AML with mutated RUNX1 (provisional entity)
▪ AML with inv(3)(q21.3q26.2) or t(3;3) (q21.3;q26.2); GATA2, MECOM	
AML with Myelodysplasia-related Changes	
Therapy-related Myeloid Neoplasms	
AML, not otherwise specified (NOS)	
▪ AML with minimal differentiation	▪ Pure erythroid leukemia (AML-M6B)
▪ AML without maturation	▪ Acute megakaryoblastic leukemia (AML-M7)
▪ AML with maturation	▪ Acute basophilic leukemia
▪ Acute myelomonocytic leukemia	▪ Acute panmyelosis with myelofibrosis
▪ Acute monoblastic/monocytic leukemia	
Myeloid Sarcoma	
Myeloid Proliferations Related to Down syndrome	
▪ Transient abnormal myelopoiesis (TAM)	▪ Acute myelogenous leukemia of Down syndrome (AML-DS) contains acquired GATA1 mutation
Acute Leukemias of Ambiguous Neoplasms	
▪ Acute undifferentiated leukemia	▪ Mixed-phenotype acute leukemia, NOS (not otherwise specified), rare types
▪ Mixed-phenotype acute leukemia with t(9;22) (q34.1;q11.2); BCR-ABL1	▪ Mixed-phenotype acute leukemia of ambiguous lineage, NOS (not otherwise specified)
▪ Mixed-phenotype acute leukemia with t(v;11q23.3); KMT2A-rearranged	
▪ Mixed-phenotype acute leukemia, B/myeloid, NOS (not otherwise specified)	
▪ Mixed-phenotype acute leukemia, T/myeloid, NOS (not otherwise specified)	

Adapted from revised 2024 WHO classification of myeloid neoplasms and acute myelogenous leukemia.

hypoxemia. Increased serum lactic dehydrogenase (LDH) is associated with central nervous system involvement. Increased number of myeloblasts in circulation can cause spurious hypoglycemia, hypokalemia and other abnormalities result from cellular metabolic activity *in vitro*.

- Hematologic findings reveal increased white blood cell count with myeloblasts in the peripheral blood smear, anemia, granulocytopenia, thrombocytopenia and disseminated intravascular coagulation.
- Imaging techniques such as radiograph, CT scan and MRI scan are performed according to symptoms related to leukemic blasts infiltration in various organs.

ACUTE MYELOGENOUS LEUKEMIA: ENTITIES

Acute myelogenous leukemia (AML) is more common in adults although it may affect any age groups. Diagnostic features, incidence, cytochemistry, immunophenotype and cytogenetics of acute myelogenous leukemia according to French-American-British classification are given in Table 9.40.

AML-M0 WITH MINIMAL DIFFERENTIATION

Acute myelogenous leukemia (AML) with minimal differentiation (AML-M0) accounts for <5% of AML cases. It is thought to be originating from hematopoietic stem cell at the earliest stage of myeloid differentiation. AML-M0 typically occurs in infants and older adults.

- AML-M0 exhibits no morphologic or cytochemical evidence of myeloid differentiation. Myeloblasts in a case of AML-M0 resemble lymphoblasts, with scant, granular cytoplasm, which show block-like periodic acid–Schiff (PAS) positivity, as is typically observed in acute lymphoblastic leukemia, but has also been reported in cases of acute myelogenous leukemia.
- In AML-M0, flow cytometric immunophenotypic analysis reveals evidence of myeloid differentiation based on expression of at least two myeloid markers such as CD13 and CD117, including CD33, HLA-DR and CD34.
- Terminal deoxynucleotidyl transferase (**TdT**) is a DNA polymerase and its positivity typically seen in cases of acute lymphoblastic leukemia (ALL).

Table 9.40 Diagnostic features, incidence, cytochemistry, immunophenotype and cytogenetics of acute myelogenous leukemias according to French-American-British classification

Feature	Cytochemistry	Immunophenotype by Flow Cytometry with Monoclonal Antibodies	Cytogenetics	Comments
AML-M0 with minimal differentiation (<5%)				
Minimal evidence of myeloid differentiation (without maturation with agranular cytoplasm)	▪ Myeloperoxidase + (<3% of blasts) ▪ Sudan black B + (<3% of blasts) ▪ Specific esterase –	▪ CD34++ ▪ HLA-DR+ ▪ CD117+++ ▪ CD33+ ▪ CD13+ ▪ CD11b+/– ▪ CD14–	No unique chromosomal abnormality	AML-M0 originates from hematopoietic precursor cell at the earliest stage associated with poor prognosis
AML-M1 without maturation (20%)				
High % of blasts without significant maturation to more mature neutrophils, blasts demonstrate Auer rods	▪ Myeloperoxidase + (<3% of blasts) ▪ Sudan black B + (<3% of blasts) ▪ Specific esterase +	▪ CD34++ ▪ HLA-DR+++ ▪ CD117+++ ▪ CD33+ ▪ CD13+ ▪ CD14+ ▪ CD11b+/–	–6, –7, –17, del 3p, +21, +8	Favorable prognosis
AML-M2 with maturation (30–40%)				
All stages of neutrophil maturation, pseudo-Pelger-Hüet anomaly, hypogranulation	▪ Myeloperoxidase + (>10%) ▪ Sudan black B + ▪ Specific esterase +	▪ CD34– ▪ HLA-DR+ ▪ CD33+ ▪ CD13+	t(8;21), del 3p, or inv 3, –5, –7,+8, t(6;9)	Favorable prognosis
AML-M3 (5–10%)				
Acute promyelocytic leukemia (APL) with hypergranular with bilobed heavy contour	Myeloperoxidase +	▪ CD34– ▪ HLA-DR– ▪ CD33+ ▪ CD13+	t(15,17)	Disseminated intravascular coagulation associated with poor prognosis
AML-M3m (5–10%)				
Acute promyelocytic leukemia (APL) with microgranular variant	Myeloperoxidase ++	▪ CD34– ▪ HLA-DR– ▪ CD33+ ▪ CD13+	t(15,17)	Favorable prognosis
AML-M4 (5–10%)				
Acute myelomonocytic leukemia (monocytes and promonocytes in the bone marrow exceed 20%)	▪ Myeloperoxidase + ▪ Esterase +	▪ CD34– ▪ HLA-DR+ ▪ CD33+ ▪ CD13+ ▪ CD14/CD64+	Inv16, t(16;16), –5, –7, t(6;9)	Favorable prognosis
AML-M4Eo (5–10%)				
Acute myelomonocytic leukemia with abnormal eosinophils (>5%)	▪ Myeloperoxidase + ▪ Esterase +	▪ CD34– ▪ HLA-DR+ ▪ CD33+ ▪ CD13+ ▪ CD11+ ▪ CD14+	Inv16	Favorable prognosis

Contd...

Table 9.40 Diagnostic features, incidence, cytochemistry, immunophenotype and cytogenetics of acute myelogenous leukemias according to French-American-British classification (*Contd...*)

Feature	Cytochemistry	Immunophenotype by Flow Cytometry with Monoclonal Antibodies	Cytogenetics	Comments
AML-M5A, acute monoblastic/monocytic leukemia-5A (5%)				
Monoblasts/monocytes dominance (≥80%), hemophagocytosis, nuclear lobulation	▪ Myeloperoxidase – ▪ Specific esterase + ▪ Nonspecific esterase +/–	▪ CD34– ▪ HLA-DR+ ▪ CD33+ ▪ CD13+/– ▪ CD11+ ▪ CD14+	t(9,11) (p21–23)	Extramedullary disease associated with poor prognosis
AML-M5B, acute monoblastic leukemia-5B (5%)				
Monoblasts/monocytes dominance	▪ Myeloperoxidase – ▪ Specific esterase + ▪ Nonspecific esterase +/–	▪ CD34– ▪ HLA-DR+ ▪ CD33+ ▪ CD13+/– ▪ CD11+ ▪ CD14+	t(9,11) (p21–23)	Extramedullary disease associated with poor prognosis
AML-M6, acute erythroid leukemia (uncommon)				
▪ >50% erythroid precursors ▪ >20% myeloblasts in the nonerythroid cell population ▪ Megakaryoblastoid morphology, ▪ Auer rods+/–	▪ PAS ++ (erythroblasts) ▪ Myeloperoxidase – ▪ Sudan black B + ▪ Nonspecific esterase –	▪ Glycophorin A+ ▪ Hemoglobin A+ ▪ CD33+++	–5, –7, –3, +8	Poor prognosis
AML-M7, acute megakaryoblastic leukemia (<5%)				
Cytopenia with or without thrombocytopenia, basophilic agranular blasts with pseudopods, micromegakaryocytes	▪ Platelet peroxidase + ▪ Myeloperoxidase – ▪ Sudan black B – ▪ PAS +/–	▪ HLA-DR+ ▪ CD117+ ▪ CD34+ ▪ CD13+ ▪ CD33+++ ▪ CD41+ ▪ CD42+ ▪ CD61+ ▪ CD11b+ ▪ Electron microscopy	+8. +21, inv/del 3p	Poor prognosis

MPO: Myeloperoxidase; PAS: Periodic acid–Schiff; CD: Cluster of differentiation; Myeloid markers: CD13, CD33; Monocyte markers: CD11, CD14.

TdT positivity is observed in 50% of cases of AML-M0, but myeloblasts are negative for B cell and T cell markers. AML-M0 may exhibit complex karyotypes and unbalanced abnormalities such as –5, del(5q), –7, del(7q), +8 and del(11q).

- Myeloblasts in AML-M0 do not express more than one cell membrane marker for myeloid lineage. Myeloblasts in AML-M0 are negative for myeloperoxidase (MPO).
- It is important to recognize that AML-M0 is of myeloid cell lineage origin, so that patients may be treated with appropriate therapy for AML. Patient presents with evidence of bone marrow failure usually associated with poor prognosis.

Laboratory Diagnosis of Acute Myelogenous Leukemia M0 (AML-M0, FAB)	
Peripheral Blood Smear	
Myeloblasts in AML-M0 are medium in size with round or slightly indented nuclei and one or two nucleoli and agranular cytoplasm. Peripheral blood smear findings in acute myelogenous leukemia M0 (AML-M0) are shown in Fig. 9.39.	
Bone Marrow Smear Examination	
Bone marrow aspirate smear is hypercellular. Myeloblasts in AML-M0 are medium in size with round or slightly indented nuclei and one or two nucleoli and agranular cytoplasm.	
Cytochemistry	
Less than 3% of myeloblasts in AML-M0 are positive for myeloperoxidase and Sudan black B stains.	
Immunophenotyping	
▪ Myeloblasts in AML-M0 demonstrate myeloid differentiation by flow cytometry for myeloid lineage (CD13 and CD33). Most AML-M0 cases are also positive for HLA-DR, CD34 and CD38. ▪ No unique chromosomal abnormalities are observed in this subcategory. Because myeloblasts in AML-M0 have no morphological differentiating features, immunophenotyping should be performed to exclude the lymphoid lineage.	
Markers	**Expression**
▪ CD34 ▪ CD38 ▪ HLA-DR ▪ CD117 ▪ CD13 ▪ CD33	▪ Positive ▪ Positive ▪ Positive ▪ Positive (some cases) ▪ Positive (some cases) ▪ Positive (some cases)
Myeloid and monocytic maturation associated antigens are not expressed such as CD11b, CD15, CD14, CD64 and CD65 in AML-M0 cases.	

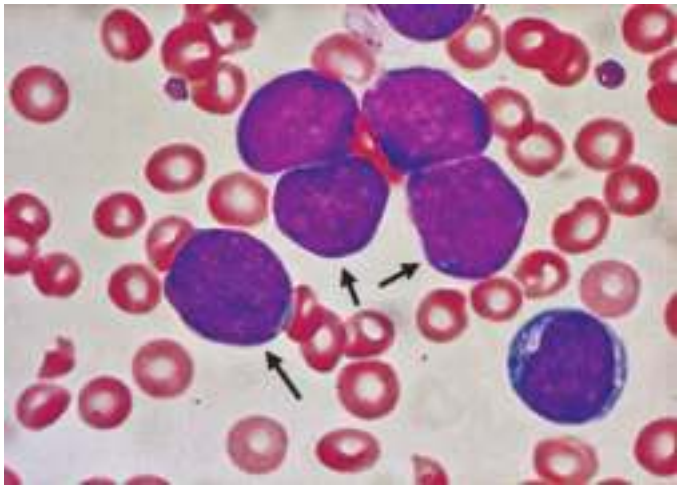


Fig. 9.39: Acute myelogenous leukemia M0 (AML-M0, FAB) in Giemsa-stained peripheral blood smear. It shows blasts in AML with minimal differentiation. The blasts are large with scant amount of cytoplasm, high nucleocytoplasmic ratio, fine nuclear chromatin and 2–4 prominent nucleoli (arrows) (1000X).

AML-M1 WITHOUT MATURATION

Acute myelogenous leukemia (AML) without maturation (AML-M1) is characterized by a high percentage (>90%) of bone marrow myeloblasts without significant evidence of maturation to more mature neutrophils.

- AML-M1 can affect any age group but most commonly in adult persons, which accounts for 5–10% of AML cases.
- Patients develop features of bone marrow failure such as anemia, thrombocytopenia and neutropenia. More than 3% of myeloblasts show myeloperoxidase (MPO) or Sudan black B positivity.
- The myeloblasts may contain azurophilic granules, Auer rods and vacuoles. Myeloblasts lacking azurophilic granules or Auer rods can resemble lymphoblasts, hence immunophenotyping helps to differentiate these blasts. Myeloblast in AML-M1 is positive for CD34, HLA-DR, CD33 and CD13. Prognosis in patients with AML-M1 is usually favorable.

Laboratory Diagnosis of Acute Myelogenous Leukemia M1 (AML-M1, FAB)	
Peripheral Blood Smear Examination	
Blasts resemble myeloblasts in some cases show azurophilic granules and Auer rods. In some cases, blasts resemble lymphoblasts. Acute myelogenous leukemia M1 (AML-M1, FAB) with minimal differentiation in Giemsa-stained peripheral blood smear is shown in Fig. 9.40.	
Bone Marrow Smear Examination	
In bone marrow aspirate smear examination, myeloblasts show presence of azurophilic granules and Auer rods. In some cases, myeloblasts resemble lymphoblasts.	
Cytochemistry	
More than 3% of myeloblasts in AML-M1 show positivity for myeloperoxidase (MPO) or Sudan black B.	
Immunophenotyping	
Myeloblasts in AML-M1 show positivity for CD13, CD33, CD117, HLA-DR and CD34.	
Markers	**Expression**
CD13 CD33 CD117 HLA-DR CD3	▪ Positive ▪ Positive ▪ Positive ▪ Positive ▪ Positive

AML-M2 WITH MATURATION

Acute myelogenous leukemia (AML) with maturation (AML-M2) is characterized by presence of ≥20% myeloblasts with evidence of maturation to more neutrophils (>10% of cells at different stages of maturation comprising of myeloblasts, promyelocytes, myelocytes

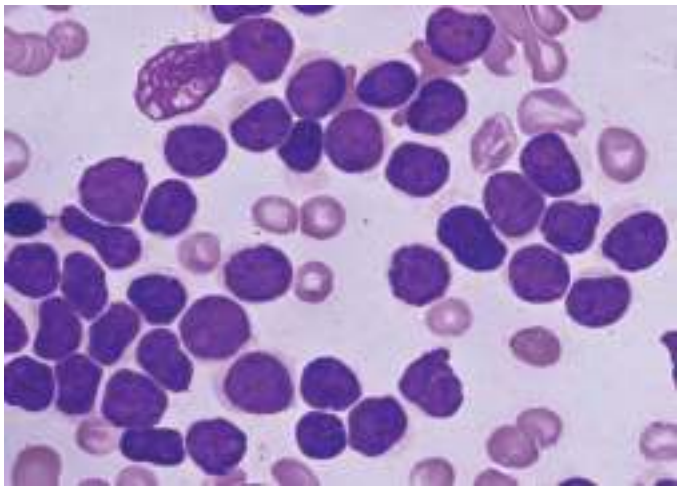


Fig. 9.40: Acute myelogenous leukemia M1 (AML-M1, FAB) with minimal differentiation in Giemsa-stained peripheral blood smear. It shows blasts with maturation with opened up chromatin with high nucleocytoplasmic ratio, 2–4 prominent nucleoli and moderate amount of cytoplasm (1000X).

and metamyelocytes) in the peripheral blood and bone marrow. Monocytes and their precursors constitute <20% of bone marrow cells.

- AML-M2 affects all age groups. It accounts for 30–40% of AML cases. Patients present with anemia, neutropenia, and thrombocytopenia.
- Myeloblasts frequently contain Auer rods. Pseudo-Pelger-Hüet anomaly and hypogranulation and binucleated cells may be present.
- About 10% of myeloblasts are positive for myeloperoxidase and Sudan black B cytochemical stain. Myeloblasts are positive for CD13, CD33 and HLA-DR, but negative for CD34.

Laboratory Diagnosis of Acute Myelogenous Leukemia M2 (AML-M2, FAB)

Peripheral Blood Smear

- Myeloblasts with Auer rods and azurophilic granules are seen along with promyelocytes, myelocytes and mature neutrophils comprising up to 10% of bone marrow cells.
- Acute myelogenous leukemia M2 (AML-M2, FAB) with maturation in Giemsa-stained peripheral blood smear is shown in Fig. 9.41.

Bone Marrow Smear Examination

In bone marrow aspirate smear examination, myeloblasts with Auer rods and azurophilic granules are seen along with promyelocytes, myelocytes and mature neutrophils comprising up to 10% of bone marrow cells.

Cytochemistry

Myeloblasts show positivity with myeloperoxidase stain. Peripheral blood smear showing MPO (myeloperoxidase) positivity in AML with maturation (AML-M2).

Immunophenotyping

Myeloblasts express positivity for CD13, CD33, CD65, CD11b and CD15.

Markers	Expression
▪ CD13	▪ Positive
▪ CD33	▪ Positive
▪ CD65	▪ Positive
▪ CD11b	▪ Positive
▪ CD15	▪ Positive

ACUTE PROMYELOCYTIC LEUKEMIA (AML-M3, FAB)

Acute promyelocytic leukemia (AML-M3) can be either hypergranular or microgranular/hypogranular, which accounts for 5–8% of AML cases. Median age of AML-M3 is 35–40 years but can occur at any age. Hypergranular variant of AML-M3 shows abnormal promyelocytes with numerous coarse azurophilic granules, folded nuclei resembling cottage loaf. Leukocyte count is decreased.

Diagnostic Criteria

Acute promyelocytic leukemia (AML-M3) is diagnosed by demonstration of >50% abnormal myelocytes with heavy cytoplasmic granules that may obscure the nuclear cytoplasmic margin, often reniform or bilobed nucleus, cells with multiple Auer rods. Abnormal promyelocytes show strong positivity for myeloperoxidase stain.

Clinical Features

Senescent leukemic cells degranulate and release thromboplastin-like substance into the peripheral blood and can cause disseminated intravascular coagulation (**DIC**) and hemorrhage before or during induction of

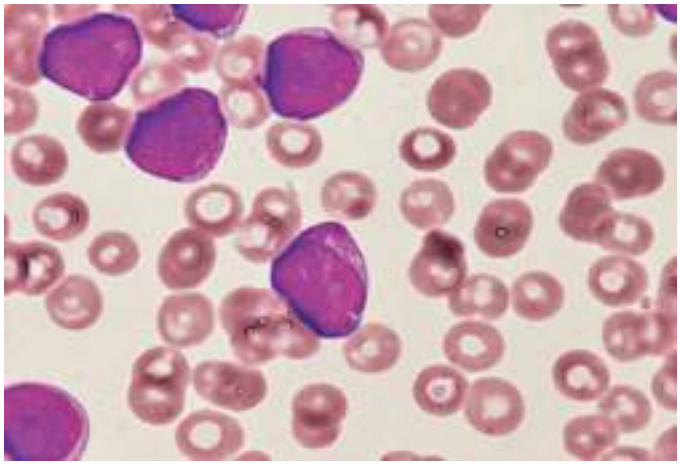


Fig. 9.41: Acute myelogenous leukemia M2 (AML-M2, FAB) with maturation in Giemsa-stained peripheral blood smear. It shows myeloblasts with promyelocyte, myelocyte and band forms of polymorphonuclear cells are showing evidence of maturation (1000X).

chemotherapy, which may cause early death. Organomegaly and extramedullary disease and skin involvement rarely occur.

Molecular Genetic Alterations

Acute promyelocytic leukemia (AML-M3) is associated with the translocation t(15;17) (q22;q21), which disrupts the retinoic acid receptor-α (RARA) gene on chromosome 17q21 and the PML gene on chromosome 15q22 and results in the formation of hybrid mRNA, that produces abnormal retinoic acid receptor leading to blockage of myeloid differentiation at the promyelocytic stage.

- Hybrid mRNA transcript is demonstrated by fluorescence *in situ* hybridization (FISH). Currently AML-M3 is classified under AML with recurrent genetic abnormalities.
- Cases without Auer rods in leukemic cells usually demonstrate additional chromosomal abnormalities. Cytogenetic abnormalities in acute promyelocytic leukemia are given in Table 9.41.

Treatment and Remission

Remission of AML-M3 is achieved by administration of oral all-*trans* retinoic acid variant of vitamin A analogue (ATRA), a form of differentiating therapy that overcomes the maturation arrest. Arsenic trioxide (ATO) for ATRA-refractory patients; induces differentiation at low doses, but higher doses cause bone marrow necrosis. All variants of AML except promyelocytic leukemia are treated by continuous infusion of cytarabine for 7 days and a 3-day course of daunorubicin followed by autologous hematopoietic stem cell transplant (HSCT) and allogeneic HSCT.

Laboratory Diagnosis of Acute Myelogenous Leukemia M3 (AML-M3,FAB)

Routine Hematologic Tests

Hemoglobin and hematocrit values (MCH, MCHC, MCV, RDW) are decreased. Total leukocyte count and erythrocyte sedimentation rate are increased.

Hemostatic Function Tests

Hemostatic function tests include prothrombin–INR time, activated partial thromboplastin time, fibrinogen levels, fibrinogen degradation products (**FDPs**), platelet count, plasma LDH level and plasma and urine lysozyme levels.

Peripheral Blood Smear Examination

- Acute myelogenous leukemia M3 (AML-M3, FAB), also called acute promyelocytic leukemia with hypergranular variant is diagnosed by demonstration of >50% abnormal promyelocytes with heavy cytoplasmic granulation that may obscure the nuclear cytoplasmic margin, often reniform or bilobed nucleus, cells with multiple Auer rods.
- Peripheral blood smear examination in AML-M3 shows either hypergranular (AML-M3, FAB) or hypogranular (AML-M3V) picture, normocytic and normochromic anemia, nucleated red blood cells, neutropenia and monocytosis.
- Acute myelogenous leukemia M3 (AML-M3, FAB), also called acute promyelocytic leukemia with hypergranular variant in Giemsa-stained peripheral blood smear is shown in Fig. 9.42.
- Acute myelogenous leukemia M3 (AML-M3V, FAB), also called acute promyelocytic leukemia with hypogranular variant in Giemsa-stained peripheral blood smear is shown in Fig. 9.43.

Bone Marrow Smear Examination

Bone marrow examination shows hypercellular marrow with high myeloid to erythroid ratio and predominant promyelocytes.

Bone Marrow Trephine Biopsy Examination

- Bone marrow trephine biopsy obtained section demonstrates numerous abnormal hypergranular promyelocytes containing abundant cytoplasm and round/oval eccentric nuclei with occasional clefts or indentations/reniform nuclei with moderate condensed chromatin and indistinct nuclei; and heavy red/purple cytoplasmic granules that may obscure nuclear borders.
- About 90% of abnormal promyelocytes contain multiple Auer rods.
- Acute myelogenous leukemia M3 (AML-M3, FAB) in hematoxylin and eosin-stained bone marrow trephine biopsy is shown in Fig. 9.44.

Cytochemistry

Leukemic promyelocytes in AML-M3 show strong positivity for myeloperoxidase stain.

Immunophenotyping

Acute promyelocytic leukemia (AML-M3) shows positivity for CD9, CD11a and CD11b.

Markers	Expression
▪ CD9	▪ Positive
▪ CD11a	▪ Positive
▪ CD11b	▪ Positive

ACUTE MYELOMONOCYTIC LEUKEMIA (AMML/AML-M4, FAB)

Acute myelomonocytic leukemia (AMML/AML-M4) is characterized by proliferation of myelocytic and monocytic precursors in the bone marrow and peripheral blood, which usually affects elderly persons. Some patients have family history of AMML.

- The cell of origin of AMML is believed to be a hematopoietic precursor cell, that has potential to differentiate into neutrophil and monocytic lineage.
- Bone marrow in AMML is hypercellular, which shows ≥20% lymphoblasts, neutrophils and their precursors, and monocytes and their precursors. Each lineage is composed of ≥20% of bone marrow cells. AMML/AML-M4 accounts for 5–10% of AML cases.

Table 9.41 Cytogenetic abnormalities in acute promyelocytic leukemia

Chromosomal Translocation	Genes Involved	All-*trans* Retinoic Acid Response
t(15;17) (q21;q11)	PML-RARA	Yes
t(11;17) (q13;q11)	NuMA-RARA	Yes
t(5;17) (q31;q11)	NPM-RARA	Yes
t(11;17) (q13;q11)	PLZF-RARA	No

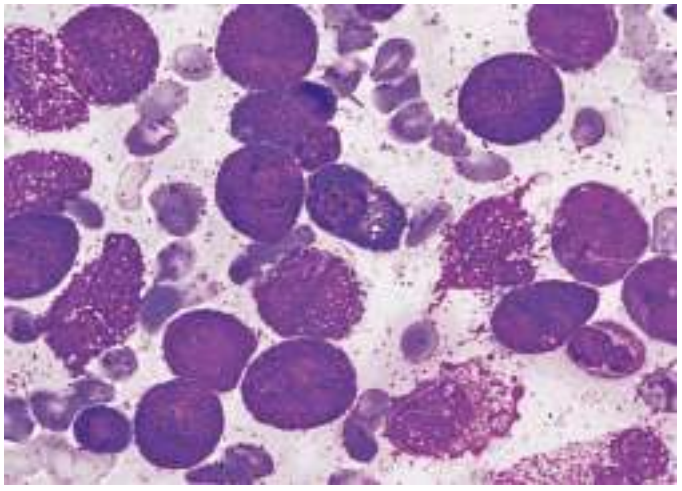


Fig. 9.42: Acute myelogenous leukemia M3 (AML-M3, FAB), also called acute promyelocytic leukemia with hypergranular variant in Giemsa-stained peripheral blood smear examination. It shows numerous promyelocytes, which contain an irregularly shaped nucleus and numerous azurophilic granules (1000X).

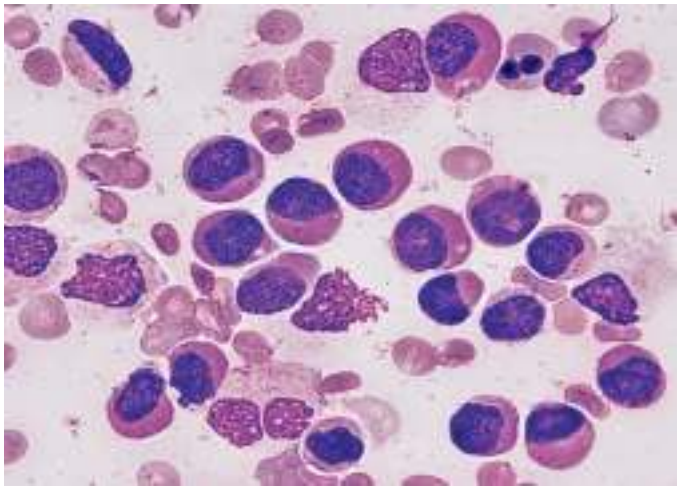


Fig. 9.43: Acute myelogenous leukemia M3 (AML-M3, FAB) also known as acute promyelocytic leukemia with hypogranular variant in Giemsa-stained peripheral blood smear examination. It shows abnormal promyelocytes, which show bilobed nuclei and absence of granules to fine granules (1000X).

- In AML-M4, extramedullary involvement is most often demonstrated. Cytogenetic analysis demonstrates inversion of 16, t(16;16), t(6;9) and loss of chromosomes 5 and 7.

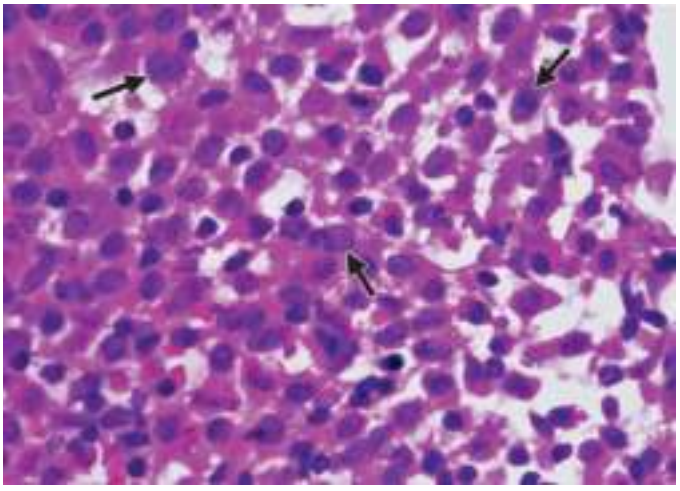


Fig. 9.44: Acute myelogenous leukemia M3 (AML-M3, FAB) in hematoxylin and eosin-stained bone marrow trephine biopsy. It shows hypercellularity and abnormal promyelocytes with moderate amount of cytoplasm and round to oval nuclei (arrows) (400X).

- Serum and urinary levels of muramidase (lysozyme) are most often elevated as a result of monocytic proliferation. Prognosis is favorable in AMML/AML-M4.
- Additional laboratory investigations of AML-M4 are essential when the bone marrow findings are as previously described, but the peripheral blood monocyte count is either $<5\times10^9$/L; or the peripheral blood monocyte count is $\geq5\times10^9$/L. In these cases, serum muramidase levels and cytochemical stains can be performed to establish AMML diagnosis.

Laboratory Diagnosis of Acute Myelomonocytic Leukemia (AMML/AML-M4, FAB)

Peripheral Blood Smear Examination

- Peripheral blood leukocyte count in acute myelomonocytic leukemia (AMML/AML-M4) is most often elevated. Monocytic lineage cells such as monoblasts, promonocytes and monocytes are elevated to $\geq5\times10^9$/dl.
- Anemia and thrombocytopenia are present in almost all cases. Myeloblasts are also present.
- Monoblasts are large cells with round to convoluted nucleus with delicate chromatin and one or more nucleoli; and abundant bluish-gray cytoplasm showing pseudopods. Monoblasts may demonstrate azurophilic granules and vacuoles.

- If peripheral blood smear examination shows monocyte count <5 × 10⁹/L; increased serum muramidase levels and cytochemical stains can be performed to establish the diagnosis of AML-M4.
- Acute myelogenous leukemia M4 (AML-M4, FAB), also known as acute myelomonocytic leukemia in Giemsa-stained peripheral blood smear examination is shown in Fig. 9.45.

Bone Marrow Smear Examination

- Bone marrow in acute myelomonocytic leukemia (AMML/AML-M4) is hypercellular and showing **≥20%** myeloblasts; neutrophils and their precursors; and monocytes and their precursors.
- Bone marrow can demonstrate hemophagocytosis by monocytes.

Cytochemistry

AMML/AML-M4 shows positivity for myeloperoxidase and non-specific esterase.

Immunophenotyping

AMML (AML-M4) demonstrates positivity for HLA-DR, CD33, CD34, CD13, CD14.CD68 and CD64.

- In normal circumstances, CD34 is a transmembrane glycoprotein expressed on 1–2% of the total bone marrow lymphohematopoietic stem cells and progenitor cells. About 40% of AMLs and ≥50% of ALLs express CD34. CD34 is also expressed in variety of nonhematopoietic neoplasms. CD34 expression in acute myelogenous leukemia M4 (AML-M4, FAB) in bone marrow trephine biopsy section is shown in Fig. 9.46.
- CD68, a hematopoietic differentiation marker of the monocytic-macrophage lineage is expressed in AML and CML. CD68 expression in acute myelogenous leukemia M4 (AML-M4, FAB) in bone marrow trephine biopsy section is shown in Fig. 9.47.

Markers	Expression
HLA-DR	Positive
CD33	Positive
CD13	Positive
CD14	Positive
CD64	Positive
CD34	Positive
CD68	Positive

ACUTE MONOBLASTIC/MONOCYTIC LEUKEMIA (AML-M5A AND AML-M5B, FAB)

Acute monoblastic/monocytic leukemia (AML-M5A, AML-M5B) is characterized by ≥80% of the leukemic cells comprising monoblasts, promonocytes, monocytes. Neutrophil component in AML-M5A/AML-M5B constitutes <20% of neutrophils.

- The majority of the monocytic cells (>80%) demonstrated in AML are monoblasts. The majority of monocytic cells in AML are promonocytes.

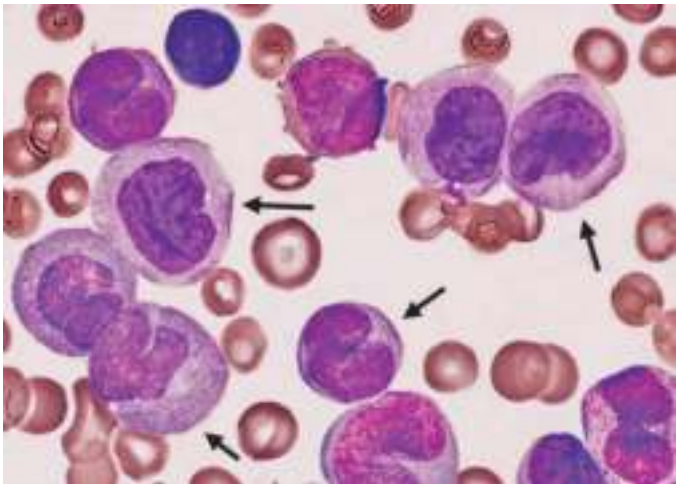


Fig. 9.45: Acute myelogenous leukemia M4 (AML-M4, FAB), also known as acute myelomonocytic leukemia in Giemsa-stained peripheral blood smear examination. It shows atypical cells of monocytic lineage (arrows) (1000X).

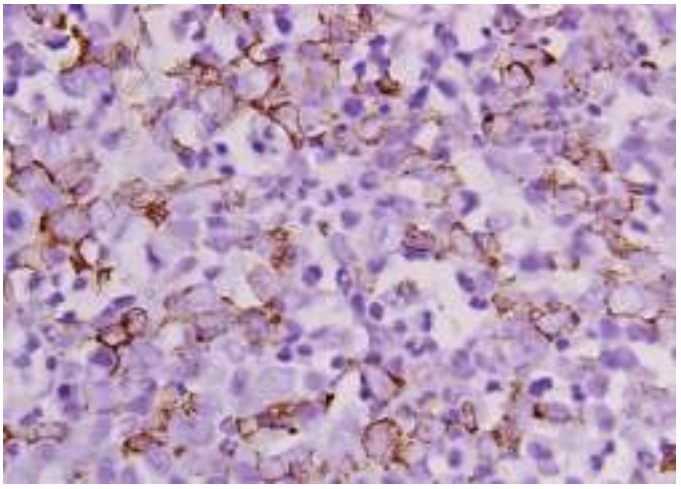


Fig. 9.46: CD34 expression in acute myelogenous leukemia M4 (AML-M4, FAB) in bone marrow trephine biopsy section. In normal circumstances, CD34 is a transmembrane glycoprotein expressed on 1–2% of the total bone marrow lymphohematopoietic cells and progenitor cells. About 40% of AMLs and over 50% of ALLs express CD34. CD34 is also expressed in variety of nonhematopoietic neoplasms (400X).

- Acute monoblastic leukemia accounts for 5–8% of AML cases. Acute monocytic leukemia accounts for 3–6% of AML cases. The disease usually affects children and young adults.
- There are two subtypes of AML-M5: (a) AML-M5A (monoblastic type with >80% blasts), and (b) AML-M5B (monocytic type with <80% blasts). AML-M5A is more common in young individuals, whereas AML-M5B commonly in adults.

Molecular Genetic Alterations

Molecular genetic alterations of the long arm of chromosome 11 with translocations or deletions are most often demonstrated in acute monocytic leukemias.

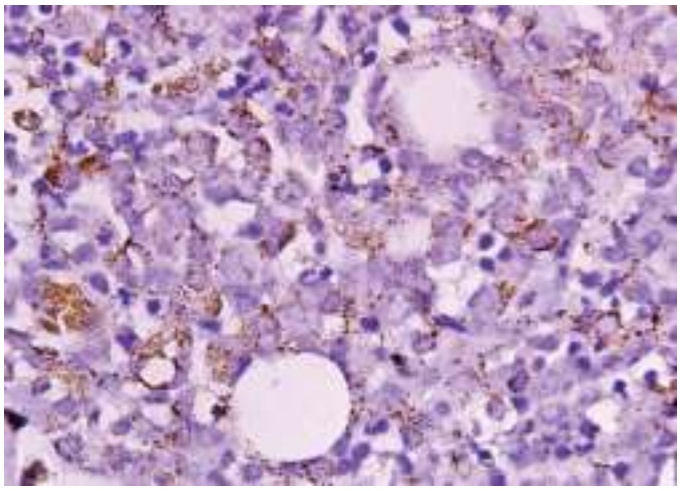


Fig. 9.47: CD68 expression in acute myelogenous leukemia M4 (AML-M4, FAB) in bone marrow trephine biopsy section (1000X). CD68, a hematopoietic differentiation marker of the monocytic-macrophage lineage is expressed in various human malignancies including acute myelogenous leukemia and chronic myelogenous leukemia (400X).

- The chromosomal t(8;16) abnormality is associated with hemophagocytosis. The expression of c-Fos proto-oncogene on chromosome 14 appears to be increased in acute leukemias with monocytic lineage.
- The expression of c-Fos proto-oncogene on chromosome 14 has been linked to normal monocyte-macrophage differentiation. The cell of origin for AML-M5 subtype is believed to be a hematopoietic stem cell committed to monocytic differentiation (CFU-M).

Clinical Features

Patient presents with weakness, bleeding gums and diffuse erythematous skin rash. In extramedullary disease, leukemic cells infiltrate gums, skin, lymph nodes, lungs and central nervous system. Extramedullary disease is associated with poor prognosis.

Laboratory Diagnosis of Acute Monoblastic/Monocytic Leukemia (AML-M5A and AML-M5B, FAB)

Biochemical Tests

Serum and urine muramidase are moderately elevated.

Peripheral Blood Smear Examination

- **AML-M5A:** In acute monoblastic/monocytic leukemia, peripheral blood smear shows increased number of monoblasts and monocytes in AML-M5A.
  - Monoblasts are large cells with round or oval nucleus with delicate chromatin and one or more nucleoli and abundant basophilic cytoplasm with pseudopod formation and scattered fine azurophilic granules and vacuoles. Auer rods are not demonstrated in leukemic cells.
  - Promonocytes contain more irregular and convoluted nucleus and nucleoli; and less basophilic cytoplasm than that of monoblasts giving ground glass appearance. Acute myelogenous leukemia (AML-M5A), also known as acute monoblastic and monocytic leukemia in Giemsa-stained peripheral blood smear examination is shown in Fig. 9.48.
- **AML-M5B:** In acute monoblastic/monocytic leukemia more mature forms of monocytic series are demonstrated in peripheral blood smear in AML-M5B. Promonocytes predominate with delicately convoluted nucleus, less basophilic cytoplasm and more obvious azurophilic granules. In Giemsa-stained peripheral blood smear examination is shown in Fig. 9.49.

Bone Marrow Smear Examination

- It is essential to evaluate both peripheral blood smear and bone marrow examination for AML-M5A and AML-M5B.
- Bone marrow aspirate smear examination shows ≥80% monoblasts and promonocytes.

Bone Marrow Trephine Biopsy Examination

- Hematoxylin and eosin-stained bone marrow trephine biopsy section shows sheets of more mature forms of monocytic series with delicately convoluted nucleus.
- Acute monoblastic/monocytic leukemia M5B (AML-M5B) in hematoxylin and eosin-stained bone marrow trephine biopsy section is shown in Fig. 9.50.

Cytochemistry

Leukemic cells in AML-M5 are positivity for specific esterase and nonspecific esterase. These cells are negative for myeloperoxidase stain.

Immunophenotyping

AML-M5 leukemic cells variably express CD13, CD33, CD15 and CD65. At least two markers of monocytic differentiation are positive such as CD14, CD11b, CD64, CD68, CD36 and lysozyme.

Markers	Expression
CD13	Positive
CD33	Positive
CD15	Positive
CD65	Positive
CD14	Positive
CD11b	Positive
CD64	Positive
CD68	Positive
CD36	Positive
Lysozyme	Positive
CD34	Negative

ACUTE ERYTHROID LEUKEMIA (AML-M6, FAB)

Acute erythroid leukemia (AML-M6, FAB) is defined as a subtype of AML-NOS with predominance of erythroid precursors. The vast majority (>90%) are of erythroid/myeloid (AML-M6A) subtype. AML-M6 is predominantly a disease of adult persons.

- Acute erythroid leukemia (AML-M6A) subtype with predominance of erythroid precursors (or acute

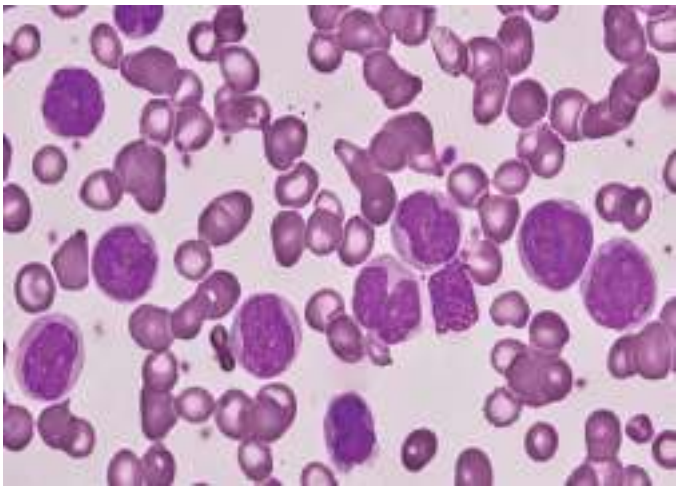


Fig. 9.48: Acute myelogenous leukemia M5A (AML-M5A), also known as acute monoblastic/monocytic leukemia in Giemsa-stained peripheral blood smear examination. It shows numerous monoblasts with round to oval nuclei with delicate chromatin, 1–2 prominent nucleoli, abundant basophilic cytoplasm with pseudopod formation and scattered fine azurophilic granules and vacuoles. Auer rods are not demonstrated in leukemic cells. Promonocytes contain more irregular and convoluted nucleus and nucleoli; and less basophilic cytoplasm than that of monoblasts giving ground glass appearance (1000X).

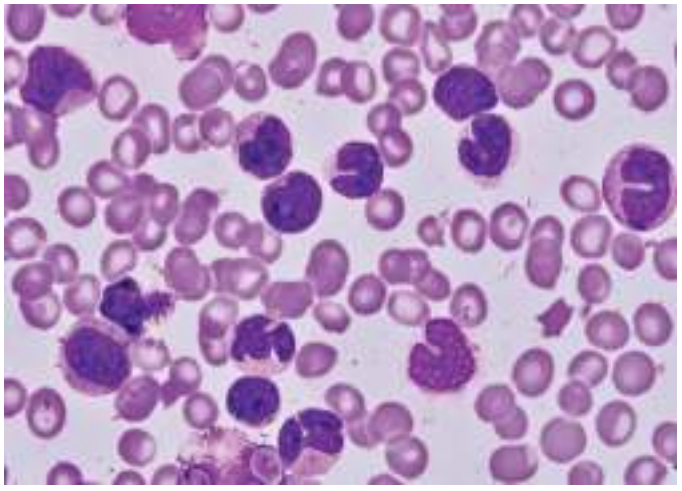


Fig. 9.49: Acute myelogenous leukemia M5B (AML-M5B), also known as acute monoblastic/monocytic leukemia in Giemsa-stained peripheral blood smear examination. It shows more mature forms of monocytic series, i.e. promonocytes and monoblasts. Promonocytes predominate with delicately convoluted nucleus, less basophilic cytoplasm and more obvious azurophilic granules; and monoblasts (1000X).

Di Guglielmo syndrome) is defined as the presence of ≥50% erythroid precursors and ≥20% lymphoblasts in the nonerythroid component affecting adults.

- The pure erythroid leukemia (AML-M6B) subtype is composed of ≥80% of more immature erythroblasts affecting any age group.
- Occasional case of CML can transform into AML-M6. Patient presents with profound anemia resulting in poor survival.

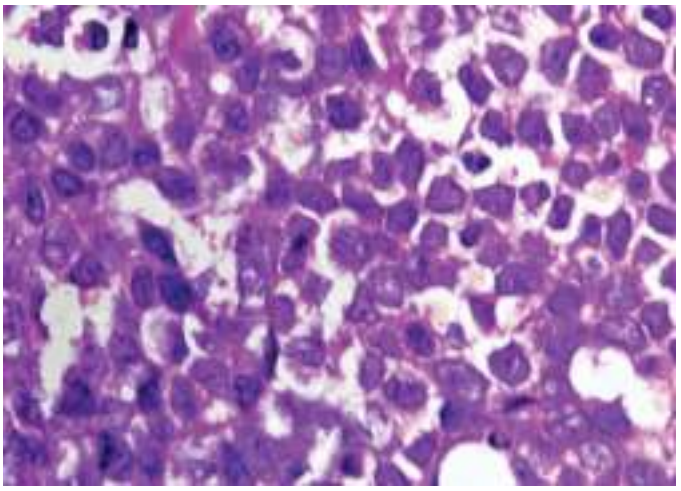


Fig. 9.50: Acute myelogenous leukemia M5B (AML-M5B), also known as acute monoblastic/monocytic leukemia in hematoxylin and eosin-stained bone marrow trephine biopsy section. It shows sheets of more mature forms of monocytic series with delicately convoluted nucleus (400X).

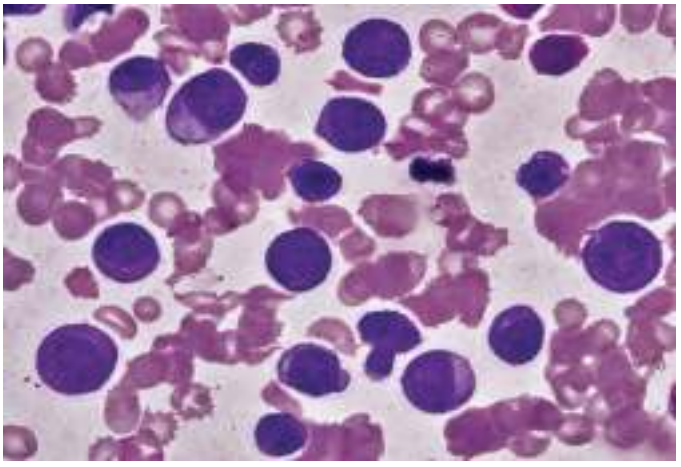


Fig. 9.51: Acute erythroid leukemia (AML-M6, FAB) in Giemsa-stained peripheral blood smear. It shows profound anemia with anisocytosis and poikilocytosis with a large number of nucleated red blood cells, that are extremely dysplastic with megaloblastoid nuclei and/or binucleated or multinucleated cells in the immature stages with vacuoles in the cytoplasm. Cytoplasmic pseudopods leukemic erythroid cells similar to those of acute megakaryocytic leukemia M7 (AMKL-M7) are demonstrated (1000X).

Laboratory Diagnosis of Acute Erythroid Leukemia (AML-M6, FAB)

Peripheral Blood Smear Examination

- Peripheral blood smear examination shows profound anemia with anisocytosis and poikilocytosis with many nucleated red blood cells, that are extremely dysplastic with megaloblastoid nuclei and/or binucleated or multinucleated cells in the immature stages with vacuoles in the cytoplasm.
- Cytoplasmic pseudopods leukemic erythroid cells similar to those in acute megakaryoblastic leukemia (AML-M7) may be present. Acute erythroid leukemia (AML-M6, FAB) in Giemsa-stained peripheral blood smear is shown in Fig. 9.51.

Bone Marrow Smear Examination

- Bone marrow examination demonstrates leukemic erythroid blasts distinctly abnormal bizarre morphologic features such as giant multilobular or multinucleated forms in AML-M6.
- Other morphologic features of leukemic erythroid blasts include nuclear budding and fragmentation, cytoplasmic vacuoles, Howell-Jolly bodies.
- Ring sideroblasts with megaloblastoid nuclei are demonstrated. Myeloblasts in mixed lineage variant contain Auer rods. Dysmegakaryopoiesis with mononuclear forms or micromegakaryoblasts are present.
- Neutrophils can demonstrate hypogranularity and pseudo-Pelger-Hüet anomaly. Leukocytes and platelets are decreased. Acute erythroid leukemia (AML-M6, FAB) in Giemsa-stained bone marrow aspirate smear is shown in Fig. 9.52.

Cytochemistry

- The leukemic erythroid blasts are myeloperoxidase (MPO) negative, but often positive for nonspecific esterase. Leukemic erythroid cells are PAS positive and forming positive lakes.
- Periodic acid–Schiff (PAS) positive erythroblasts are occasionally demonstrated in myelodysplastic syndrome (MDS), other subgroups of AML, thalassemia, iron deficiency anemia, severe hemolytic anemia and sometimes in megaloblastic anemia. Periodic acid–Schiff (PAS) stain positivity in various hematologic disorders is given in Table 9.42.
- Myeloblastic component in acute erythroleukemia demonstrates reactions similar to those found in other subtypes of AML in both morphological features and chromosomal aberrations.

Immunophenotyping

- Flow cytometry is performed to diagnose AML-M6 cases. Erythroblasts usually react positively with monoclonal antibodies to glycophorin A (CD71), hemoglobin A and CD33.
- The erythroid component in AML-M6 lacks myeloperoxidase (MPO), CD34, CD45 and pan-myeloid markers. CD117 and CD43 are often positive, when myeloblasts are present.
- AML-M6A (mixed lineage) cell of origin is thought to a primitive progenitor cell committed to the erythroid lineage (BFU-E or CFU-E).

Markers	Expression
▪ Glycophorin A	▪ Positive
▪ Hemoglobin A	▪ Positive
▪ CD33	▪ Positive

ACUTE MEGAKARYOBLASTIC LEUKEMIA (AMKL-M7, FAB)

Acute megakaryoblastic leukemia (AMKL-M7), subgroup of AML is defined by presence of 50% of the leukemic blasts of megakaryoblastic lineage, which accounts for <5% of AML cases. It occurs in adults and children.

- Acute megakaryoblastic leukemia (AMKL-M7) excludes AML with myelodysplasia-related changes such as AML with t(1;22) (p13;q13), inv(3)(q21;q26), t(3;3)(q21;q26.2), and Down syndrome-related cases. Patient presents with cytopenia, thrombocytopenia and sometimes with increased platelet count.
- There is no significant hepatosplenomegaly. Neutrophils and platelets can demonstrate dysplastic features. AML-M7 is associated with poor prognosis.

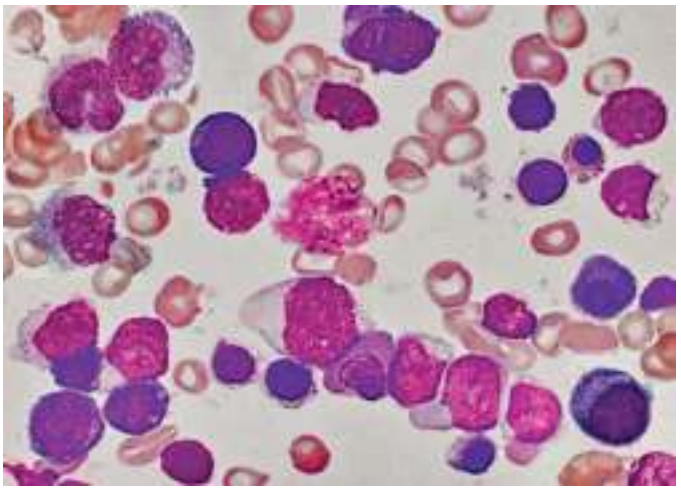


Fig. 9.52: Acute erythroid leukemia (AML-M6, FAB) in Giemsa-stained bone marrow aspirate smear. It shows myeloblast, promyelocyte and normoblasts with evidence of dyserythropoiesis (1000X).

Table 9.42 Periodic acid–Schiff (PAS) stain positivity in various hematologic disorders

Acute erythroid leukemia (AML-M6)
Myelodysplastic syndrome (MDS)
Acute myelogenous leukemia of other subgroups
Iron deficiency anemia
Thalassemia
Severe hemolytic anemia
Megaloblastic anemia (some case)

Laboratory Diagnosis of Acute Megakaryoblastic Leukemia (AMKL-M7, FAB)

Peripheral Blood Smear Examination

- On careful examination of the peripheral blood smear shows micromegakaryocytes and undifferentiated blasts.
- Demonstration of cytoplasmic blebs suggests that these cells are megakaryocytes. Megakaryocytic fragments can also be present.
- Acute megakaryoblastic leukemia (AMKL-M7, FAB) in Giemsa-stained bone marrow aspirate smear is shown in Fig. 9.53.

Bone Marrow Smear Examination

- Bone marrow smear examination shows medium to large size megakaryoblasts with basophilic often agranular cytoplasm, which usually exhibit distinct pseudopod formation. Small size megakaryoblasts can also be demonstrated.

- Morphology of megakaryoblasts is highly pleomorphic varying from small to large in size.
- Small megakaryoblasts are round in shape containing scanty cytoplasm and nucleus with dense chromatin resembling lymphoblasts.
- Large megakaryoblasts contain moderate amount of basophilic cytoplasm and nucleus with fine chromatin and prominent nucleoli. Some megakaryoblasts show cytoplasmic blebs.
- Dysplasia of all cell lineages is a common finding. No unique chromosomal abnormality is associated with acute megakaryoblastic leukemia in adults.

Cytochemistry

Megakaryocytes are positive for periodic acid–Schiff (PAS) and peroxidase stains; and negative for myeloperoxidase (MPO) and Sudan black B stains.

Bone Marrow Trephine Biopsy Examination

- Bone marrow aspiration usually results in dry tap due to bone marrow fibrosis associated with an expanded megakaryocyte lineage.
- In these cases, bone marrow trephine biopsy is performed to demonstrate increased fibroblasts and/or reticulin as well as ≥20% blasts.
- It has been proposed that those megakaryocytes synthesize a number of mitogenic factors that stimulate proliferation of fibroblasts.
- Hematoxylin and eosin-stained bone marrow trephine biopsy section shows extensive infiltration by blasts with sparse cytoplasm, frequent convoluted nuclei with fine chromatin and distinct nucleoli. Megakaryoblasts may be difficult to identify in bone marrow trephine biopsy, which may be present in small or large clusters surrounded by a network of reticulin fibers (Fig. 9.54).

Immunophenotyping

Monoclonal antibodies (CD41, CD42, CD61, HLA-DR, CD117, CD34, CD13, CD33, CD11b) are most commonly used to demonstrate megakaryoblasts by flow cytometry.

Markers	Expression
▪ CD41	▪ Positive
▪ CD42	▪ Positive
▪ CD61	▪ Positive
▪ HLA-DR	▪ Positive
▪ CD117	▪ Positive
▪ CD34	▪ Positive
▪ CD13	▪ Positive
▪ CD33	▪ Positive
▪ CD11b	▪ Positive

MYELOID SARCOMA

Myeloid sarcoma, also known as extramedullary myeloid tumor, that arises from hematopoietic stem cell affecting adults in 5–15% cases of AML.

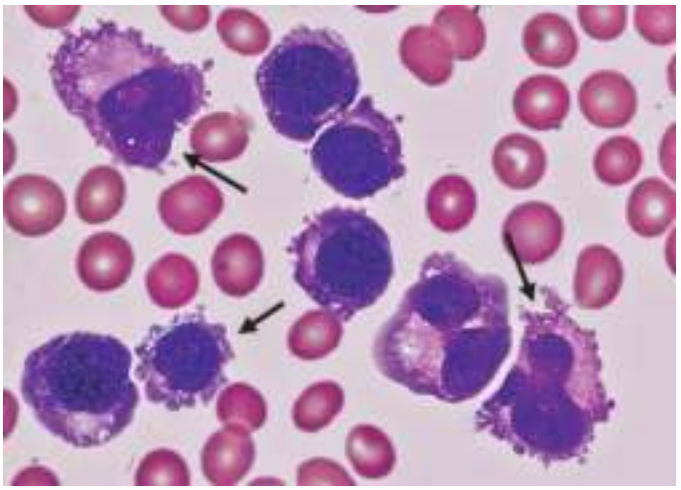


Fig. 9.53: Acute megakaryoblastic leukemia M7 (AMKL-M7, FAB) in Giemsa-stained bone marrow aspirate smear. It shows megakaryoblasts of variable in size. Some megakaryoblasts show cytoplasmic budding (arrows) (1000X).

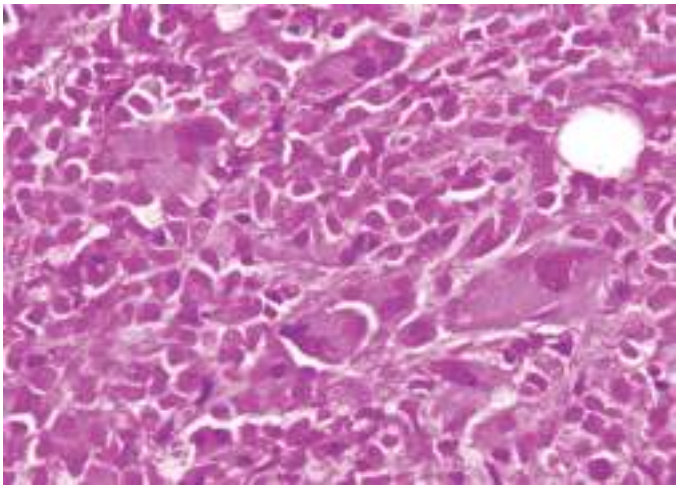


Fig. 9.54: Acute megakaryoblastic leukemia M7 (AMKL-M7, FAB) in hematoxylin and eosin-stained bone marrow trephine biopsy section. It shows extensive infiltration by blasts with sparse cytoplasm, frequent convoluted nuclei with fine chromatin and distinct nucleoli. Megakaryoblasts may be difficult to identify in bone marrow trephine biopsy. These may be present in small or large clusters surrounded by a network of reticulin fibers (400X).

- Cell of origin of myeloid sarcoma is believed to be the common myeloid progenitor cell. Cytogenetic abnormalities include t(8;21), inv(16), t(19;11)(p21;q23) and trisomy 8.
- Myeloid sarcoma tumor mass is composed of myeloblasts with or without maturation present in tissues outside bone marrow such as bone (periosteum) orbit, paranasal sinuses, skin, breast, gastrointestinal tract, mediastinum, pleura, ovary and lymph nodes.
- Myeloid sarcoma can occur *de novo* or initial manifestation of relapse in a patient previously diagnosed

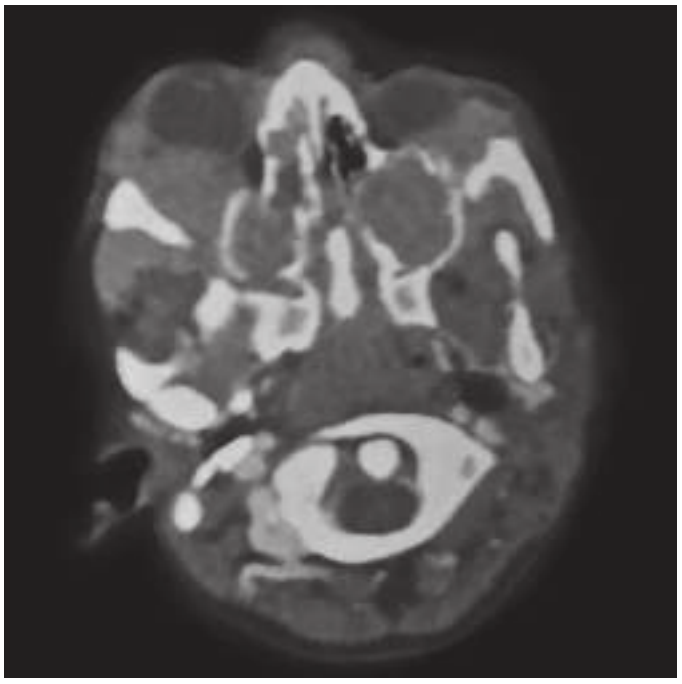


Fig. 9.55: Myeloid sarcoma involving nose and paranasal sinuses on CT scan. Axial contrast enhanced computed tomography (CECT) of the nose and paranasal sinuses reveals an enhancing soft tissue mass in bilateral nasal cavities and maxillary sinus with erosion of walls of maxillary sinus, extension into ethmoid sinuses, retroconal space of bilateral orbits and premaxillary space.

as AML, or chronic myelogenous leukemia (CML) with blast phase and/or other myelodysplastic/myeloproliferative disorders. It has varied clinical presentation depending on site involved.

- Diagnosis of myeloid sarcoma is challenging, that relies on radiology, histology, immunophenotyping and molecular analysis. Myeloid sarcoma involving nose and paranasal sinuses on CT scan is shown in Fig. 9.55.
- Patients of myeloid sarcoma are treated by surgery and radiotherapy. Allogeneic hematopoietic stem cell transplantation is beneficial in patients who achieve complete remission with AML-induction protocols.

Laboratory Diagnosis of Myeloid Sarcoma

Light Microscopy

Myeloid sarcoma tumor mass is composed of sheets of tumor cells arranged in cords admixed with or without promyelocytic or neutrophilic maturation that effaces the tissue architecture. Myeloid sarcoma in bone marrow trephine biopsy section from orbit is shown in Fig. 9.56.

Cytochemistry

Myeloperoxidase (MPO) positivity in myeloid sarcoma in bone marrow trephine biopsy is shown in Fig. 9.57.

Immunophenotyping

Panel of monoclonal antibodies is used to demonstrate tumor cells of myeloid sarcoma, which include cMPO, CD68, CD117, CD99, CD34, lysozyme, TdT, CD13, CD14, CD15, and CD33.

Markers	Expression
▪ cMPO	▪ Positive
▪ CD68	▪ Positive
▪ CD117	▪ Positive
▪ CD99	▪ Positive
▪ CD34	▪ Positive
▪ Lysozyme	▪ Positive
▪ TdT	▪ Positive
▪ CD13	▪ Positive
▪ CD14	▪ Positive
▪ CD15	▪ Positive
▪ CD33	▪ Positive

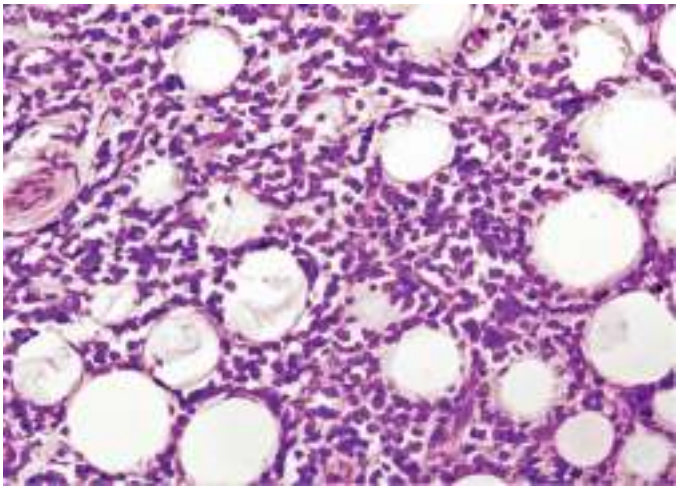


Fig. 9.56: Myeloid sarcoma. Bone marrow trephine biopsy section from orbit. It shows sheets of atypical cells infiltrating into surrounding adipose tissue (hematoxylin and eosin, 400X).

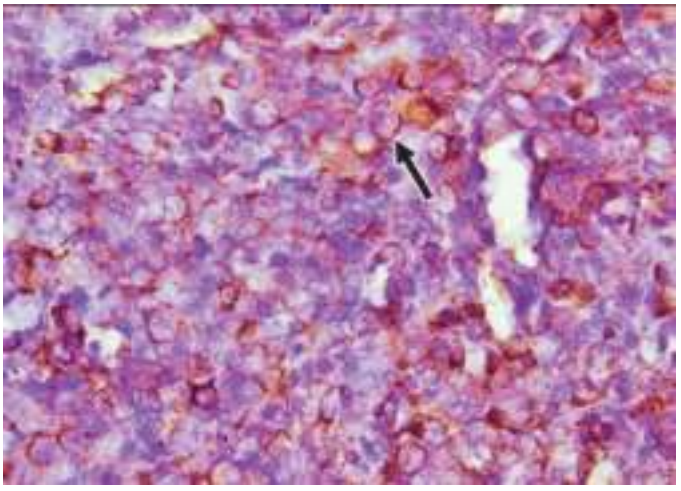


Fig. 9.57: Myeloperoxidase (MPO) positivity in myeloid sarcoma in bone marrow trephine biopsy (arrow) (300X).

ACUTE MYELOGENOUS LEUKEMIA, NOT OTHERWISE SPECIFIED (AML-NOS)

Leukemic myeloblasts in acute myelogenous leukemia, not otherwise specified (AML-NOS) are usually demonstrated on the Wright-Giemsa or May-Grünwald-Giemsa-stained peripheral blood smear, bone marrow aspirate smear; and hematoxylin and eosin-stained sections of bone marrow trephine biopsy. The results can be particularly informative, and attempts should be made to secure these records.

- A comprehensive clinical history should be taken of antecedent hematologic disorders including cancer diagnosis in the patient or family together, with history of occupational exposure to radiation, medication in treatment of cancer such as chemotherapy, radiation, or immunosuppressive drugs. The clinical history of the patient should be communicated to the pathologist together with results of relevant examination, laboratory, radiographic and other findings.
- Immunophenotyping by multiparameter flow cytometry, karyocyte (metaphase cytogenetics +/–FISH), molecular analysis (including NPM1, FLT3, KIT, CEBPA, TP53, RUNX1).
- Lumbar puncture should be performed if CNS symptoms appear. Immunophenotyping with a panel of monoclonal antibodies is particularly useful for distinguishing acute myelogenous leukemia from acute lymphoblastic leukemia (ALL) and for identification of acute myelogenous leukemia (AML) subtypes.
- Morphologic diagnosis of AML can be supported by the presence of Auer rods in the cytoplasm of myeloblasts, positive cytochemistry with Sudan black, and staining for myeloperoxidase and nonspecific esterase.
- Cytogenetic and molecular genetic alterations associated with the morphologic subtypes of acute myelogenous leukemia (AML) can further support the diagnosis and assist predict treatment outcomes.

Laboratory Diagnosis of Acute Myelogenous Leukemias, Not Otherwise Specified (AML-NOS)

Hematologic investigations of acute myelogenous leukemia include hemoglobin, total leukocyte count, differential leukocyte count, peripheral blood smear and bone marrow aspiration and trephine bone marrow biopsy. The quality of bone marrow specimen is critical for all subsequent analysis of molecular genetic alterations.

Hemoglobin and Blood Counts

- Hemoglobin is low. Total leukocyte count is 20×10^9/dl in acute myelogenous leukemia (AML) except in subleukemic leukemia.
- Platelet count is markedly reduced.

Peripheral Blood Smear Examination

Red blood cells

- Red blood cells are decreased, and hemoglobin <10 g/dl is common finding.
- Peripheral blood smear shows macrocytic picture because of their inability to successfully compete with the leukemic cells for folate or cobalamin and /or early release of reticulocytes.
- Red blood cell distribution (RDW) is usually elevated.
- Red blood cells demonstrate Howell-Jolly bodies.
- Pappenheimer bodies and basophilic stippling are indicative of red blood cell maturation defects.
- Nucleated red blood cells can be present in proportion to the anemia or bone marrow damage.

White blood cells

- Peripheral blood picture is quite variable in AML. Although, it is traditional to describe leukemias having increased white blood cells. About 50% of AML cases demonstrate decreased leukocyte count within the reference interval at the diagnosis.
- Leukocyte count ranges $<1 \times 10^9$/L to $>100 \times 10^9$/L. Regardless of the total leukocyte count, the presence of myeloblasts in the peripheral blood is suggestive of acute leukemia.
- The revised 2024 WHO classification of acute leukemia requires ≥20% blasts in the bone marrow or peripheral blood smears. These AML cases must be classified based on morphologic features, cytogenetic, molecular genetic diagnostic techniques.
- Myeloblasts in AML measure 20 µm in diameter and contain large nucleus composed of dispersed chromatin (euchromatin or transcriptionally active) with prominent 3–5 nucleoli.
- Myeloblasts contain linear Auer rods composed of crystallized azurophilic granular material that form elongated needles in the cytoplasm demonstrated under light microscopic examination. Auer rods are composed of fused lysosomes and contain lysosomal enzymes, peroxidase and larger crystalline inclusions. Auer rods are demonstrated in AML-M2, AML-M3 and CML with blast phase. The presence of Auer rods in myeloblasts in AML cases excludes ALL.
- Buffy coat should be prepared to demonstrate blasts in cases with low leukocyte count $<2 \times 10^9$/L.

Platelets

- Peripheral blood picture shows thrombocytopenia. Hypogranular platelets and occasional giant platelets can be present.
- As the disease advances, more immature platelets such as megakaryocytic fragments can be demonstrated.
- The platelet count might not correlate with the potential complication of bleeding because of the presence of qualitative platelet defects.

Other abnormal hematologic findings

Other abnormal hematologic findings demonstrated on the peripheral blood smear include monocytosis and neutropenia. Monocytosis usually precedes overt leukemia.

- Neutrophils can exhibit signs of dysplasia including hypo-segmentation, hypogranulation and small nuclei increased condensed chromatin. Signs of myelodysplasia are especially demonstrated in acute promyelocytic leukemia. There can be mild to moderate increase in the eosinophils and basophils.
- Presence of basophil differentiates AML from leukemoid reactions. Absolute basophil is absent in leukemoid reactions.
- Blast of chronic myelogenous leukemia (CML) should be excluded by evaluating BCR-ABL1.

Bone Marrow Smear Examination

Bone marrow analysis should include bone marrow aspiration and trephine bone marrow biopsy. The quality of bone marrow specimen is critical for all subsequent analysis such as cytogenetic and molecular genetic analysis.

- **Cellularity:** Bone marrow is hypercellular with high myeloid to erythroid ratio; and with decreased fat content. Bone marrow contains predominant myeloblasts and sometimes an increase in fibrosis.
- **Myeloid lineage:** Bone marrow demonstrates predominance of blasts. According to revised 2024 WHO classification, cut off percentage of blasts is ≥20%, which represents a change from original guidelines where ≥30% blast cells were considered as per FAB classification criteria. Frequently the blast count is close to 100%. Auer rods are demonstrated in bone marrow myeloblasts in 50% of cases. Differentiating features of myeloblast and lymphoblast in acute leukemias are given in Table 9.43.
- **Erythroid lineage:** Erythroid precursors are reduced as a result of increase in number of myeloblasts or suppression of hematopoietic stem cell.
- **Megakaryocytic lineage:** Megakaryopoiesis is markedly reduced.

Bone Marrow Trephine Biopsy Examination

- Bone marrow trephine biopsy is performed in patients with dry tap in bone marrow aspiration in acute myelogenous leukemia, not otherwise specified (AML-NOS) with pancytopenia. It demonstrates clumps of myeloblasts, occasionally forming sheets of infiltrate that disturb the usual bone marrow architecture.
- Bone marrow fibrosis is demonstrated by reticulin stain. In addition to the light microscopic morphologic evaluation, bone marrow samples should be analyzed for flow cytometry, cytogenetic and molecular genetic alterations analysis.
- Immunophenotyping is performed using anti-MPO, CD13, CD33, CD15, CD11b, and HLA-DR to confirm diagnosis.

DIAGNOSTIC APPROACH

A diagnosis of acute myelogenous leukemia, not otherwise specified (AML-NOS) requires the presence of at least ≥20% blasts in the bone marrow or peripheral blood. It is important to recognize that promonocytes are considered blast equivalents for the purpose of defining an acute myelogenous leukemia. Diagnostic tools for diagnosing acute myelogenous leukemia include cytomorphologic study, cytochemistry, immuno-phenotyping, cytogenetic and molecular alterations analysis.

Cytochemistry

The common cytochemical stains used in diagnosing AML-NOS include the myeloperoxidase (MPO), Sudan black B (SBB), naphthol AS-D chloroacetate esterase (specific esterase) and α-naphthyl acetate esterase (nonspecific esterase).

- Granulocytic cells are positivity for myeloperoxi-dase and with Sudan black B stain, whereas lympho-blasts are negative. Thus, cytochemical stains help in differentiating acute myelogenous leukemia from acute lymphoblastic leukemia.
- The esterase stains help in differentiating precursor granulocytic cells from precursor monocytic cells. Granulocytic cells stain positive with naphthol AS-D chloroacetate esterase (specific esterase), and cells of monocytic lineage stain positive with α-naphthyl acetate esterase (nonspecific esterase).
- Cytochemical stains are useful in cases not defined by immunophenotyping and cytogenetic analysis being used as first-line tests to define cell lineage. Cytochemistry in acute myelogenous leukemia according to French-American-British classification is given in Table 9.44.

Immunophenotyping

Immunophenotyping by low cytometry or immuno-histochemistry can be used to demonstrate terminal deoxynucleotidyl transferase (TdT) in cells. TdT was thought to be specific marker for lymphoid cell lineage. But TdT is also demonstrated on more immature hematopoietic stem cells, sometimes including those of myeloid lineage. Therefore, TdT cannot be the sole determinant of lymphoid lineage.

- Immunophenotyping by flow cytometry is an essential diagnostic tool in acute leukemia classification by using a specific sequence of testing with monoclonal antibodies. Commonly cell markers used in identification include CD2, CD3, CD4, CD5, CD10, CD13, CD14, CD15, CD16, CD19, CD22, CD33, MPO, HLA-DR, CD34, CD45, CD56, CD64 and CD117.
- The first panel of monoclonal antibodies used in flow cytometry should differentiate AML from ALL, and ALL-B cell origin from ALL-T cell origin.
- Monoclonal antibodies (CD19, CD20, CD22, CD79a) used in flow cytometry demonstrate B lymphoid cell antigens.

Table 9.43 Differentiating features of myeloblast and lymphoblast in acute leukemias

Parameters	Myeloblast in AML	Lymphoblast in ALL
Morphologic features		
Size of blast	Large size	Small size
Cytoplasm	Scant to moderate	Scant basophilic cytoplasm
Cytoplasmic granules	Cytoplasmic granules present	▪ Cytoplasmic granules rarely present ▪ Negative for myeloperoxidase stain and toluidine
Nuclear chromatin	Fine chromatin, delicately dispersed	▪ Fine chromatin, with dispersed ▪ Very condensed in small lymphoblasts
Nuclear membrane	Fine	Relatively dense
Nucleoli number	3–5	1–2
Auer rods	Present, especially in acute promyelocytic leukemia (hypergranular form)	Always absent
Cytochemistry		
Myeloperoxidase	Positive	Negative
Sudan black B (SBB)	Positive	Negative
Periodic acid–Schiff stain	Negative	Positive
Acid phosphatase	Negative	Positive
Nonspecific esterase	Negative, positive in AML-M4, AML-M5	Negative
Lineage markers		
Cluster of differentiation	CD13, CD15, CD33, CD11b, TdT	▪ B cell: CD10, CD19, CD79a, TdT ▪ T cell: CD3
Therapeutic response		
Chemotherapeutic agents	Cytosine, arabinoside, anthracycline, 6-thioguanine	Vincristine, anthracycline, prednisone, L-asparaginase
Response to therapy	Remission of short duration	Remission of long duration
Median survival	12–18 months	Children with CNS involvement for 5 years; and adults for 8–12 years

Table 9.44 Cytochemistry in acute myelogenous leukemia according to French-American-British classification

FAB Class and Features	Morphologic Features	Cytochemistry
AML-M0 minimal differentiated	Minimal evidence of myeloid differentiation (without maturation with agranular cytoplasm)	Myeloperoxidase stain positive (<3%)
AML-M1 without maturation	▪ High percentage of myeloblasts without significant maturation to more mature neutrophils, blasts demonstrate ▪ Auer rods are present in myeloblasts	Myeloperoxidase stain positive (>3%)
AML-M2 with maturation	All stages of neutrophil maturation, pseudo-Pelger-Hüet anomaly, hypogranulation	Myeloperoxidase stain positive (>10%)
AML-M3	Acute promyelocytic leukemia (APL) with hypergranular cytoplasm with bilobed heavy contour	Myeloperoxidase stain positive
AML-M3m	Acute promyelocytic leukemia (APL) with microgranular variant	Myeloperoxidase stain positive
AML-M4	Acute myelomonocytic leukemia (monocytes and promonocytes in the bone marrow exceed 20%)	▪ Myeloperoxidase stain positive ▪ Nonspecific esterase stain positive

Contd...

Table 9.44 Cytochemistry in acute myelogenous leukemia according to French-American-British classification (*Contd...*)

FAB Class and Features	Morphologic Features	Cytochemistry
AML-M4Eo	Acute myelomonocytic leukemia with abnormal eosinophils (>5%)	▪ Myeloperoxidase stain positive ▪ Nonspecific esterase stain positive
AML-M5A (acute monoblastic leukemia without differentiation)	Monoblasts/monocyte dominance (≥80%), hemophagocytosis, nuclear lobulation	Nonspecific esterase (NSE) stain positive
AML-M5B (acute monoblastic leukemia with differentiation)	Monoblasts/monocyte dominance	Nonspecific esterase (NSE) stain positive
AML-M6 (acute erythroid leukemia)	▪ >50% erythroid precursors ▪ >20% myeloblasts in the nonerythroid cell population ▪ Megakaryoblastoid morphology ▪ Auer rods +/– in leukemic blasts	▪ Periodic acid–Schiff (PAS) positive ▪ Glycophorin positive
AML-M7 (acute megakaryocytic leukemia)	Cytopenia with or without thrombocytopenia, basophilic agranular blasts with pseudopods, micro-megakaryocytes	Platelet peroxidase positive

MPO: Myeloperoxidase; PAS: Periodic acid–Schiff; CD: Cluster of differentiation; Myeloid markers: CD13, CD33; Monocyte markers: CD11, CD14.

- T lymphoid cell antigens are demonstrated by using monoclonal antibodies (CD2, CD3, CD5, CD7). Human leukocyte antigen DR (HLR-DR) antigens are present on both myeloid and lymphoid cells.
- Several aberrant antigens are sometimes demonstrated on the leukemic myeloid cells in AML (CD7, lymphoid cell antigen). CD34 marker is also demonstrated on the least differentiated myeloid cells and early lymphoid cells.
- The monoclonal antibodies that react with most cases of AML include CD13, CD15, CD33, CD64 and CD117.
- In addition, monoclonal antibody that identifies myeloperoxidase (MPO) is employed especially when cytochemistry for MPO is negative.
- Differentiation of acute myelogenous leukemia from acute lymphoblastic leukemia using immunophenotyping with selected monoclonal antibodies is given in Table 9.45. Expression of cell-surface and cytoplasmic markers for the diagnosis of acute myelogenous leukemia and mixed-phenotype acute leukemia are given in Table 9.46.

Cytogenetic Analysis

About 66% of AML patients have detectable cytogenetic abnormalities that include aneuploidies (variation in number of chromosomes) and chromosomal translocation. Commonly demonstrated aneuploidies include trisomy 8, trisomy 21, monosomy 7, and loss of an X or Y chromosome.

- Chromosomal translocations result in fusion genes, that are either beneficial for proliferation and survival of cells or disrupting differentiation and maturation of myeloid cells. Additional genetic abnormalities can develop in subclones as the disease progresses.
- Characteristics of nonrandom cytogenetic abnormalities are demonstrated in the revised 2024 WHO classification of acute myelogenous leukemia with recurrent genetic alterations and in therapy-related myeloid neoplasms. These genetic abnormalities are analyzed by cytogenetic analysis, fluorescence *in situ* hybridization (FISH) or molecular genetic studies.

Table 9.45 Differentiation of acute myelogenous leukemia from acute lymphoblastic leukemia using immunophenotyping with selected monoclonal antibodies

Leukemia Type	Cell Marker				
	HLA-DR	CD13, CD33, CD15, CD11b	CD19, CD20, CD22, CD79a	CD10	CD2, CD3, CD5, CD7
AML	+	+	–	–	–
ALL-B cell origin	+	–	+	+	–
ALL-T cell origin	–	–	–	+/–	+

Flow cytometry or immunohistochemistry can be used to demonstrate terminal deoxynucleotidyl transferase (TdT) in cells. TdT was thought to be a lymphoid specific marker. But TdT is also demonstrated on more immature hematopoietic cells, sometimes including those of myeloid lineage. Therefore, TdT cannot be the sole determinant of lymphoid lineage.
AML: Acute myelogenous leukemia; ALL: Acute lymphoblastic leukemia.

Table 9.46 Expression of cell-surface and cytoplasmic markers for the diagnosis of acute myelogenous leukemia and mixed-phenotype acute leukemia

Cell Lineage	Expression of Marker
Diagnosis of acute myelogenous leukemia	
Precursor cells	CD34, CD38, CD117, CD133, HLA-DR
Granulocytic markers	CD13, CD15, CD16, CD33, CD65, cytoplasmic myeloperoxidase (cMPO)
Monocytic markers	CD11b, CD11c, CD14, CD64, CD4, CD36, NG2 homologue, lysozyme, nonspecific esterase (NSE)
Megakaryocyte markers	CD41 (glycoprotein IIb/IIIa), CD61 (glycoprotein IIIa), CD42 (glycophorin Ib)
Erythroid cell markers	CD235a (glycophorin A member of glycoprotein family)
Diagnosis of mixed-phenotype acute myelogenous leukemia (MPAL)	
Myeloid lineage markers	MPO or evidence of monocytic differentiation (at least two of the following: NSE, CD11c, CD14, CD64, lysozyme)
B cell lineage markers	CD19 (strong positivity) with at least one of the following: CD79, cCD22, CD10
T cell lineage markers	cCD3, or surface CD3

- Molecular genetic alterations and morphologic features used to classify acute myelogenous leukemia (AML) with recurrent cytogenetic abnormalities are given in Table 9.47. Morphologic features in AML with t(8;21) (q22;q22) are given in Table 9.48. Salient features for laboratory diagnosis of acute myelogenous leukemia (AML) with inv (16) (p13q22) or t(16;16(p13; q22) are given in Table 9.49.

Table 9.47 Cytogenetic and morphologic features used to classify acute myelogenous leukemia (AML) with recurrent cytogenetic abnormalities

AML with Recurrent Molecular Genetic Alterations	Morphologic Features, Cytogenetics, Immunophenotypes	Comments
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1 fusion gene involved in activation of other genes and cellular proliferation	▪ Similarity to FAB AML-M2 affecting children and adults <60 years ▪ Subcategory accounts for 5–10% of AML cases ▪ Maturation in the neutrophilic lineage (promyelocytes, myelocytes and mature neutrophils) with dysplasia in bone marrow ▪ Auer rods in myeloblasts ▪ Pseudo-Pelger-Hüet nuclear abnormality ▪ Immunophenotyping: CD34+, CD30+, HLA-DR+, MPO++, CD15+, CD33+ ▪ Aberrant markers: CD19+, PAX5+, cCD79a+	▪ Secondary genetic defects: deletion of Y chromosome and 9q ▪ Young persons may present with myeloid sarcoma ▪ Good response to chemotherapy ▪ Poor prognosis with KIT mutations
AML with inv(16) (13; q22) or t(16;16)(p13;q22) or t(16;16)(p13.1;q22); CBFβ-MYH11	▪ Similarity to FAB AML-M4 ▪ Subcategory accounts for 7% of AML cases ▪ CBFβ-MYH11 fusion proteins binds to RUNX1 and represses its function as transcription factor ▪ Maturation in both neutrophil and monocytic lineage, abnormal eosinophils (AMMLE0) ▪ Eosinophils ≥5% with abnormal eosinophilic granules present in promyelocyte or myelocyte stage ▪ Immunophenotyping: blasts with granulocyte differentiation: CD13, CD33, CD65+, MPO+ ▪ Immunophenotyping: blasts with monocytic differentiation: CD14, CD11b, CD11c, CD64, CD36, lysozyme ▪ Aberrant marker: CD2	▪ Secondary genetic defects: +22, +8, 21+ deletion 7q ▪ Young persons may present with myeloid sarcoma ▪ Good prognosis with +22 ▪ Poor prognosis with KIT mutations

Contd...

Table 9.47 Cytogenetic and morphologic features used to classify acute myelogenous leukemia (AML) with recurrent cytogenetic abnormalities (*Contd...*)

AML with Recurrent Molecular Genetic Alterations	Morphologic Features, Cytogenetics, Immunophenotypes	Comments
AML with t(9;11) (p22;q23); MLLT3-MLL	▪ Similarity to FAB AML-M4, AML-M5 usually associated with monocytic features usually affecting children and any age group ▪ MLL gene (11q23) protein, a DNA binding protein that interacts with other nuclear proteins and help other transcription factors ▪ Monoblasts and promonocytes predominate, can demonstrate granules and vacuoles ▪ Immunophenotyping: CD34+ CD33+, CD65, HLA-DR++, CD13+, CD14+/– ▪ Aberrant marker: CD2	▪ Secondary genetic defect: +8 ▪ Children present with gingival infiltration by leukemic cells and clinical manifestations of disseminated intravascular coagulation ▪ Intermediate prognosis
Acute promyelocytic leukemia with t(15;17) (q22;q12);PML-RARA and various chromosomal translocations	▪ Similarity to FAB AML-M3, AML-3v associated with abnormal promyelocytes ▪ Hypergranular variant with predominant promyelocytes and multiple Auer rods in one cell ▪ Microgranular variant with bilobed nuclei without visible granularity ▪ Immunophenotyping in homozygous: CD33+ CD13+; and heterozygous ▪ Immunophenotyping in heterozygous: HLA-DR+, CD34–	▪ Secondary genetic defect: chromosome 8+, FLT3-ITD ▪ Adult persons present with clinical manifestations of disseminated intravascular coagulation such as bleeding treated by heparin therapy
AML with t(6;9)(p23;q34); DEK-NUP214	▪ Resembles FAB AML-M2, AML-M4 ▪ With or without monocytic features and often associated with basophilia and multilineage dysplasia ▪ Immunophenotyping: MPO+, CD13+, CD33+, CD38++, HLA-DR ▪ Aberrant marker: TdT	▪ Secondary genetic defects: FLT3-ITD ▪ Children and adults present with pancytopenia or anemia with thrombocytopenia ▪ Poor prognosis
AML with inv(3)(q21;q26) or t(3;3) (q21;q26.2); RPN1-EV11	▪ Resembles FAB AML-M1, AML-M4, AML-M7 ▪ Associated with elevated platelets and atypical megakaryocytes (monolobed or multilobed nuclei) with multilineage dysplasia ▪ Immunophenotyping: CD34+, HLA-DR+, CD13+, CD33+, CD41, CD61+ ▪ Aberrant marker: CD7	▪ Secondary genetic defect: monosomy 7, deletion 5q ▪ Disorder in adults arises *de novo* or MDS presents with hepatosplenomegaly with aggressive clinical course
AML with t(1;22)(p13;q13); RBM15-MLK1	▪ Resembles FAB AML-M7 ▪ Megakaryocytic lineage with maturation (small and large size) ▪ Immunophenotyping:CD34++, HLA-DR, CD41+, CD61+, CD42+, CD13+, CD45– ▪ Aberrant marker absent	▪ Secondary genetic defects: absent ▪ Infants and young children in <3 years present with hepatosplenomegaly ▪ Patients respond to intensive chemotherapy
AML with mutation in nucleophosmin 1 (NPM1) associated with other gene abnormalities, FLT-ITD	Myelomonocytic or monocytic features present in older people with high blood counts and a normal karyocyte	Secondary genetic defects: chromosome 8+, 4+, 21+, loss of Y, deletion of 9q
AML with biallelic CEBPα mutation, tumor suppressor gene encodes transcription factor involved in granulopoiesis	Myeloid leukemia with or without maturation, can demonstrate myelomonocytic or monoblastic features	Prognosis favorable

Table 9.48 Morphologic features in acute myelogenous leukemia (AML) with t(8;21) (q22;q22)

French-American-British (FAB) AML-M2 morphology
Mature and immature myeloid cells contain abundant Auer rods
Myeloid cells contain salmon-colored granules in the cytoplasm and a rim of basophilic cytoplasm
Pseudo-Chédiak-Higashi anomaly present
Cells contain pink, waxy cytoplasmic globules
Large size blasts with prominent Golgi area
Cells contain cytoplasmic vacuoles
Bone marrow eosinophilia (>5%)

PROGNOSTIC FACTORS

Powerful prognostic factors established over decades include age over 60 years, cytogenetic abnormalities, prior myelodysplastic syndrome, and treatment-related AML supplemented by molecular genetic abnormalities. Prognostic factors variable associated with treatment outcomes include age of the patient, AML arising from pre-existing MDS, treatment related-AML, performance status, extramedullary disease, and comorbid conditions. Prognostic factors of acute myelogenous leukemia according to French-American-British classification are given in Table 9.50.

- The best pretreatment prognostic predictors of long-term outcome, together with age include chromosomal and molecular genetic alterations in the leukemic cells in AML cases.
- Cytogenetic analysis is the basis of stratification of AML patients into three risk categories with different response to chemotherapy and long-term outcomes. Stratification of acute myelogenous leukemia (AML) patients into three risk groups based on molecular genetic alterations is given in Table 9.51.

Table 9.49 Salient features for laboratory diagnosis of acute myelogenous leukemia (AML) with inv(16)(p13;q22) or t(16);16(p13;q22)

Karyotypic Analysis AML with inv(16)(p13;q22) or t(16);16(p13;q22)
Molecular Characterization of CBFb-MYH11 Fusion Gene by Following Techniques ▪ Fluorescence *in situ* hybridization (FISH) ▪ Real-time reverse transcriptase (RT-PCR)
Bone Marrow ▪ Myeloblasts and monoblasts ≥20% in the bone marrow; <20% acceptable when typical karyotype or molecular pattern is demonstrated ▪ Eosinophils >5% in the bone marrow, absence of eosinophilia; and eosinophils with abnormality in cytoplasmic granules and cytochemical stain

Contd...

Immunophenotyping by Flow Cytometry	
Leukemic Cells	**Markers**
Myelomonocyte markers	▪ CD13+ ▪ CD33+ ▪ CD14+ ▪ CD11b+ ▪ CD11c+ ▪ Myeloperoxidase (MPO)+
Immature cell markers	▪ CD34+ ▪ CD117+
Marker expressed by myeloid and T cell	CD2+

Table 9.50 Prognostic factors of acute myelogenous leukemia according to French-American-British classification

Characteristics	Favorable Prognosis	Poor Prognosis
Age group	<40 years	<2 years and >60 years in AML-M0, AML-M6, AML-M7
Sex distribution	Females	Males
Origin	*De novo*	Therapy-induced or in MDS
Infections	Absent	Present (AML-M5)
Total leukocyte count	<25000/cumm	>100,000/cumm
Serum LDH	<400 IU/L	>400 IU/L
Extramedullary involvement	Absent	Present
CNS involvement	Absent	Present
Cytogenetics	▪ AML-M2; t(8.21) ▪ AML-M3; t(15;17) ▪ AML-M4; inversion (16)	▪ AML-M2; t(6;9) ▪ AML-M5; translocation or deletion of chromosome 5

Contd...

Table 9.50 Prognostic factors of acute myelogenous leukemia according to French-American-British classification (*Contd...*)

Characteristics	Favorable Prognosis	Poor Prognosis
Molecular genetics	▪ NPM1 gene mutation (5q35) ▪ Biallelic CEBPA gene mutation (19q13.1)	▪ FLT3 gene mutation (13q12) ▪ WT1 gene mutation (11p13) ▪ KIT mutation (4q11, q12) ▪ BALLC overexpression (8q22.3) ▪ ERG overexpression (21q22.3) ▪ EVI1 overexpression (3q;26) ▪ MN1 overexpression (22q.12.1)
Immunophenotype	▪ CD34 negative ▪ CD14 negative ▪ HLA-DR negative	▪ CD34 positive ▪ HLA-DR positive

Immunophenotype markers are studied in bone marrow smears and bone marrow trephine biopsy specimens. Other parameters such as angiogenesis and proliferative index are also studied.

Table 9.51 Stratification of acute myelogenous leukemia patients into three risk groups based on molecular genetic alterations

Risk Status	Molecular Genetic Alterations in AML
Favorable prognosis	▪ AML with inv(16)(p13.1;q22) or t(16;16)(p13.1;q22);CBFB-MYH11 ▪ AML with t(8;21)(q22;q22); RUNX-RUNX1T1 ▪ AML with t(15;17) ▪ AML with NPM1 mutation in absence of FLT3-ITD ▪ AML with isolated biallelic CEBPα mutation
Intermediate prognosis	▪ AML with normal cytogenetics ▪ AML with chromosome +8 ▪ AML with t(9;11) ▪ AML with t(8;21), inv(16), t(16;16) with c-KIT mutation ▪ AML with not defined as better- or poor-risk status ▪ AML with NPM1 and FLT3-ITD (normal karyotype) and wild-type NPM1 ▪ AML with FLT3-ITD (normal karyotype) and wild type NPM1 without FLT3-ITD
Adverse prognosis	▪ AML with complex karyotype (≥ clonal chromosomal abnormalities) ▪ AML with monosomal karyotype ▪ AML with complex karyotype –5, del(5q)–7,17q ▪ AML with 11p23-non t(9;11) ▪ AML with inv(3)(q21;q26.2) or t(5,3)(q21;q26.2); RPN1-EVI1 ▪ AML with t(6;9)(p23;q34); DEK-NUP214 ▪ AML with t(v;11)(v;q23); MLL rearranged ▪ AML with t(0;22) ▪ AML with normal cytogenetics with FLT3-ITD mutation ▪ AML with TP53 mutation

NPM1: Nucleophosmin 1; FLT3-ITD: Fms-like tyrosine 5-internal tandem duplications; c-KIT; CD117 receptor tyrosine kinase; CEBPA or CCAAT/enhancer binding protein α.

- About 40–50% of AML cases demonstrate a normal karyotype at the time of diagnosis. Absence of NPM1 or FLT3-ITD or isolated biallelic CEBPA gene mutations, is linked to intermediate risk group. Frequency and prognostic impact of gene mutations in acute myelogenous leukemia (AML) is given in Table 9.52. These gene mutations provide substantial insights into the pathogenesis of AML and assist in identification of molecular targets for treatment of these patients.

TREATMENT

Basic aim of management of AML patients is to achieve remission after initial therapy. Except for acute

Table 9.52 Frequency and prognostic impact of gene mutations in acute myelogenous leukemia (AML)

Gene Mutation	Frequency	Comments
Favorable prognosis		
NPM1*	25–30%	Favorable prognosis in natural killer cell-based immunotherapy for AML
CEBPα (biallelic)*	≤5%	Favorable prognosis in isolated biallelic CEBPA mutation in AML
Intermediate prognosis		
KIT	≤5%	Intermediate prognosis when AML is associated with t(8;21), inv(16) karyotype
Poor prognosis		
FLT3-ITD*	20–25%	Poor prognosis in natural killer cell-based immunotherapy for AML but no prognostic significance in acute promyelocytic leukemia (APL)
TP53	≤6%	Poor prognosis in natural killer cell-based immunotherapy for AML
WT1	≤6%	Resistant AML disease associated with poor prognosis
RUNX1	9–10%	Resistant AML disease associated with poor prognosis
Undefined prognosis		
DNMT3A	18–25%	Unclear prognostic significance in AML, and may be demonstrated in healthy persons
IDH1 and IDH2	17–20%	Prognosis of AML varies based on combinations of genotypes (DNMT3 mutations)
TET2	≤10%	Co-mutated with NPM1, chromatin-spliceosome genes in AML

**Mutations of genes are already demonstrated within clinical guidelines.*
CEBPA or CCAAT/enhancer binding protein alpha; FLT3-ITD: Fms-like tyrosine 5-internal tandem duplications; IDH1/2: Isocitrate dehydrogenase 1 and 2; NPM1: Nucleophosmin 1; RUNX1: Runt-related transcription factor 1; CEBPA or CCAAT/enhancer binding protein alpha; TET2: ten-eleven-translocation 2; WT1: Wilms' tumor 1, DNMT3 alpha; DNA-methyltransferase 3A.

promyelocytic leukemia, it is challenge for the clinician to determine if the patient is candidate for intensive cytotoxic therapeutic agents. The patients are treated in two phases: remission induction and post-remission therapy.

- The goal of remission induction phase is to achieve a hematologic complete remission with incomplete hematologic recovery currently defined as meeting response criteria for at least four weeks, i.e. <5% of blasts in the bone marrow, no blasts with Auer rods, normal maturation of all cellular components in the bone marrow and absence of extramedullary disease.
- Acute promyelocytic leukemia M3 (AML-M3) is a well-defined disease with characteristic morphology and potentially life-threatening coagulopathy, i.e. disseminated intravascular coagulation (DIC) at presentation, but overall better prognosis than another AML subgroup.
- AML-M3 is treated by targeted therapy directed against the leukemogenic event, t(15.17) PML-RARA fusion transcript to improved outcome. Acute promyelocytic leukemia now represents most curable leukemia treated by administration of nontoxic cytotoxic chemotherapeutic drugs such as all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO).

Initial Management

The early management of AML patients should be organized and implemented by an experienced team. Tests/procedures in the initial management of acute myelogenous leukemia are given in Table 9.53.

Newer Treatment Modalities

The traditional chemotherapy for AML is designated to reduce the tumor load as rapidly as possible. Most commonly used therapeutic agents in patients <60 years include a combination of cytarabine by intravenous infusion over 7 days with 3 days of an anthracycline (daunorubicin or idarubicin) regimen. Complete remission is achieved in 60–85% in patients younger than 60 years. However, complete remission varies depending on subtype of AML.

- Newer treatment modalities for AML patients include molecular targeted therapies (e.g. ATRA), epigenetic-targeted therapies (demethylating agents, histone deacetylase inhibitors), and autologous or allogeneic hematopoietic stem cell (HSC) transplantation and infusion of donor lymphocytes with total body irradiation to enhance leukemic cell destruction. Current research is being focused on novel therapies

Table 9.53 Tests/procedures in the initial management of acute myelogenous leukemia

Tests to Establish Diagnosis
Complete blood counts and differential blood counts
Examination of peripheral blood smear
Bone marrow aspiration for morphology, cytochemistry, cytogenetics, molecular genetics and flow cytometry
Bone marrow trephine biopsy for pretreatment leukemic bone marrow sample and mandatory with a dry bone marrow aspirate
Immunophenotyping
Cytogenetic abnormalities such as RUNX1-RUNX1T1, CRFB-MYH11, PML-RARA or other fusion gene screening should be performed if chromosome morphology is of poor quality
Additional Tests/Procedures at Diagnosis
History and clinical examination
Performance status (ECOG/WHO score)
Analysis of comorbidities
Serum biochemistry includes glucose, sodium, potassium, calcium, uric acid, total glycerides, creatinine phosphokinase (CPK)
Evaluation of renal and kidney function tests: urea, creatinine, aspartate aminotransferase (AST), alanine aspartate transferase (ALT), alkaline phosphatase, lactic dehydrogenase, bilirubin, total serum proteins
Coagulation tests include prothrombin time, international normalized ratio (INR), activated partial thromboplastin time (APTT), fibrinogen, D-dimer
Urine analysis includes pH, glucose, proteins, red blood cells, leukocytes, nitrite
Serum pregnancy test in women with childbearing age
Information on oocyte and sperm cryopreservation: cryopreservation to be performed in accordance with consent of the patient
Human leukocyte antigen (HLA) typing and cytomegalovirus of patients eligible for allogeneic hematopoietic stem cell transplantation: ▪ Hepatitis A, B, C, herpes simplex virus (HSV), cytomegalovirus and HIV-1 testing ▪ Chest radiograph ▪ Electrocardiogram
Lumbar puncture in extramedullary disease in patients at high risk of CNS involvement (symptoms of intracranial bleeding, leptomeningeal disease, and mass lesion) especially in FAB AML-M4 or AML-M5
Biobanking should be performed in general practice
Prognostic/Predictive Marker Assessment
NAM1, CEBPA, FLT3 gene mutation: strongly encouraged in AML with normal karyotype
WT1, RUNX1, MLL, KIT, RAS, TP53, TET2, IDH1 gene mutation
ERG, MN1, EV11, BAALC gene expression
Detection of minimal residual disease (MRD)

including the use of monoclonal antibodies and gene therapy and destruction of the bone marrow cellular matrix that supports the leukemic cells.

- Peripheral blood smear examination is essential for blood counts such as leukocytes and platelets during chemotherapy.
- Patients treated by chemotherapeutic agents, development of pancytopenia, severe granulocytopenia and/or thrombocytopenia should be monitored, so that early intervention can be initiated by blood transfusions and administration of hematopoietic growth factors to induce hematopoiesis.
- Allogeneic or autologous HSC transplantation remains the only therapeutic choice that currently provides the prolonged ≥10 years disease-free survival for most of AML patients.

MYELODYSPLASTIC SYNDROMES

MYELODYSPLASTIC SYNDROMES: OVERVIEW

Myelodysplastic syndromes (MDSs) are considered primary neoplastic, pluripotent hematopoietic stem cell (HSC) disorders of the bone marrow, which most often involve the myeloid progenitor lineage cells; however, the lymphoid lineage cells are rarely affected.

- Myelodysplastic syndrome (MDS) is characterized by one or more peripheral blood cytopenias, with prominent maturation abnormalities (dyspoiesis or dysplasia) in the bone marrow and genetic instability.
- Peripheral blood cytopenias result from ineffective erythropoiesis and increased apoptosis of hematopoietic cells as evidenced by an accompanying bone marrow hyperplasia. Morphologic dysplastic changes can be demonstrated in the erythroid cells, myeloid cells and megakaryocytes with genetic instability.
- Myelodysplastic syndromes most affect middle-aged and elderly persons. However, these are diagnosed occasionally in children.
- The term myelodysplasia is more appropriate until the patient actually develops overt leukemia. Approximately 30–50% cases of myelodysplastic syndrome may develop acute myelogenous leukemia (AML).
- Myelodysplastic changes may be detected in healthy persons during aging process. Some persons may show persistent cytopenia without dysplastic change due to unknown etiology, which are considered as pre-phases of MDS. These persons may later develop hematologic neoplasms in life.
- Recently, molecular, genetic and flow cytometry-based techniques may help in the delineation of these cases. Some authors proposed classification of pre-phase of MDS cases with minimum diagnostic criteria to delineate from myelodysplastic syndrome and myelodysplastic neoplasms.
- Revised 2024 WHO classification of myelodysplastic syndromes and myelodysplastic neoplastic disorders is given in Table 9.54.
- Diagnostic criteria of myelodysplastic syndrome are based on consideration of pre-requisite criteria, major criteria, and co-criteria. Diagnostic criteria of myelodysplastic syndrome are given in Table 9.55.

FRENCH-AMERICAN-BRITISH (FAB) AND REVISED 2024 WHO CLASSIFICATION OF MDS

Chronic myelomonocytic leukemia (CMML), a clonal hematopoietic stem cell malignancy has been included in the category of myelodysplastic syndrome according to WHO classification 2024.

- Chronic myelomonocytic leukemia is stratified into myelodysplastic (white blood cell count $<13\times10^9$/L) and proliferative (white blood cell count $>13\times10^9$/L).
- Diagnostic criteria for myelodysplastic syndrome (MDS) entities according to revised 2024 WHO classification of hematologic neoplasms are given in Table 9.56.

Table 9.54 Revised 2024 WHO classification of myelodysplastic syndromes and myelodysplastic neoplastic disorders

Myelodysplastic Syndromes
Myelodysplastic syndrome with single lineage dysplasia (MDS-SLD)
Myelodysplastic syndrome with ring sideroblasts (MDS-RS) and single lineage dysplasia (MDS-RS-SLD)
Myelodysplastic syndrome with ring sideroblasts and multilineage dysplasia (MDS-RS-MLD)
Myelodysplastic syndrome with multilineage dysplasia (MDS-MLD)
Myelodysplastic syndrome with excess blasts (MDS-EB) and erythroid predominance or fibrosis
Myelodysplastic syndrome with isolated del(5q)
Myelodysplastic syndrome unclassifiable
Childhood myelodysplastic syndrome: refractory cytopenia of childhood
Myelodysplastic/Myeloproliferative Neoplasms
Chronic myelomonocytic leukemia (CMML)
Atypical chronic myelogenous leukemia, BCR-ABL1 negative
Juvenile myelomonocytic leukemia
Myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis
Myelodysplastic/myeloproliferative neoplasms, unclassifiable

Adapted from revised 2024 WHO classification of hematologic neoplasms.

Myelodysplastic Syndrome with Single Lineage Dysplasia

Myelodysplastic syndrome with single lineage dysplasia (MDS-SLD) presents with unexplained anemia, cytopenia or bicytopenia, with 10% dysplastic cells in one myeloid lineage. Some patients present with persistent unexplained neutropenia or thrombocytopenia. Peripheral blood and bone marrow are the principal sites of involvement.

Table 9.55 Diagnostic criteria of myelodysplastic syndrome

Pre-requisite Diagnostic Criteria of Myelodysplastic Syndrome must be Fulfilled
Persistent (for 4 months duration) peripheral blood cytopenia/s in 1–3 cell lineages (erythroid, neutrophils and megakaryocytes). There is presence of abnormal phenotype of bone marrow cell 1–3 lineages analyzed by flow cytometry
Exclusion of hematopoietic and nonhematopoietic disorders of primary cytopenia/dysplasia
Major Diagnostic Criteria of Myelodysplastic Syndrome (at least one of the following major diagnostic criteria must be fulfilled)
Dysplasia in at least 10% of cells in 1–3 cell lineages (erythroid, neutrophils and megakaryocytes) in the bone marrow. Bone marrow is usually hypercellular
Presence of ≥15% ring sideroblasts (Perl Prussian blue stain positivity for iron) in bone marrow or ≥5% ring sideroblasts in the presence of SF3B1 gene mutation
Presence of 5–19% myeloblasts in the bone marrow or 2–19% myeloblasts in peripheral blood
Presence of cytogenetic abnormalities in chromosome (3, 5 and del 5q, 7 and del 7q, and trisomy 8) demonstrated by conventional karyotyping or fluorescence *in situ* hybridization (FISH)
Diagnostic Co-criteria of Myelodysplastic Syndrome
Patients fulfill pre-requisite diagnostic criteria, but not fulfill major diagnostic criteria of MDS. Clinical manifestations include macrocytic anemia and transfusion-dependent clinical course
Trephine bone marrow biopsy and immunohistochemical analysis show clonal population of cell lineage. Molecular sequencing studies demonstrate MDS-related gene mutations

Myelodysplastic Syndrome with Multilineage Dysplasia

Myelodysplastic syndrome with multilineage dysplasia is characterized by one or more cytopenias and dysplastic changes in two or more of the hematopoietic stem cell lineages (erythroid, granulocytic and megakaryocytic).

- Peripheral blood smear examination shows **<1% blasts** and $<1 \times 10^9$/L monocytes. Bone marrow demonstrates <5% blasts. Auer rods are absent in cytoplasm of myeloid cells.
- The recommended values for defining cytopenias are those suggested in the International Prognostic Scoring System (**IPSS**): (a) hemoglobin concentration <10 g/dl, (b) platelet count $<100 \times 10^9$/L and (c) absolute neutrophil count **$<1.8 \times 10^9$/L.** Blood and bone marrow are involved.
- Patient presents with evidence of bone marrow failure. Most patients have unicytopenia or bicytopenia. Some patients present with pancytopenia or milder cytopenias above the levels in the International Prognostic Scoring System (IPSS).

Myelodysplastic Syndrome with Ring Sideroblasts

Myelodysplastic syndrome (MDS) with ring sideroblasts (MDS-RS) is characterized by cytopenias, morphologic dysplasia and ring sideroblasts most often constituting ≥15% of erythroid precursor cells in bone marrow. Secondary causes of ring sideroblasts must be excluded.

- MDS-RS is associated with SF3B1 gene mutation in majority of cases. MDS-RS diagnosis is established by presence of ≥5% ring sideroblasts in bone marrow.

Table 9.56 Diagnostic criteria for myelodysplastic syndrome (MDS) entities according to revised 2024 WHO classification of hematologic neoplasms

MDS Entities	Number of Dysplastic Lineage	Number of Cytopenias	Ring Sideroblasts as % of Bone Marrow Erythroid Elements	Molecular Genetic Alterations	Myeloblasts in Bone Marrow and Peripheral Blood
MDS with single lineage dysplasia (MDS-SLD)	1	1–2	<15%	SF3B1 gene (<5%)	▪ Bone marrow <5% ▪ Peripheral blood <1% ▪ Auer rods (absent)
MDS with multilineage dysplasia (MDS-MLD)	2–3	1–3	<15%	SF3B1 gene (<5%)	▪ Bone marrow <5% ▪ Peripheral blood <1% ▪ Auer rods (absent)
MDS with ring sideroblasts and single lineage dysplasia (MDS-RS-SLD)	1	1–2	≥15%	SF3B1 gene (<5%)	▪ Bone marrow <5% ▪ Peripheral blood <1% ▪ Auer rods (absent)
MDS with ring sideroblasts and multilineage dysplasia (MDS-RS-MLD)	2–3	1–3	≥15%	SF3B1 gene (<5%)	▪ Bone marrow <5% ▪ Peripheral blood <1% ▪ Auer rods (absent)

Contd...

Table 9.56 Diagnostic criteria for myelodysplastic syndrome (MDS) entities according to revised 2024 WHO classification of hematologic neoplasms (*Contd...*)

MDS Entities	Number of Dysplastic Lineage	Number of Cytopenias	Ring Sideroblasts as % of Bone Marrow Erythroid Elements	Molecular Genetic Alterations	Myeloblasts in Bone Marrow and Peripheral Blood
MDS with isolated del(5q)	1–3	1–2	None or any	del(5q)	▪ Bone marrow <5% ▪ Peripheral blood <1% ▪ Auer rods (absent)
MDS with excess blasts and erythroid predominance (MDS-EB1)	1–3	1–3	None or any	Not applicable	▪ Bone marrow 5–9% ▪ Peripheral blood 2–4% ▪ Auer rods (absent)
MDS with excess blasts and fibrosis (MDS-EB2)	1–3	1–3	None or any	Not applicable	▪ Bone marrow 10–19% ▪ Peripheral blood 5–9% ▪ Auer rods (present)
MDS-U with 1% blood blasts	1–3	1–3	None or any	Not applicable	▪ Bone marrow <5% ▪ Peripheral blood 1% ▪ Auer rods (absent)
MDS unclassifiable with single lineage dysplasia and pancytopenia	1	3	None or any	Not applicable	▪ Bone marrow <5% ▪ Peripheral blood 1% ▪ Auer rods (absent)
MDS unclassifiable based on defining cytogenetic abnormality	0	1–3	15%	*Complex karyotype (three or more abnormalities)	▪ Bone marrow <5% ▪ Peripheral blood 1% ▪ Auer rods (absent)
Refractory cytopenia of childhood			None	Not applicable	▪ Bone marrow <5% ▪ Peripheral blood <2% ▪ Auer rods (absent)

Complex karyotype (three or more abnormalities), unbalanced abnormalities such as 7/del(7q), dl(5q)/5t(5q), i(17q)t(17q), –13/del(13q), and del(11q) and balanced abnormalities such as t(11;16(q23.3;p13/3), t(3;21)(q26.2;q22.1) and 5(1;3)(p36.3;q21.2).

- Myeloblasts account for **<5%** of the nucleated bone marrow cells and <1% of peripheral blood leukocytes. Auer rods are absent in myeloblasts.
- MDS with ring sideroblasts (MDS-RS) is of two types: (a) MDS with single lineage dysplasia, and (b) MDS with multilineage dysplasia. The peripheral blood and bone marrow are the principal sites of involvement.
- Patient presents with anemia (most often), neutropenia or thrombocytopenia.

Myelodysplastic Syndrome with Excess Blasts

Myelodysplastic syndrome (MDS) with excess blasts is characterized by 5–19% myeloblasts in the bone marrow or 2–19% blasts in the peripheral blood (but <20% in both bone marrow and peripheral blood).

- Two subtypes of MDS with excess blasts include MDS-EB1 and MDS-EB2. MDS-EB1 is defined by 5–8% myeloblasts in the bone marrow or 2–4% myeloblasts in peripheral blood and MDS-EB2 is defined by 10–19% blasts in the bone marrow or 5–19% myeloblasts in the peripheral blood.
- The presence of **Auer rods in myeloblasts** designates any MDS case as MDS-EB2 irrespective of myeloblast percentage.

Myelodysplastic Syndrome with Isolated Deletion of Chromosome 5q

Myelodysplastic syndrome with isolated del(5q) is characterized by anemia (with or without cytopenias and/or thrombocytosis), which most often occurs in elderly women.

- Cytogenetic abnormality del(5q) in myelodysplastic syndrome occurs in isolation or with monosomy 7 or del(7q). Myeloblasts constitute <5% of the nucleated cells in bone marrow and <1% nucleated cells in the peripheral blood. Auer rods are absent in myeloblasts. Peripheral blood and bone marrow are affected.
- Peripheral blood smear examination shows severe macrocytic anemia and elevated platelet count. Thrombocytosis is demonstrated in 50% of cases. Thrombocytopenia and pancytopenia are rare.
- It is recommended that cases otherwise fulfilling the MDS criteria, but with pancytopenia (hemoglobin

concentration <10 g/dl, platelet count $<100 \times 10^9$/L and absolute neutrophil count $<1.8 \times 10^9$/L) be categorized as MDS, unclassifiable, because their clinical course is uncertain.

Myelodysplastic Syndrome Unclassifiable

Myelodysplastic syndrome unclassifiable (MDS-U) lacks appropriate findings for classification into any other MDS category. The peripheral blood and bone marrow are affected. Patient presents with symptoms similar to those seen in other MDS such as anemia, neutropenia or thrombocytopenia induced clinical manifestations.

PATHOGENESIS

Myelodysplastic syndromes are considered pluripotent hematopoietic stem cell disorders of the bone marrow, which most often involves the myeloid progenitor cells. Lymphoid lineage is rarely affected.

- Many of the afflicted hematopoietic stem cells do differentiate into mature cells but exhibit ineffective progression so that their number decrease in the peripheral blood; and phenomenon is known as 'ineffective hematopoiesis'.
- In addition to decreased number of cells, the myelodysplastic cells develop into abnormal morphologic features with compromised functions.
- Gradually pluripotent hematopoietic stem cells lose their ability to differentiate; and in some persons, dysplastic cells undergo acute myelogenous leukemia. About 50% of MDS patients with normal chromosomes present with genetic instability of the myeloid precursor cell contributing to the development of MDS.
- Primary myelodysplastic syndrome originates in *de novo*.
- Secondary myelodysplastic syndrome most often occurs as a result of administration of chemotherapeutic alkylating drugs or radiotherapy in treating various cancers after 5–7 years.
  - Exposure to tobacco smoke, benzene or viruses may cause secondary MDS.
  - Other predisposing factors for developing secondary MDS include paroxysmal nocturnal hemoglobinuria (PNH), aplastic anemia, neurofibromatosis type 1, Shwachman-Diamond syndrome, Kostmann agranulocytosis type 3, Down syndrome, Fanconi syndrome, ataxia-telangiectasia, and gene mutations in N-RAS gene, TP53 gene, IRF gene and BCL-2 gene.
  - Predisposing factors and epidemiologic associations of myelodysplastic syndromes are given in Table 9.57.

Table 9.57 Predisposing factors and epidemiologic associations of myelodysplastic syndromes (MDSs)

Hereditary Predisposition to MDS*
Constitutional genetic disorders [Down syndrome (trisomy 21), trisomy 8 mosaicism, familial monosomy]
Diamond-Blackfan anemia
Neurofibromatosis type 1 (NF1)
Germ cell tumors (embryonal dysgenesis)
Congenital neutropenia (Kostmann syndrome, Shwachman-Diamond syndrome)
Germline gene mutations (GATA2 gene mutation, germline predisposing mutations in RUNX1, ANKRD26, or ETV6 genes with pre-existing platelet disorder, germline predisposing mutations in CEBPA or DDX41 genes without pre-existing condition or organ dysfunction)
DNA repair deficiencies and telomere biology disorders (ataxia-telangiectasia, Bloom syndrome, Fanconi anemia, xeroderma pigmentosum, dyskeratosis)
Acquired Causes of MDSs
Therapeutic chemotherapy in cancers (alkylating agents, DNA topoisomerase II inhibitors)
Radiation exposure to hematopoietic bone marrow, β-emitters (phosphorus 32)
Autologous pluripotent stem cell transplantation
Exposure to environmental agents (benzene, tobacco smoke, viruses)
Stem cell disorders [paroxysmal nocturnal hemoglobinuria (PNH), aplastic anemia]
Gene mutations (N-RAS gene, TP53 gene, BCL-2 gene, IRF2 gene)

**Primary myelodysplastic syndrome (MDS) originates in de novo.*

Molecular Genetic Alterations

Molecular genetic alterations, epigenetics and single gene mutations contribute to pathogenesis of myelodysplastic syndrome (MDS). *In vitro* studies reveal that excessive premature intramedullary death of hematopoietic stem cells via apoptosis leads to ineffective hematopoiesis and peripheral blood cytopenias in early MDS. Increased levels of tumor necrosis factor α (TNF-α) in MDS patients have been linked to increased apoptosis of MDS clone as well as normal hematopoietic stem cells. Progression of MDS to AML is associated with a reduction in apoptosis, therefore permitting the expansion of the neoplastic clone.

- Nearly every chromosome exhibits genomic aberrations in MDS.
- MDS clone is characterized by altered function of genes because of chromosomal abnormalities, gene silencing or single gene mutation.

- Unbalanced molecular genetic alterations are responsible for ineffective hematopoiesis. Most encountered abnormal karyotypes in MDS are the loss of 5/del (5q), loss of 7/del(7q), trisomy 8, del(20q), and loss of Y chromosome suggesting that genes located in these regions play an important role in the pathogenesis of MDS.
- Examples of molecular genetic alterations in myelodysplastic syndrome and prognosis are given in Table 9.58. Abnormal karyotyping in myelodysplastic syndrome (MDS) is given in Table 9.59. Molecular genetic alterations in primary and therapy-related myelodysplastic syndrome with approximate frequency are given in Table 9.60. Common somatic gene mutations in at least 5% cases of myelodysplastic syndrome are given in Table 9.61. Significant gene mutations in myelodysplastic syndrome are given in Table 9.62.

Table 9.58 Examples of cytogenetic abnormalities in myelodysplastic syndrome and prognosis

Prognosis	Cytogenetic Abnormalities
Very good prognosis	Loss of Y chromosome or del(11q)
Good prognosis	Normal or del(5q)
Intermediate prognosis	del(7q) or double independent clones
Poor prognosis	inv(3) or double loss including 1 and 7 del(7q)
Very poor prognosis	Complex: >3 abnormalities

Table 9.59 Abnormal karyotyping in myelodysplastic syndrome

MDS Associated with Chromosome 5 and Deletion (5q)
Chromosome 5 and del(5q) is most frequent acquired genetic abnormality demonstrated in 10–20% of MDS cases. Three commonly deleted regions (CDRs) on chromosome 5q31–33 include EGR1 gene (early growth response gene 1), NPM1 gene (nucleophosmin 1) and RPS14 (ribosomal subunit)
Normally, EGR1, NPM1 and RPS14 genes encode proteins, that control cell signaling, transcription, cell cycle and apoptosis.
Loss of commonly deleted regions (CDRs) on chromosome 5 plays significant role in the pathogenesis of MDS. Whole or partial losses of chromosome 5 are somatically acquired.
These patients are treated with radiotherapy, alkylating agents, or DNA topoisomerase II antagonists. 5q syndrome manifests as anemia, with or without mild neutropenia and platelet counts either preserved or elevated
The prognosis is relatively good. Several cytokines, growth factors, and their receptors are found at the 5q locus
MDS Associated with Chromosome 7 and Deletion (7q)
Chromosome 7 and del(7q) genetic abnormality is most frequently demonstrated ≤20% of MDS cases. Three commonly deleted regions (CDRs) are demonstrated on chromosome 7 at 7q22, 7q31–32 and 7q36
This cytogenetic abnormality is most often demonstrated in children
Impaired neutrophilic function is associated with recurrent infections
MDS Associated with Trisomy 8
Trisomy 8 is demonstrated in about 10% of MDS cases. In contrast to chromosome 5 and del(5q) and chromosome 7 and del(7q), trisomy 8 in MDS and AML is not associated with prior treatment with radiation, alkylating agents or DNA topoisomerase II antagonists
It has been suggested that effect of trisomy is influenced by the overexpression of the proto-oncogene Myc located on chromosome 8q24
MDS Associated with Chromosome Deletion (20q)
L3MBTL1 gene is located on chromosome 20q12.13, that encodes a transcriptional repressor
Deletion of chromosome in the region has been demonstrated in 5% of cases
Loss of L3MBTL1 gene is likely to be involved in the neoplastic state of MDS
MDS Associated with Loss of Y Chromosome
Loss of the Y chromosome is variable in MDS as well as healthy aging persons
Loss of chromosomal Y is likely an age-related abnormality, independent of a neoplastic process
MDS Associated with Complex Karyotype
Complex karyotype (involving >3 chromosomal abnormalities such as whole or partial deletions of chromosome 5 and chromosome 7) is demonstrated in 20% of primary MDS cases and 90% of treatment related MDS patients

Table 9.60 Molecular genetic alterations in primary and therapy-related myelodysplastic syndrome (MDS) with approximate frequency

Molecular Genetic Alterations	Primary Myelodysplastic Syndrome	Therapy-related Myelodysplastic Syndrome
Complex karyotype	15–20%	80–90%
Deletion (5q) monosomy 5 (favorable prognosis)	15–20%	30–40%
Deletion (7q) monosomy 7 (unfavorable prognosis)	10–15%	40–50%
Trisomy 8 (intermediate prognosis)	10–15%	10–15%
Deletion (20q) (favorable prognosis)	5–10%	Nil
Loss of Y chromosome	5%	Nil
Deletion (11q)	<5%	Nil
Deletion (12p)	<5%	Nil
Deletion (17q)	<5%	Nil
Deletion (9q)	1–2%	Nil
Idic(X)(q13)	1–2%	Nil

Table 9.61 Common somatic gene mutations in at least 5% cases of myelodysplastic syndrome

Gene Mutation	Pathway	Frequency	Prognosis
RNA splicing factors			
SF3B1	RNA splicing factor	20–30%	Favorable prognosis
SRSF2	RNA splicing factor	≤15	Adverse prognosis
U2AF1	RNA splicing factor	5–10%	Adverse prognosis
ZRSR2	RNA splicing factor	5–10%	Adverse prognosis
Transcriptional factors			
N-RAS	Transcription factor	≤5%	Adverse prognosis
BCOR1	Transcription factor	≤5%	Adverse prognosis
RUNX1	Transcription factor	≤10	Adverse prognosis
ETV6	Transcription factor	<5%	Adverse prognosis (transformation to chronic myelomonocytic leukemia)
TP53 (tumor suppressor gene)	Transcription factor	5–10%	Adverse prognosis
GATA2	Transcription factor	<5%	Adverse prognosis (recurrent infection)
Growth factor signaling			
N-RAS/K-RAS	Growth factor signaling	5–10%	Adverse prognosis
JAK2	Growth factor signaling	<5%	Adverse prognosis
CBL*	Growth factor signaling	≤5%	Adverse prognosis
Epigenetic regulators			
TET2*	Epigenetic regulator (DNA methylation)	20–30%	Either neutral prognostic impact or conflicting data
DNMT3A*	Epigenetic regulator (DNA methylation)	≤10	Adverse prognosis
ASXL*	Epigenetic regulator (histone modification)	15–20%	Adverse prognosis
EZH2	Epigenetic regulator (histone modification)	5–10%	Adverse prognosis
STAG2	Cohesion complex	5–7%	Adverse prognosis
IDH1/IDH2	DNA methylation	≤5%	Either neutral prognostic impact or conflicting data

**Clonal hematopoiesis of indeterminate potential.*

Table 9.62 Significant gene mutations in myelodysplastic syndrome (MDS)

Gene	Locus on Chromosomes	Normal Function of Protein	Gene Mutation Frequency in MDS	Significance
ASXL1 (additional sex combs like 1) gene	20q11.1	Gene product regulates the expression of many genes, that play important role before birth	≤11%	Gene mutation causes myeloproliferation (prevalent in MDS/MPN)
EGR1 (early growth response 1) gene	5q31	Gene product regulates the transcription of numerous target genes, thereby regulating the response to growth factors, which regulate cell proliferation, survival and apoptosis	5–10%	Putative tumor suppressor gene, initial event in MDS
NPM1 (nucleoplasmin 1) gene	5(q35)	NPM1 gene protein 'nucleophosmin functions as tumor suppressor gene	5%	Putative proto-oncogene that inactivates p53
EVI1 (ecotropic viral integration site 1) gene	3q26.2	EVI1 gene product regulates normal development of hematopoietic cells and leukemogenesis	≤5%	Putative tumor suppressor signaling and apoptosis
JAK2V617F (Janus kinase 2) gene	9p	JAK2 gene protein tyrosine kinase controls production of blood cells from hematopoietic stem cells	5%	JAK2 gene mutation causes hypersensitivity to cytokines
N-RAS (neuroblastoma rat sarcoma) gene	1p13.2	N-RAS protein regulates cell division	≤15%	Activating gene mutations stimulate MARK signaling
RUNX1 (runt-related transcription factor 1) gene	21q22.12	RUNX1 gene protein activates genes that regulates hematopoiesis	20%	Important for differentiation of hematopoietic cells
K-RAS (Kristen rat sarcoma) gene	12p12.1	K-RAS protein regulates controlled cell proliferation	≤40% in advanced MDS	Associated with a worse prognosis and increased risk of progression to AML
FLT3 (Fms-related tyrosine kinase 3) gene encodes tyrosine kinase, that regulates proliferation, differentiation, apoptosis of hematopoietic stem cells	13.q12	FLT3 protein transmits signals from the cell surface into the cell through signal transduction	5–10%	FLT3 gene mutation associated with progression of AML
TP53 gene	17p13.1	p53 gene protein regulates cell cycle	20% and 25% in secondary MDS	Encodes a checkpoint protein (p53), rapid progression to AML with poor prognosis

CLINICAL FEATURES

Patient presents with fatigue and weakness related to anemia, that is nonresponsive to treatment. Less commonly symptoms are bleeding tendencies and related to thrombocytopenia and recurrent infections related to neutropenia $<1\times10^9$/L) as a result of apoptosis of hematopoietic precursor cells. Bleeding and recurrent infection are most common causes of death in MDS patients.

- Many patients are asymptomatic and detected on routine hematologic investigations. Splenomegaly and hepatomegaly are uncommon.
- Synthesis of transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) cause bone marrow fibrosis resulting in pancytopenia, extramedullary hematopoiesis in spleen and liver. MDS patient may develop acute myelogenous leukemia (AML) in about 40–50% of cases.

LABORATORY DIAGNOSIS

Patient with MDS presents with a range of abnormal morphologic features demonstrated on stained peripheral blood and bone marrow.

- Laboratory investigations performed to diagnose myelodysplastic syndrome include peripheral blood smear examination, bone marrow trephine biopsy examination, bone marrow aspiration, biochemical investigations, molecular diagnostics and immunophenotyping.
- Salient features of myelodysplastic syndrome are given in Table 9.63. Hematologic abnormalities in

Table 9.63 Salient features of myelodysplastic syndrome
Peripheral blood picture of cytopenia in 1–3 cell lineages
Dysplasia in 1–3 cell lineages demonstrated in peripheral blood and bone marrow
Bone marrow usually hypercellular
Increased ring sideroblasts
Increase in myeloid precursor antigens (CD34, CD117, HLA-DR) and immature granulocytic antigens (CD13, CD33)
Decrease in mature granulocytic antigens (CD10, CD11b, CD16, CD64)
Aberrant expression of nonmyeloid markers (CD7, CD19, CD56)
Identification of cell lineage and distribution by immunohistochemistry
Cytogenetic abnormalities as discussed in this chapter

myelodysplastic syndrome are given in Table 9.64. Characteristics of flow cytometric analysis of myelodysplastic syndrome are given in Table 9.65.

PROGNOSTIC CRITERIA

Accurate classification and prognosis of MDS is essential to plan therapy. MDS is categorized into three risk groups on the basis of survival time and evolution to AML. The comprehensive cytogenetic prognostic scoring system for myelodysplastic syndrome is given in Table 9.66.

MOLECULAR DIAGNOSTICS

Fluorescence *in situ* hybridization (FISH) provides diagnostic and prognostic information and monitor response to therapy in MDS cases. Specific FISH probes demonstrate commonly encountered abnormal karyotypes. Molecular diagnostic testing includes gene expression analysis by microarray, next generation sequencing and whole genome analysis to elucidate the complicated genetic profile of MDS.

Table 9.65 Characteristics of flow cytometric analysis of myelodysplastic syndrome (MDS)
Decreased granulocytic side scanner as a result of hypogranularity of granulocyte cytoplasm
Reduced expression of CD15, CD10, CD16, CD11b and CD13 on myeloid progenitors and maturing myeloid cells
Loss of synchronized expression on granulocytes including the failure of CD64/CD33, CD16/CD13, and CD16/CD11b antigens to co-express as expected
Characteristics lymphoid antigens such as CD2, CD5, CD7, CD19 and CD56 inappropriately expressed on granulocytes and/or monocytes
Decreased or absent CD4, CD13, and HLA-DR antigen expression on monocytes
Retention of an abnormally immature phenotype as a result of expansion of CD34+ and CD117+ immature cells

Table 9.64 Hematologic abnormalities in myelodysplastic syndrome

Erythroid Series	Myeloid Series	Thrombopoietic Series
Peripheral blood smear findings		
Red blood cell changes	**White blood cell changes**	**Platelet changes**
▪ Anemia ▪ Anisocytosis ▪ Poikilocytosis ▪ Macrocytes, oval macrocytes ▪ Basophilic stippling ▪ Nucleated RBCs ▪ Howell-Jolly bodies ▪ Sideroblasts ▪ Reticulocytopenia	▪ Neutropenia ▪ Hypogranulation, abnormal granulation ▪ Shift to the left ▪ Nuclear abnormalities (pseudo-Pelger-Hüet, ring nuclei) ▪ Monocytosis	▪ Thrombocytopenia or thrombocytosis ▪ Giant forms ▪ Hypogranulation ▪ Micromegakaryocytes ▪ Functional abnormalities
Bone marrow		
Erythropoiesis	**Myelopoiesis**	**Megakaryopoiesis**
▪ Megaloblastoid erythropoiesis ▪ Nuclear fragmentation and budding ▪ Nuclear karyorrhexis ▪ Multiple nuclei in erythroid cells ▪ Defective hemoglobinization ▪ Vacuolization in erythroid cells ▪ Ring sideroblasts	▪ Abnormal granules in promyelocytes ▪ Increase in granular and agranular blats ▪ Absence of secondary granules ▪ Nuclear abnormalities ▪ Decreased myeloperoxidase ▪ Auer rods in myeloblasts	▪ Micromegakaryocytes ▪ Megakaryocytes with multiple separated nuclei ▪ Large mononuclear megakaryocytes ▪ Hypogranular or large abnormal granules in megakaryocytes

Table 9.66 The comprehensive cytogenetic prognostic scoring system and recommended values for defining cytopenias are those suggested in the International Prognostic Scoring System (IPSS) for myelodysplastic syndrome

Parameters	Very Good Prognosis	Good Prognosis	Intermediate Prognosis	Poor Prognosis	Very Poor Prognosis
The recommended values for defining cytopenias are those suggested in the International Prognostic Scoring System (IPSS)					
Myeloblasts (%)	≤2%	>2–<5%	5–10%	>10%	>10%
Hb (g/dl)	≥10 g/dl	8–<10 g/dl	–	–	–
Platelet count	$\geq 100 \times 10^9$/L	$\leq 50–100 \times 10^9$/L	–	–	–
Absolute neutrophil count	$\geq 1.8 \times 10^9$/L	–	–	–	–
Defining cytogenetic abnormalities and prognostic significance					
Cytogenetic abnormalities	▪ Loss of Y chromosome ▪ del(11q)	▪ del(5q) ▪ del(12p) ▪ del(20q) [single/ double deletion including del(5q)]	▪ del(7q) ▪ Gain(8,19) ▪ Isochromosome 17q ▪ Single or double abnormalities, not specified in other subgroups ▪ Two or more independent non-complex clones	▪ Loss (7) ▪ inv(3), t(3q) or del(3q) ▪ Double including loss of chromosome 7 or del(7q) ▪ Complex (>3) cytogenetic abnormalities	▪ Complex (>3) cytogenetic abnormalities

Laboratory Diagnosis of Myelodysplastic Syndrome (MDS)

Biochemical Investigations

- Serum iron and serum ferritin levels are normal or increased.
- Total iron binding capacity (TIBC) is normal or decreased, a feature distinguishing MDS from iron deficiency anemia.
- Serum vitamin B_{12} and folic acid levels are normal or increased, a feature distinguishing MDS from megaloblastic anemia.
- Lactic dehydrogenase and uric acid levels are increased as a result of ineffective hematopoiesis.

Peripheral Blood Smear Examination

- Peripheral blood smear examination demonstrates cytopenias and dysplasia. In cytopenias, patient has hemoglobin <10 g/dl, absolute neutrophil count,1.8×10^9/L and platelets $<100 \times 10^9$/L.
- Anemia is most consistent finding in >80% of cases. Bicytopenia is demonstrated in 30% and pancytopenia in 15% of cases. Functional abnormalities of hematologic cells are common.
- Dysplastic features of one or more cell lineages are demonstrated. Higher degree and number of cytopenias are associated with poor prognosis.
- Peripheral blood smears should be prepared within two hours of anticoagulated blood. Morphologic changes from prolonged exposure to anticoagulant in blood sample can easily be confused with dysplastic changes in hematopoietic cells.

Red blood cells

Peripheral blood smear shows macrocytic picture with oval cells similar to those seen in megaloblastic anemia. Dimorphic anemia is also common finding. In addition to anemia, qualitative abnormalities indicative of dyserythropoiesis is demonstrated such as anisocytosis, poikilocytosis, basophilic stippling, Howell-Jolly bodies and nucleated erythrocytes.

Leukocytes

Neutropenia accompanied by shift to the left with myelocytes and metamyelocytes is the second most common cytopenia demonstrated in 50% MDS cases.

- Abnormalities in granulocytes indicative of dysgranulopoiesis is the hallmark finding in MDS, in which the granulocytes are agranular or hypogranular with persistent basophilic cytoplasm and hyposegmentation (pseudo-Pelger-Hüet abnormality).
- Neutrophils show decreased myeloperoxidase and leukocyte alkaline phosphatase (LAP). In some MDS cases, neutrophils show severe functional impairment including defective bactericidal, phagocytic or chemotactic properties.
- Absolute monocytosis is a constant finding even in leukemic conditions.
- MDS-related pancytopenia in Giemsa-stained peripheral blood smear is shown in Fig. 9.58. MDS-related band form segmented neutrophils in Giemsa-stained peripheral blood smear is shown in Fig. 9.59. MDS-related Pelger-Hüet cell abnormality in Giemsa-stained peripheral blood smear is shown in Fig. 9.60. MDS-related abnormal monocytoid cells in Giemsa-stained peripheral blood smear is shown in Fig. 9.61.

Platelets

- Qualitative and quantitative platelet abnormalities are demonstrated in MDS. About 25–50% of patients present with mild to moderate thrombocytopenia.
- Peripheral blood smear examination shows giant platelets, hypogranular platelets and platelets with large, fused platelets. Qualitative abnormalities include abnormal platelets adhesion and aggregation.
- Sometimes, peripheral blood smear examination shows micromegakaryocytes in MDS.
  - Micromegakaryocytes represent abnormal megakaryocytes with reduced ability to undergo endomitosis.

- Micromegakaryocytes contain single-lobed nucleus with attached one or more platelets. Nuclear structure of micromegakaryocytes is variable with dense clumped chromatin demonstrated by Wright-Giemsa stain.

Bone Marrow Smear Examination

Bone marrow smear examination is essential to demonstrate dyshematopoiesis, cellularity and diagnosing of myelodysplastic syndrome. Bone marrow shows increased iron demonstrated by Perls' Prussian blue stain. Myelodysplastic syndrome related iron overload demonstrated by Perls' Prussian blue reaction in bone marrow aspirate smear is shown in Fig. 9.62.

- **Cellularity:** Hypercellular bone marrow with peripheral blood picture of cytopenia occurs because of premature hematopoietic cells apoptosis and ineffective erythropoiesis. Bone marrow contains <20% myeloblasts. At least 10% of hematopoietic lineage cells should be dysplastic to establish diagnosis.
- **Dyserythropoiesis:** Nuclear-cytoplasmic asynchrony similar to megaloblastic erythropoiesis is the most common finding in MDS.
  - However, the chromatin of dyserythropoietic cells show hypocondensation of chromatin. Patients with MDS are unresponsive to vitamin B_{12} or folic acid therapy. Giant multinucleated erythroid precursors can be demonstrated.
  - Cytoplasm of erythroid precursor cells shows defective hemoglobinization, basophilic staining and vacuoles. Ring sideroblasts reflecting the abnormal erythrocyte metabolism are commonly demonstrated.
  - Ring sideroblasts contain at least five mitochondrial iron deposits encircle at least one-third or more of the circumference of the nucleus.
- **Dysgranulopoiesis:** Abnormal granulocyte maturation is almost always demonstrated.
  - In dysgranulopoiesis, bone marrow examination shows abnormal staining of granules in the promyelocytes and myelocytes. Sometimes, granules may be larger than normal.
  - Secondary granules are absent in myelocytes and mature neutrophils resulting in hypogranular neutrophils with irregular cytoplasmic basophilia demonstrated in the bone marrow.
- **Dysmegakaryopoiesis:** Bone marrow examination may show decreased, normal or increased megakaryocytes.
  - Presence of micromegakaryocytes, mononuclear megakaryocytes and megakaryocytes with abnormal nuclear configurations such as hyperlobulated or hypolobulated and multiple widely separated nuclei reflect abnormalities in maturation.
  - Dysplastic micromegakaryocytes containing single eccentric nucleus are demonstrated in MDS with chromosome del(5q) and abnormality of chromosome 3.
  - MDS-related dysmegakaryopoiesis in hematoxylin and eosin-stained bone marrow trephine biopsy section is shown in Fig. 9.63.

Bone Marrow Trephine Biopsy Examination

- Bone marrow trephine biopsy is helpful in diagnosing MDS in difficult cases. Abnormal localization of immature precursors can be demonstrated in bone marrow trephine biopsy before an increase in number of myeloblasts is demonstrated in bone marrow smears. ALIP has been associated with increased risk of AML in MDS cases.
- Presence of <5% of myeloblasts and abnormal localization of immature precursors (ALIP) indicates evolution to a more aggressive MDS.
- Bone marrow trephine biopsy provides a better assessment of cellularity and amount of bone marrow fibrosis. Reticulin stain demonstrates slight increase in reticulin fibers.
- Bone marrow trephine biopsy also shows the ring sideroblasts, nuclear fragmentation and budding. Auer rods, irregular cytoplasmic basophilia and abnormal granules in promyelocytes are more easily demonstrated in bone marrow smears.
- Thus, bone marrow aspiration and bone marrow trephine biopsy preparations are essential for accurate diagnosis of MDS.

Immunophenotyping

- Immunophenotyping is used in diagnosing and predicting prognosis of MDS patients. It is most useful in MDS cases, where bone marrow aspiration, bone marrow trephine biopsy and cytogenetics are inconclusive. It helps in distinguishing MDS cases with hypoplastic bone marrow morphology from other cause of bone marrow failure disorder such as aplastic anemia.
- Aberrant antigenicity demonstrated in MDS includes lymphoid antigens on myeloid cells, increased or decreased expression of expected antigens, the presence of immature antigens on mature cells, and expression of mature antigens on immature cells.

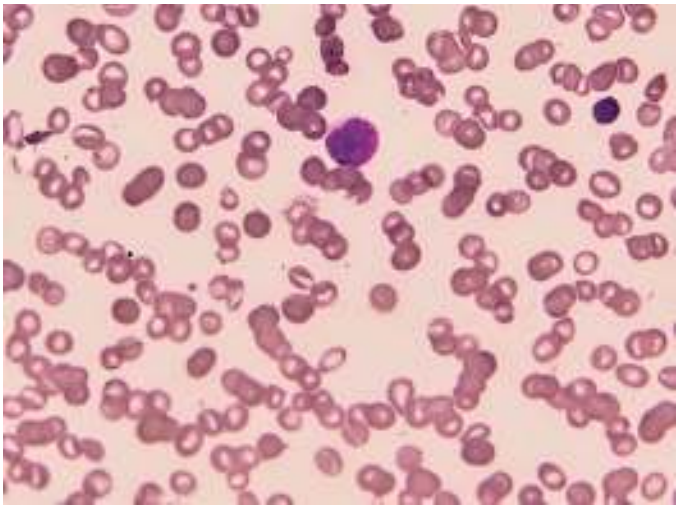


Fig. 9.58: Myelodysplastic syndrome related pancytopenia in Giemsa-stained peripheral blood smear. It shows pancytopenia with blast cells (1000X).

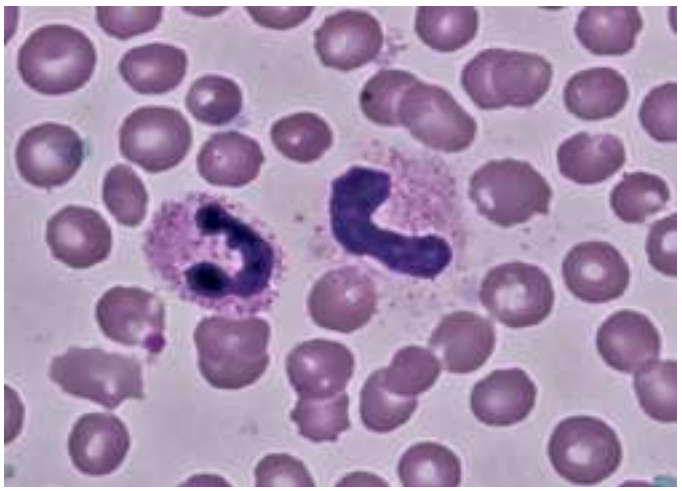


Fig. 9.59: Myelodysplastic syndrome related band form segmented neutrophils in Giemsa-stained peripheral blood smear. It shows band form segmented neutrophils (1000X).

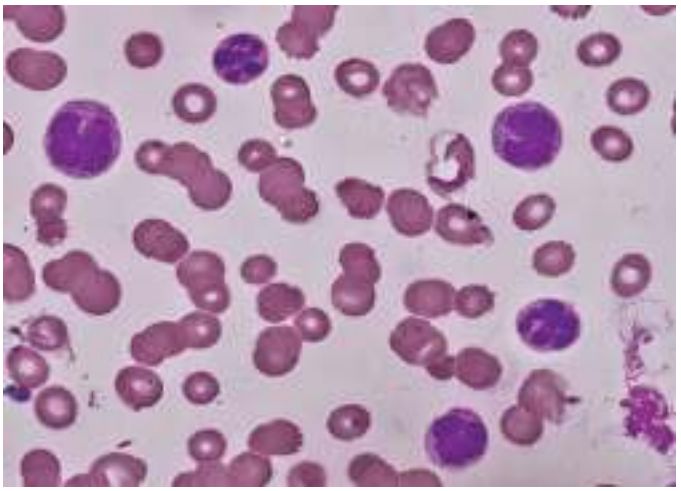


Fig. 9.61: Myelodysplastic syndrome related abnormal monocytoid cells in Giemsa-stained peripheral blood smear (1000X).

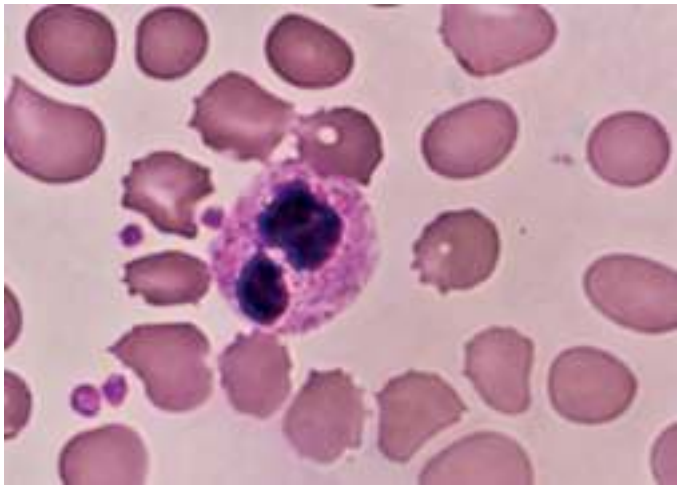


Fig. 9.60: Myelodysplastic syndrome related Pelger-Hüet cell abnormality in Giemsa-stained peripheral blood smear. It shows MDS related Pelger-Hüet cell abnormality (1000X).

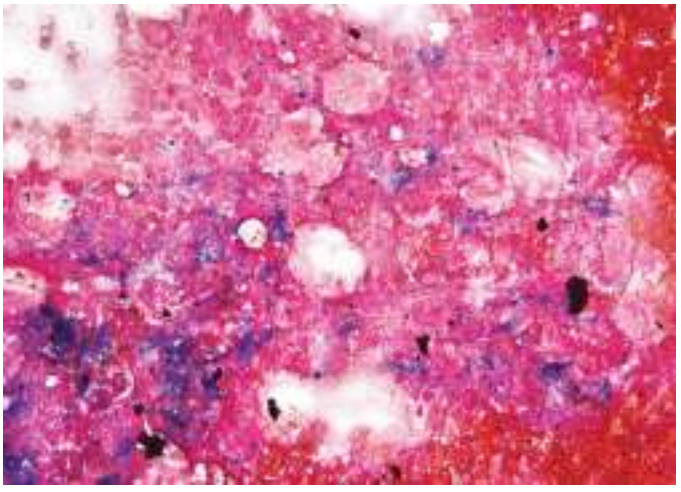


Fig. 9.62: Myelodysplastic syndrome related iron overload demonstrated by Perls' Prussian blue reaction in bone marrow aspirate smear (1000X).

TREATMENT

Therapeutic strategies of myelodysplastic syndrome (MDS) include supportive care, suppression of MDS clone and its leukemia progeny, improvement bone marrow function and curative allogeneic hematopoietic stem cell transplantation. It is essential to segregate patients into low-risk and high-risk before making therapeutic decision in these cases.

Supportive Care

Cytopenias-related recurrent infections cause death in MDS patients. Recurrent infections in the setting of neutropenia must be treated aggressively by administration of granulocyte-colony stimulating factor **(G-CSF)** and erythropoietin. These therapeutic agents do not hasten leukemic process. Thrombopoietin administration may hasten leukemic process. Recently, thrombopoietin receptor agonist 'Eltrombopag' agent is promising therapeutic agent.

Allogeneic Hematopoietic Stem Cell Transplantation

Allogeneic hematopoietic stem cell transplantation is the only curative in MDS. Decision for hematopoietic stem cell transplantation demands balancing the probability of disease progression and procedural abnormality and mortality. Favorable outcomes of hematopoietic stem cell transplantation are seen in younger patients with HLA-identical siblings.

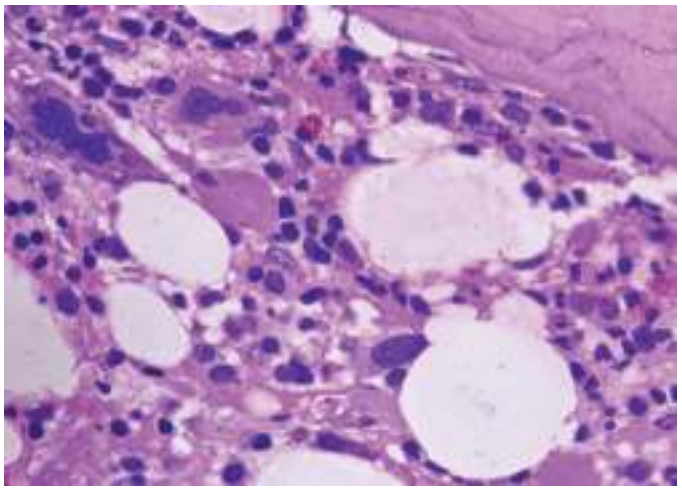


Fig. 9.63: Myelodysplastic syndrome (MDS) related dysmegakaryopoiesis in hematoxylin and eosin-stained bone marrow trephine biopsy. It shows dysmegakaryopoiesis (1000X).

Disease-modifying Hypermethylation Agents (DNA Methyltransferase Inhibitors)

DNA methyltransferases participate in hypermethylation of CpG promoter regions of many tumor suppressor genes resulting in decreased gene expression without changing the DNA sequence. Acquired hypermethylation of tumor suppressor genes downregulates expression and increasing the potential for dysplastic growth. '**Azacytidine**' is currently approved therapeutic agent to treat patients with all MDS subtypes.

Immunomodulatory Agents and Immunosuppression

Lenalidomide is an oral analog of thalidomide with greater potency and established efficacy. It is superior, and safe in treating MDS. Lenalidomide is administered in MDS patients with chromosome deletion 5q, with or without other cytogenetic abnormalities.

MYELODYSPLASTIC/MYELOPROLIFERATIVE NEOPLASMS

MYELODYSPLASTIC/MYELOPROLIFERATIVE NEOPLASMS: ENTITIES

Myelodysplastic/myeloproliferative neoplasms have features of both myelodysplastic/myeloproliferative neoplasms.

- Normally, bone marrow makes enough healthy red blood cells, white blood cells and platelets.
- In myelodysplastic/myeloproliferative neoplasms, hematopoietic stem cells do not mature into healthy red blood cells, white blood cells and platelets. Majority of these immature cells die within the bone marrow or soon after they enter in the blood circulation. As a result, there is presence of fewer healthy red blood cells, white blood cells and platelets in the blood.
- In myeloproliferative neoplasms (MPNs), bone marrow produces excessive cells in one or more types of the cell lineages. Some of these cells may show morphologically and/or functionally dysplastic features.
- Cytopenia may be demonstrated due to ineffective cell production in one or more of cell lineages in the bone marrow. The blast percentage in the bone marrow and peripheral blood is always <20%.
- A myeloblastic/myeloproliferative neoplasm may develop into acute myelogenous leukemia (AML).
- There are three types of myelodysplastic/myeloproliferative neoplasms: (a) chronic myelomonocytic leukemia (**CMML**), (b) juvenile myelomonocytic leukemia (**JMML**), and (c) atypical chronic myelogenous leukemia (**aCML**).
- Schematic representation of origin of bone marrow myeloproliferative neoplasms is shown in Fig. 9.64. Features of myelodysplastic/myeloproliferative neoplasms are given in Table 9.67.

CHRONIC MYELOMONOCYTIC LEUKEMIA

Chronic myelomonocytic leukemia (CMML) is a clonal hematopoietic stem cell neoplasm associated with persistent relative >10% of absolute monocytosis with monocyte count ($>1 \times 10^9$/L) in the peripheral blood. In bone marrow, some of monoblasts do not develop into mature monocytes, and persists for >3 months. CMML has features of both myeloproliferative neoplasm and myelodysplastic syndrome.

- The monocytes and immature cells (promonocytes and monoblasts) crowd out the other lineage precursor cells in the bone marrow with suppression of erythroid cells and megakaryocytes.
- Although the peripheral blood finding of absolute monocytosis is essential for CMML diagnosis, yet other hematologic findings are remarkably variable such as leukocytosis, neutrophilia or neutropenia.
- Dyshematopoiesis can range from minimal expression in single cell lineage to marked dysplasia in all the lineages. Blood and bone marrow are invariably involved in CMML.
- Spleen, liver, skin and lymph nodes are the most common extramedullary sites of involvement, CMML as inherent risk for transformation to acute myelogenous leukemia.

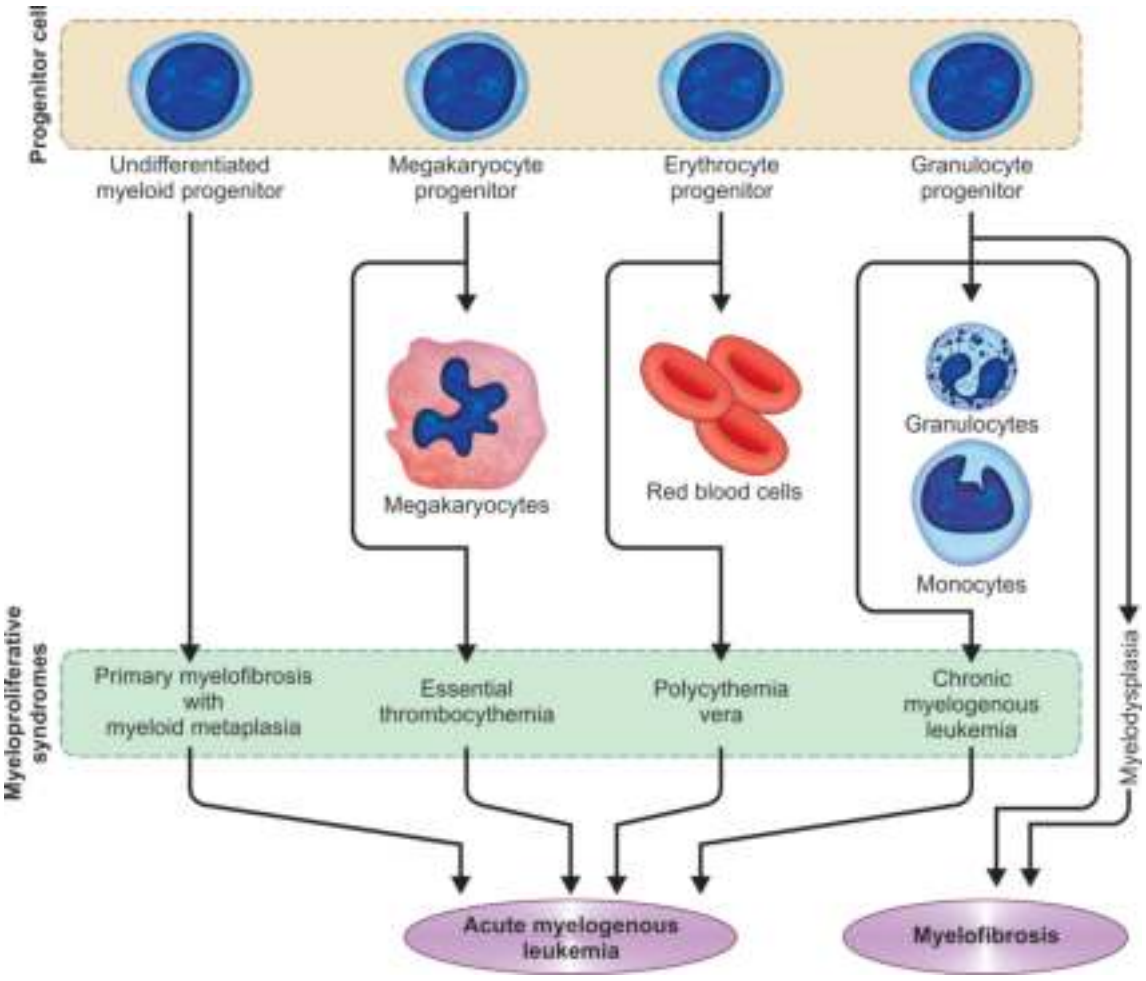


Fig. 9.64: Schematic representation of origin of bone marrow myeloproliferative neoplasms. Each malignant disorder is derived from particular type of myeloid progenitor cells. The myeloproliferative neoplasms are clonal hematopoietic disorders characterized by an overproduction of differentiated hematopoietic cells.

- CMML most often occurs in persons over 60 years of age, which is more common in men than in women. About 20–40% of persons have changes in certain chromosomes.

Molecular Genetic Alterations

The cell of origin of chronic myelomonocytic leukemia (CMML) is believed to be the hematopoietic stem cell (HSC). The clonal molecular genetic alterations are demonstrated in 20–40% of patients, however, none of these clonal molecular genetic alterations is specific.

- Most frequently molecular genetic alterations include chromosome +8, –7/del(7q), del(12p), del(20p), isochromosome 17q, and complex karyotype.
- Mutations in N-RAS/K-RAS, RUNX1, TET2, CBL, or ASX1 genes are demonstrated in 40% of patients.

Diagnostic Criteria

Diagnostic criteria for chronic myelomonocytic leukemia according to revised 2024 World Health Organization (WHO) recommendations are given below:

- Peripheral blood smear examination shows monocytosis $>1 \times 10^9$/L, and <20% of cells (e.g. myeloblasts, monoblasts and promonocytes) in the peripheral blood for at least 3 months including exclusion of all other reactive causes of monocytosis.
- Bone marrow examination shows monoblasts and monocytes, that constitute <20% including dysplasia in one or more myeloid lineages.
- Molecular genetic study shows absence of Philadelphia chromosomal abnormality or BCR-ABL1 fusion gene, and without rearrangement of PDGFRA and PDFGRB genes.
- Some CMMLs are more myeloproliferative in nature with white blood cell count 13×10^9/L, whereas others demonstrate more dysplastic characteristics with white blood cell count $<13 \times 10^9$/L. Diagnostic criteria of chronic myelomonocytic leukemia are given in Table 9.68.

Table 9.67 Features of myelodysplastic/myeloproliferative neoplasms

Classification	Peripheral Blood Smear Examination	Bone Marrow Examination	Genetics	Immunophenotyping
Chronic myelomonocytic leukemia (CMML-1)	▪ Monocytosis >1 × 10^9/L ▪ Blasts <5% including monocytes	▪ Blasts <10% including promonocytes ▪ Dysplasia of ≥1 myeloid lineage	BCR-ABL1 negative	▪ CD33+ ▪ CD13+ ▪ CD14 variable ▪ CD68 variable ▪ CD64 variable ▪ Lysozyme +
Chronic myelomonocytic leukemia (CMML-2)	▪ Monocytosis >1 × 10^9/L ▪ Blasts 5–19% including monocytes	▪ Blasts <10–19% including promonocytes or ▪ When Auer rods present, regardless of blast/promonocyte count	BCR-ABL1 negative	▪ CD33+ ▪ CD13+ ▪ CD14 variable ▪ CD68 variable ▪ CD64 variable ▪ Lysozyme +
Juvenile myelomonocytic leukemia (JMML)	▪ Monocytosis >1 × 10^9/L ▪ Blasts <20%	▪ Bone marrow hypercellular ▪ Blasts <20%, dysgranulopoiesis (occasional dyserythropoietic cell)	BCR-ABL1 negative	▪ CD33+ ▪ CD13+ ▪ CD14 variable ▪ CD68 variable ▪ CD64 variable ▪ Lysozyme +
Atypical chronic myelogenous leukemia (aCML, BCR-ABL1 negative)	▪ Leukocytosis >13 × 10^9/L ▪ Blasts <5% ▪ Promyelocytes, myelocytes, metamyelocytes: 10–20%	▪ Bone marrow hypercellular ▪ Blasts <20%; dysgranulopoiesis, occasional dyserythropoiesis	BCR-ABL1 negative	▪ CD33+ ▪ CD13+ ▪ Myeloperoxidase +
Myelodysplastic/ myeloproliferative neoplasm, unclassifiable (MDS/MPN, U)	▪ Anemia ▪ Leukocytosis and/or thrombocytosis ▪ Blasts <20%	▪ Bone marrow hypercellular ▪ Proliferation in any or all myeloid lineages	BCR-ABL1 negative	Nondiagnostic

Table 9.68 Diagnostic criteria of chronic myelomonocytic leukemia (CMML)

Peripheral Blood Smear Examination

- Persistent peripheral monocytosis >1 × 10^9/L for at least 3 months, monocyte count ≥10% of the leukocytes; and exclusion of all other reactive causes of monocytosis
- Monoblasts and monocytes constitute <20% containing Auer rods
- CMML-0 shows <2% monoblasts
- CMML-1 shows <5% monoblasts
- CMML-2 shows 5–19% monoblasts or Auer rods

Bone Marrow Examination

- Monoblasts and monocytes constitute <20%
- CMML-0 shows <2% monoblasts
- CMML-1 shows <10% monoblasts
- CMML-2 shows <20% monoblasts or Auer rods
- Dysplasia in one or more of myeloid lineages (if dysplasia is absent or minimal, diagnosis of CMML can still be made if the other requirements are met and an acquired cytogenetic or molecular abnormality is present)

Not Meeting WHO Criteria

- BCR-ABL1 positivity as demonstrated in chronic myelogenous leukemia
- Primary myelofibrosis
- Polycythemia vera
- Essential thrombocythemia

No Evidence of Gene Mutation

- PDGFRA
- PDFGRB
- PCM1-JAK2
- EGFR1 rearrangement

Table 9.69 Revised 2024 WHO subclassification for chronic myelomonocytic leukemia (CMML)

CMML Variants in Comparison to AML	Peripheral Blood Smear Examination	Bone Marrow Examination
CMML-0	<2% blasts	<2% blasts
CMML-1	<5%blasts	5–9% blasts
CMML-2	5–19% blasts or Auer rods	10–19% blasts or Auer rods
Acute myelogenous leukemia	≥20% blasts	≥20% blasts

CMML Subcategories

Currently, chronic myelomonocytic leukemias (CMMLs) can be divided into three subcategories (CMML-0, CMML-1 and CMML-2) depending on the number of blasts present in the peripheral blood and bone marrow.

- **Chronic myelomonocytic leukemia 0 (CMML-0):** It is defined by the presence of <2% blasts in peripheral blood and <2% blasts in the bone marrow.
- **Chronic myelomonocytic leukemia 1 (CMML-1):** It is defined by the presence of <5% blasts (i.e. 2–4 blasts) in the peripheral blood and <10% blasts (i.e. 5–9% blasts) in the bone marrow.
- **Chronic myelomonocytic leukemia 2 (CMML-2):** It is defined by the presence of 5–19% blasts (i.e. 5–19) or Auer rods in the peripheral blood and <20% (i.e. 10–19) blasts in the bone marrow or whenever Auer rods are present in blasts. Revised 2024 WHO subclassification for chronic myelomonocytic leukemia is given in Table 9.69.

Clinical Features

Patient presents with fever, weight loss, pallor, generalized weakness, recurrent infections, shortness of breath, easy bruising, and hepatosplenomegaly. Certain factors affect the prognosis and treatment options, which include: the number of white blood cells or platelets produced in the bone marrow or blood, presence or absence of anemia, percentage of blasts in the bone marrow or blood, the amount of hemoglobin in the red blood cells and certain alterations in the chromosomes.

Prognostic Factors

Prognosis of CMML is poor, with a median overall survival of 2–3 years.

- Approximately 15–30% of CMML cases have increased risk of transformation to acute myelogenous leukemia (AML) associated with high white blood cell count, thrombocytopenia, ASXL1 (frameshift or nonsense mutations) and certain molecular genetic alterations including trisomy 8, alterations of chromosome 7 and a complex karyotype.
- Allogeneic hematopoietic stem cell transplantation remains the only curative treatment option to treat CMML.

Laboratory Diagnosis

The various tests are performed to diagnose or rule out myelodysplastic/myeloproliferative neoplasms, which include: (a) complete blood count to assess the number and quality of white blood cells (leukocytosis or leukopenia with relative monocytosis and neutrophilia or neutropenia), red blood cells (anemia) and platelets (thrombocytopenia or rarely thrombocytosis), (b) blood biochemistry tests to know how well certain organs are functioning (increased serum lactate dehydrogenase), (c) bone marrow aspiration, and bone marrow trephine biopsy to confirm whether or not person has myelodysplastic/myeloproliferative neoplasm, (d) molecular genetic tests on a sample of blood or bone marrow cells to look for certain changes to chromosomes, and (e) fluorescence *in situ* hybridization (FISH) to analyze specific alterations in chromosomes and some abnormalities that are too small for standard molecular cytogenetic testing to find chromosomal alterations.

Laboratory Diagnosis of Chronic Myelomonocytic Leukemia

Peripheral Blood Smear Examination

- Giemsa-stained peripheral blood smear examination shows relative and absolute monocytosis, variable increased immature monocytes (promonocytes and monoblasts), which may show neutrophilia or neutropenia with variable dysplastic neutrophils (hypogranular, hyperlobated).
- There may be left shift of granulocytes accounting for <10% of leukocytes and blasts are <20% by definition. There may be presence of variable anemia and thrombocytopenia.
- Chronic myelomonocytic leukemia (CMML) in Giemsa-stained peripheral blood smear is shown in Fig. 9.65.

Cytochemistry

Myeloperoxidase (MPO) stain positivity in chronic myelomonocytic leukemia (CMML) in Giemsa-stained peripheral blood smear is shown in Fig. 9.66.

Bone Marrow Smear Examination

- Giemsa-stained bone marrow examination shows hypercellular marrow and myelomonocytic proliferation resulting in increased myeloid:erythroid ratio but an increased erythroid precursor cells in some cases.

- Monocytic proliferation and dysgranulopoiesis are always present. Dysmegakaryopoiesis is observed in ≤80% of cases. Bone marrow shows mild to moderate reticulin fibrosis.

Histopathologic Examination

Splenic red pulp is usually infiltrated by leukemic cells in cases of chronic myelomonocytic leukemia.

JUVENILE MYELOMONOCYTIC LEUKEMIA

Juvenile myelomonocytic leukemia (JMML) is an aggressive childhood myeloproliferative hematopoietic stem cell disorder affecting younger children of >4 years of age.

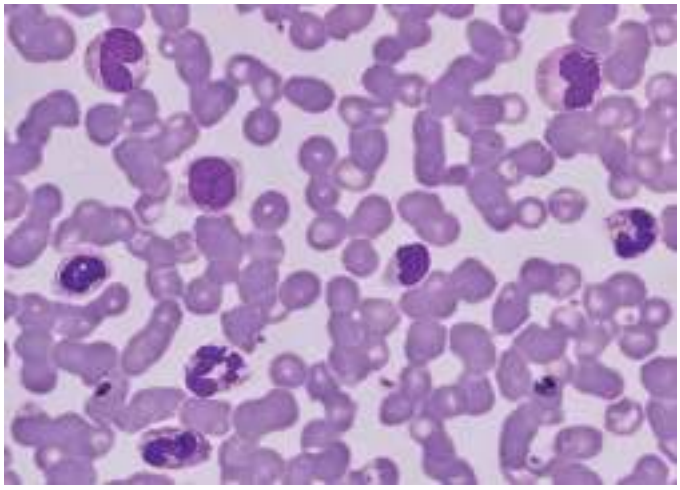


Fig. 9.65: Chronic myelomonocytic leukemia (CMML) in Giemsa-stained peripheral blood smear. It shows persistent peripheral monocytosis >1 × 10⁹/L for at least 3 months, monocyte count ≥10% of the leukocytes; and exclusion of all other reactive causes of monocytosis. Although the peripheral blood finding of absolute monocytosis is essential for diagnosis, yet other hematologic findings are remarkably variable. Dyshematopoiesis can range from minimal expression in a single lineage to marked dysplasia in all the lineages (1000X).

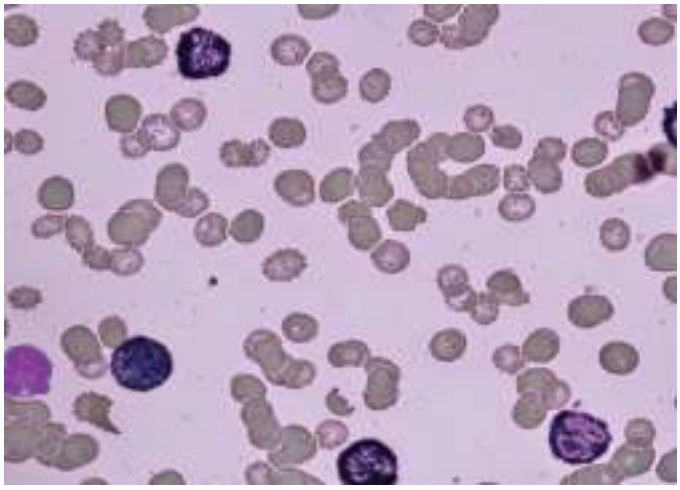


Fig. 9.66: Myeloperoxidase (MPO) stain positivity in chronic myelomonocytic leukemia in Giemsa-stained peripheral blood smear (1000X).

- JMML is characterized by proliferation and immaturity of the granulocytic and monocytic lineages in the peripheral blood and bone marrow.
- Bone marrow examination demonstrates dysmyelopoiesis and dyserythropoiesis; and rarely dysmegakaryopoiesis. JMML patient presents with anemia and thrombocytopenia.

Molecular Genetic Alterations

The cell of origin of juvenile myelomonocytic leukemia (JMML) is believed to be the hematopoietic stem cell (HSC). The gain-of-function mutations in N-RAS and K-RAS genes and disruption of tumor suppressor genes like NF1 are essential in the pathogenesis of JMML.

- Moreover, somatic **PTPN11 gene mutation** is demonstrated in 30–40% of JMMC. PTPN11 proto-oncogene encodes Src homology tyrosine phosphatase with a role in RAS/MAPK signal transduction and hematopoiesis. These molecular changes have been included in the diagnostic criteria of JMML.
- A significant association with neurofibromatosis type 1 (NF1) occurs in JMML. Overall prognosis of JMML is poor due to leukemic infiltration in organs.

Diagnostic Criteria

Salient features for diagnosis of juvenile myelomonocytic leukemia include: (a) peripheral blood smear shows monocytosis (>1 × 10⁹/L), <20% blasts (monoblasts and promonocyte) in peripheral blood and bone marrow; (b) absence of Philadelphia chromosome or BCR-ABL1 fusion gene, (c) increase in fetal hemoglobin for age, (d) immature granulocytes, leukocyte count >10 × 10⁹/L, (e) clonal chromosomal abnormality, and (f) GM-CSF hypersensitivity of myeloid progenitors *in vitro*. Recent WHO diagnostic criteria for juvenile myelomonocytic leukemia are given in Table 9.70.

Clinical Features

Patient presents with hemorrhagic tendencies, lymphadenopathy and skin rashes. Marked splenomegaly is less frequent finding. Patient has a poor response to therapy with fatal outcome.

Laboratory Diagnosis of Juvenile Myelomonocytic Leukemia (JMML)

Peripheral Blood Smear Examination

- Peripheral blood smear examination shows leukocytosis (>10 × 10⁹/L), monocytosis, absolute monocyte count (>1 × 10⁹/L) and thrombocytopenia unlike adult chronic myelogenous leukemia.

- Monoblasts and promonocytes constitute <20% of white blood cells.
- Neutrophil alkaline phosphatase score is low. Juvenile myelomonocytic leukemia (JMML) in a 10-month-old boy in Giemsa-stained peripheral blood smear is shown in Fig. 9.67.

Bone Marrow Smear Examination

Bone marrow is hypercellular. Monoblasts and promonocytes constitute <20% of cells. Megakaryocytes are decreased.

Prognostic Factors

Certain factors affect prognosis and treatment options of juvenile myelomonocytic leukemia. Prognosis depends on the age of the child at diagnosis, number of platelets in the blood and the amount of certain type of hemoglobin in the red blood cells. Poor prognostic factors for juvenile myelomonocytic leukemia include thrombocytopenia and increased fetal hemoglobin.

ATYPICAL CHRONIC MYELOGENOUS LEUKEMIA (aCML), BCR-ABL1 NEGATIVE

Atypical chronic myelogenous leukemia (aCML), BCR-ABL1 negative is a rare myelodysplastic syndrome/myeloproliferative neoplasm for which no current standard of management exists. Peripheral blood smear examination shows prominent immature granulocytosis and granulocytic dysplasia.

Table 9.70 Recent WHO diagnostic criteria for juvenile myelomonocytic leukemia with absence of BCR-ABL-1

Clinical and Hematologic Features (All Mandatory)
Peripheral monocytosis ($\geq 1 \times 10^9$/L)
Peripheral blood and bone marrow demonstrate <20% blasts (monoblasts and promonocyte)
Splenomegaly
Absence of Philadelphia chromosome (BCR-ABL1 rearrangement)
Genetic Analysis (One Finding Sufficient)
Somatic mutation in PTPN11 (35%) or K-RAS or N-RAS
Clinical diagnosis of neurofibromatosis type 1 due to NF1 gene mutation
Germline CBL gene mutation and loss of heterozygosity of CBL.
For Patients without Genetic Features, besides Clinical and Hematologic Features (Criteria must be fulfilled)
Monosomy 7 or any other chromosomal abnormality or at least two of the following mentioned below:
Hemoglobin F increased for age
GM-CSF hypersensitivity of myeloid progenitors *in vitro*
Hyperphosphorylation of STAT5
Presence of myeloid or erythroid precursors in the peripheral blood

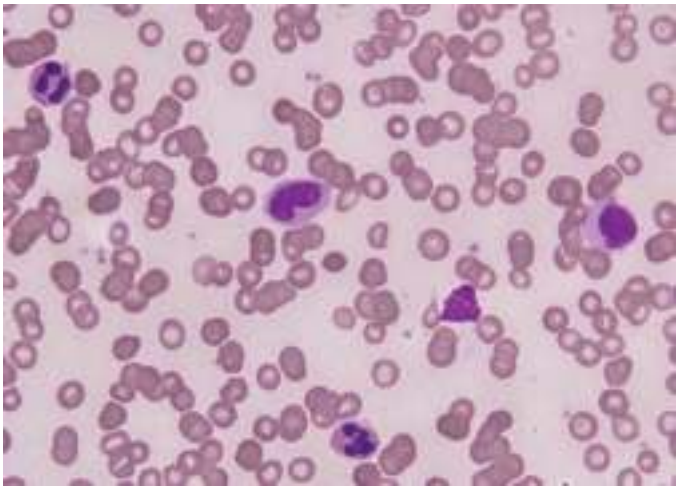


Fig. 9.67: Juvenile myelomonocytic leukemia (JMML) in a 10-month-old boy in Giemsa-stained peripheral blood smear. Complete blood count showed WBC of 10×10^9/L with 28% monocytes and significant myeloid left-shift. Molecular analysis showed evidence of a somatic PTPN11, N-RAS or K-RAS or NF1 gene mutations, all of which encode components of RAS dependent pathways in 30–40% of juvenile myelomonocytic leukemia and atypical chronic leukemia (aCML)(1000X).

- The cell of origin of atypical chronic myelogenous leukemia (aCML), BCR-ABL1 negative is believed to be the common myelogenous progenitor (CMP) cell. Molecular genetic alterations in aCML are similar as demonstrated in chronic myelomonocytic leukemia.
- Atypical chronic myelogenous leukemia shares clinical and laboratory features of chronic myelogenous leukemia but lacks the BCR-ABL1 fusion.
- Molecular genetic analysis reveals mutation of SETBP1 gene encoding Gly870Ser. Patient with SETBP1 gene mutation has higher total leukocyte count associated with poor prognosis.
- Atypical chronic myelogenous leukemia affects elderly persons with male predominance. Majority of patients die due to bone marrow failure.

Molecular Genetic Alterations

Molecular genetic alterations and molecular genetic studies are essential in the diagnosis of atypical myelogenous leukemia, to exclude t(9;22) or BCR-ABL1 positive chronic myelogenous leukemia. No definite molecular genetic alteration is known in atypical chronic myelogenous leukemia, but del(20p) (q11), del(12p), del(12p), trisomy 8 and 13, and i(17q); and complex karyotype have been reported. Mutations of the N-RAS/K-RAS, RUNX1, TET2, CBL, or ASX1 proto-oncogenes are demonstrated in about 30% of patients.

Revised 2024 WHO Diagnostic Criteria for Atypical CML (BCR-ABL1 Negative)

Recent 2024 WHO diagnostic criteria for atypical chronic myelogenous leukemia, BCR-ABL1 negative include the peripheral white blood cells (WBCs) count must be $>13 \times 10^9/L$ with increased dysplastic neutrophils and their precursors. Blasts constitute <20%; and promyelocytes, myelocytes and metamyelocytes together constitute >10% in the peripheral blood.

- Absolute monocytosis and basophilia are absent. Dysplasia and ineffective hematopoiesis in other cell lines frequently result in anemia and thrombocytopenia. Prognosis of aCML is very poor ranging between 11 and 25 months.
- Revised 2024 WHO diagnostic criteria for atypical chronic myelogenous leukemia, BCR-ABL1 negative are given in Table 9.71.

Clinical Features

Patients with atypical chronic myelogenous leukemia have some features of usual chronic myelogenous leukemia, BCR-ABL1 positive with splenomegaly, an elevated white blood cells count of predominantly granulocytic cells, anemia and normal or decreased platelet count.

- Atypical chronic myelogenous leukemia, BCR-ABL1 negative appears to be more aggressive disease than usual chronic myelogenous leukemia with progression occurring within two years.
- Patients may develop acute myelogenous leukemia or bone marrow failure secondary to marked bone marrow fibrosis. Majority of patients die due to bone marrow failure.

Table 9.71 Recent 2024 WHO diagnostic criteria for atypical chronic myelogenous leukemia, BCR-ABL1 negative

Peripheral Blood Leukocytosis
Peripheral blood leukocytosis occurs due to increased neutrophils number and their precursors (i.e. promyelocytes, myelocytes, metamyelocytes) constituting >10% of leukocytes
Number of blasts <20%
Dysgranulopoiesis, which may include abnormal chromatin clumping
No or minimal absolute basophils; basophils constitute usually <2% of leukocytes
No or minimal absolute monocytosis; monocytes constitute <10% of leukocytes
Hypercellular Bone Marrow Associated with Findings
▪ Myeloid to erythroid ratio is about 10:1
▪ Granulocytic proliferation and granulocytic dysplasia, with or without dysplasia in the erythroid and megakaryocytic lineages
▪ Blasts <20%
Absence of the Following Molecular Genetic Alterations
▪ PDGFRA
▪ PDGFRB
▪ FGFR1 rearrangement
▪ PCM1-JAK2
Not Meeting WHO Criteria for Following
▪ Chronic myelogenous leukemia, BCR-ABL1 positive
▪ Polycythemia vera (PV)
▪ Essential thrombocythemia (ET)
▪ Primary myelofibrosis (PMF)

CHRONIC MYELOPROLIFERATIVE NEOPLASMS

Chronic myeloproliferative neoplasms are heterogenous group of disorders, which include polycythemia vera (PV), chronic myelogenous leukemia, BCR-ABL1 positive, chronic myelomonocytic leukemia (CMML), chronic neutrophilic leukemia, chronic eosinophilic leukemia (CEL), not otherwise specified, chronic basophilic leukemia (CBL), primary myelofibrosis (PMF), essential thrombocytosis, mast cell disease (mastocytosis), and chronic myeloproliferative neoplasm (MPN) unclassifiable. Classification of chronic myeloproliferative neoplasms by prominent cell type is given in Table 9.72. The revised 2024 WHO classification of myeloproliferative neoplasms is given in Table 9.73. Chronic myeloproliferative neoplasms associated with gene mutation/rearrangement of receptor tyrosine kinase genes are given in Table 9.74. Genetic assessment of chronic myeloproliferative neoplasms is given in Table 9.75.

CHRONIC MYELOGENOUS LEUKEMIA, BCR-ABL1 POSITIVE

Chronic myelogenous leukemia (CML), BCR-ABL1 positive is myeloproliferative neoplasm characterized by clonal proliferation of granulocytes. CML arises from pluripotent hematopoietic stem cell in the bone marrow with 9(9;22) (q34.1;q11.2) chromosomal translocation and formation of the Philadelphia chromosome, containing the BCR-ABL1 fusion gene. Myeloid lineage is most often affected with elevation of granulocytes in the peripheral blood. Erythroid and megakaryocytic cell lineages as well as B cells can also expand, without T cells.

Table 9.72 Classification of chronic myeloproliferative neoplasms by prominent cell type

Involved Cell Line	Chronic Myeloproliferative Neoplasms
Erythroid cell lineage	Polycythemia vera (PV)
Myeloid cell lineage	▪ Chronic myelogenous leukemia (CML), BCR-ABL1 positive ▪ Chronic myelomonocytic leukemia (CMML) ▪ Chronic neutrophilic leukemia (CNL) ▪ Primary myelofibrosis (sometimes)
Megakaryocyte cell lineage	Essential thrombocythemia (ET)
Eosinophil cell lineage	Chronic eosinophilic leukemia (CEL)
Basophil cell lineage	Chronic basophilic leukemia (CBL)
Mast cell lineage	Mast cell disease (mastocytosis)
Variable cell lineage	Myeloproliferative disease, unclassified (MPD-U)
Fibroblasts*	Chronic idiopathic myelofibrosis (CIMF) with extramedullary hematopoiesis

**The fibroblast in chronic idiopathic myelofibrosis is not a part of neoplastic process but is increased due to reactive process.*

- Natural clinical course of CML disease occurs in three different phases: chronic phase (CP), accelerated phase (AP) and blast phase (BP). In chronic phase involves blood, bone marrow, spleen and liver. Chronic phase of CML progresses to accelerated phase. In blast phase, spleen, liver, lymph nodes, skin and soft tissues are the most common extramedullary sites of involvement.

Table 9.73 The revised 2024 World Health Organization classification of chronic myeloproliferative neoplasms

Chronic myelogenous leukemia (CML), BCR-ABL1 positive
Chronic neutrophilic leukemia (CNL)
Polycythemia vera (PV)
Primary myelofibrosis (PMF) ▪ Primary myelofibrosis prefibrotic/early stage ▪ Primary myelofibrosis overt fibrotic stage
Essential thrombocythemia (ET)
Chronic eosinophilic leukemia (CEL), NOS
Chronic myeloproliferative neoplasm (MPN) unclassifiable

Adapted from revised 2024 World Health Organization (WHO) classification of hematopoietic neoplasms.

- Chronic phase of CML responds well to therapy, which maintains normal health for many months and years. Majority of cases progress from chronic phase to accelerated phase then to blast phase (acute myelogenous leukemia or acute lymphoblastic leukemia) in 3–5 years.
- CML accounts for 15–20% of all leukemias, which can affect any age group with male predominance. Peak incidence is observed between 35 and 90 years of age. Incidence of CML increases with median age of diagnosis at 65 years. Disease is relatively rare in pediatric age group. Disease has an insidious onset. Ionizing radiation is the only known etiological factor. CML usually occurs within 6–8 years of radiation exposure.

Table 9.74 Chronic myeloproliferative neoplasms associated with gene mutation/rearrangement of receptor tyrosine kinase genes

Disorder	Gene Mutation	
Chronic myelogenous leukemia	BCR-ABL1	
Polycythemia vera (PV)	▪ JAK2 V617 (97%) ▪ JAK exon 12 ▪ CALR (0%)	▪ MPL (<1%) ▪ Triple negative: JAK2 V617, CALR, MPL
Chronic neutrophilic leukemia (CNL)	CSF3R T6181	
Primary myelofibrosis (PMF)	▪ JAK2 V617 (55–65%) ▪ CALR (20–25%) ▪ MPL (7%)	▪ Triple negative: JAK2 (V617F), CALR, MPL in 5–10%
Essential thrombocythemia (ET)	▪ JAK2 V617 (50–60%) ▪ CALR (20–25%) ▪ MPL (3–5%)	▪ Triple negative: JAK2 (V617F), CALR, MPL in 10–15%
Mastocytosis	KIT D816V	
Myeloid neoplasms with eosinophilia	▪ PDGFRA ▪ PDGFRB	▪ FGFR1 ▪ PCM1-KAK2

1. JAK2 V617 gene mutation is demonstrated in polycythemia vera, primary myelofibrosis, essential thrombocythemia, acute myelogenous leukemia, myelodysplastic syndrome, chronic myelomonocytic leukemia and myelodysplastic syndrome/myeloproliferative neoplasms.
2. JAK exon 12 mutation is demonstrated only in polycythemia vera.

Table 9.75 Genetic assessment of chronic myeloproliferative neoplasms

Unique 'Driver Molecular Genetic Alterations*	Comments	Most Frequent Molecular Genetic Alterations	Cytogenetic Alterations
Chronic myelogenous leukemia (CML) BCR-ABL1 positive			
BCR-ABL1	BCR-ABL1 fusion product demonstrated in CML	▪ TP53 ▪ RB1 ▪ RAS ▪ Myc ▪ AML1 ▪ EVI1 ▪ CDKN2A	▪ +8 ▪ +9 ▪ Philadelphia duplication ▪ Iso(17q) +
Chronic neutrophilic leukemia (CNL)			
CSF3R T6181 or CSF3R activating mutations positive	▪ Activating mutation in gene encoding the receptor for the colony stimulating factor (CSF3R) ▪ Patient carrying JAK activating CSF3R has marked improvement after administration of the JAK1/JAK2 inhibitor ruxolitinib	▪ Chromatin modifiers (ASXL1, EZH2) ▪ Spliceosome (SRSF2, SF3B1)	▪ +8 ▪ +9 ▪ del(20q) ▪ del(13q) ▪ del(5q)
Polycythemia vera (PV)			
▪ JAK2 V617F positive (>97%) ▪ JAK2 exon 12 (3%) ▪ MPL (1%) ▪ Triple negative (0–1%)	JAK2 allele burden is linked to transformation to acute leukemia (100% cases)	DNA methylation (TET2, DNMT3A, IDH1, IDH2, TP53)	▪ 7/del(7q) ▪ Complex karyotype ▪ del(17p)
Primary myelofibrosis (PMF)			
JAK2 (V617F) positive (60–65%)	JAK2 positivity has relatively low survival rate in comparison to CALR positivity	Activating signaling pathways (RAS, CBL)	No cytogenic aberration
CALR exon 9 positive (20–25%)	CALR positivity occurs in younger age with indolent clinical course and better survival than JAK2 positive	Activating signaling pathways (RAS, CBL)	No cytogenic aberration
MPL exon 10 positive (5–8%)	MPL exon 10 positivity has greater risk of thrombosis in JAK2 positive cases of primary myelofibrosis	Activating signaling pathways (RAS, CBL)	No cytogenic aberration
Triple negative: JAK2 (V617F), CALR, MPL (5–10%)	Triple negative status linked to inferior outcome and increase risk for transformation to acute leukemia	Activating signaling pathways (RAS, CBL)	No cytogenic aberration
Essential thrombocythemia (ET)			
JAK2 (V617F) positive (50–60%)	Higher rate of thrombosis in JAK2 positive patients	No molecular mutations	No cytogenic aberration
CALR exon 9 positive (20–25%)	Higher platelet count and high rate of fibrotic transformation in CALR positive essential thrombocytosis patients	No molecular mutations	No cytogenic aberration
MPL exon 10 positive (3–5%)	Survival rate is short in MPL negative mutated patients	No molecular mutations	No cytogenic aberration
Triple negative: JAK2 (V617F), CALR, MPL (10–15%)	Survival rate is longest for triple negative	No molecular mutations	No cytogenic aberration

Contd...

Table 9.75 Genetic assessment of chronic myeloproliferative neoplasms (*Contd...*)

Unique 'Driver Molecular Genetic Alterations*	Comments	Most Frequent Molecular Genetic Alterations	Cytogenetic Alterations
Mastocytosis			
c-KIT positive (frequent)	Abnormal growth and accumulation of atypical mast cells in the bone marrow and viscera	No molecular mutations	No cytogenic aberration
Chronic eosinophilic leukemia (CEL)			
No distinctive genotype	Persistent increase in eosinophils in peripheral blood, bone marrow and tissues	No molecular mutations	No cytogenic aberration
Myeloproliferative neoplasia unclassifiable			
No distinctive genotype	MPN unclassifiable with ring sideroblasts	No molecular mutations	No cytogenic aberration

**Some molecular genetic alterations can precede the 'driver' mutation or acquired during disease progression. Some cytogenetic abnormalities are associated with accelerated phase and/or leukemic transformation.*

- Children and young adults tend to have a more aggressive clinical presentation than older adults, more frequent in accelerated or blast phase. Prognosis is very poor in transformation of accelerated phase to blast phase and associated with survival of <6 months by using traditional chemotherapy.
- Three different phases, approximate length of phase and treatment status of chronic myelogenous leukemia (CML) BCR-ABL1 are given in Table 9.76.

PATHOPHYSIOLOGY

Molecular genetic analysis has increased our understanding the role of Philadelphia chromosome in the pathogenesis of chronic myelogenous leukemia (CML) BCR-ABL1 positive.

- Philadelphia chromosome (Ph) is an acquired chromosomal translocation that results in fusion gene called BCR-ABL1. Reciprocal chromosomal translocation t(9;22)(q34;q11) leads to the formation of the BCR-ABL1 chimeric fusion gene, which is

Table 9.76 Three different phases, approximate length of phase and treatment status of chronic myelogenous leukemia (CML), BCR-ABL1 positive

CML Phases and Features	Approximate Length of Phase	Treatment Status with Chemotherapy
CML chronic phase		
▪ Presence of blasts <10% in bone marrow and peripheral blood ▪ Massive splenomegaly (>90% of cases)	2–5 years	Highly treatable phase
CML accelerated phase		
▪ Presence of blasts 10–19% in bone marrow and peripheral blood ▪ Progressive splenomegaly ▪ Associated with additional chromosomal and molecular abnormalities	6–18 months	Resistance to chemotherapy develops
CML blast phase		
▪ Presence of blasts ≥20% in the bone marrow and peripheral blood ▪ Proliferation of blasts in extramedullary sites such as lymph nodes, skin, nervous system and other sites ▪ Blasts are present in clusters in bone marrow trephine biopsy ▪ Associated with additional chromosomal and molecular abnormalities	3–4 months	Generally unresponsive to chemotherapy

responsible for production of the oncoprotein BCR-ABL1. BCR-ABL1 oncoprotein has unrestricted tyrosine kinase activity, making abnormal pluripotent hematopoietic stem cell initiate excessive production of all myeloid cell lineages.

- Once the Philadelphia chromosome is detected, its rise and fall reflects the tumor burden; and hence can be used as measure of the disease progression, remission and relapse.
- All the three cell lineages of the hematopoietic stem cells are involved in CML, the original neoplastic cell is pluripotent hematopoietic stem cell (HSC). Pathogenesis of chronic myelogenous leukemia (BCR-ABL1 positive) is shown in Fig. 9.68.

Pathology Pearls: CML, BCR-ABL1 Positive Pathogenesis

First Hypothesis

According to first hypothesis, the acquisition of BCR-ABL1 may occur in a pluripotent hematopoietic stem cell with little or no T cells differentiation capacity in chronic myelogenous leukemia.

Second Hypothesis

- According to second hypothesis, T cells bearing BCR-ABL1 may be systematically eliminated.
- Unregulated proliferation of BCR-ABL1 positive pluripotent hematopoietic stem cell is responsible for massive expansion, primarily in granulocyte production, which leads to leukocytosis.
- Extramedullary granulocytic proliferation in the spleen and liver induces splenomegaly and hepatomegaly respectively, that reflects progression of disease.

Philadelphia Chromosome

Philadelphia chromosome is formed by balanced reciprocal translocation between long arms of chromosomes 9 and 22, t(9;22) (q34;q11.2) in CML,BCR-ABL1 positive. Lengthened Philadelphia chromosome is chromosome 22. Chronic myelogenous leukemia exhibiting balanced reciprocal translocation 9/22 known as Philadelphia chromosome is shown in Fig. 9.69. Exon maps of the normal BCR-ABL1 genes, formed by the Philadelphia chromosome translocation in CML and *de novo* acute leukemias are shown in Fig. 9.70.

Pathology Pearls: BCR-ABL1 Fusion Gene in Chronic Myelogenous Leukemia (CML)

- Exon maps of the normal BCR-ABL1 genes, formed by the Philadelphia chromosome translocation in CML and *de novo* acute leukemias.
- Philadelphia chromosome is formed by balanced reciprocal translocation between long arms of chromosomes 9 and 22, t(9;22) (q34;q11.2) in CML.
- Chromosome 22 is known as Philadelphia chromosome. Normally, ABL1 gene is located on chromosome 9 and BCR gene is located on chromosome 22. Due to reciprocal chromosomal translocation, BCR-ABL1 fusion gene is formed in CML and *de novo* acute leukemias.
- Breakpoints in the ABL1 gene occur upstream of exon 2 (e2 downstream arrow). Breakpoints in major breakpoint cluster region (**M-BCR**) of the BCR gene occur in exon 13 (e13) and exon 14 (e14).
- Breakpoints can also take place in the minor breakpoint cluster region (M-BCR, 3' of exon 1) in acute lymphoblastic leukemia. Following BCR-ABL1 fusion gene, the resulting protein of 210 (p210; e13a2 or e14a2) or 190 (p190; e1a190; e1a2) kilodalton is formed.

- **Normal ABL1 and BCR gene:** Normally, **ABL1 gene** is located on chromosome 9 and **BCR gene** is located on chromosome 22. Breakpoints in major breakpoint cluster region (M-BCR) of the BCR gene occur in exon 13 (e13) and exon 14 (e14), in which receptor tyrosine kinase domains reside. BCR-ABL1 fusion gene is formed by Philadelphia chromosome contain BCR-ABL1 fusion gene due to reciprocal chrosomal translocation found in CML and *de novo* acute leukemias.
- **Breakpoints in the ABL1 gene and BCR gene:** Breakpoints in the ABL1 gene occur upstream of exon 2 (a2 downstream arrow) in major breakpoint cluster region (M-BCR) of the BCR gene in exon 13 (e13) and exon 14 (e14). Breakpoints can also take place in the minor breakpoint cluster region (M-BCR, 3' of exon 1) in acute lymphoblastic leukemia. Following BCR-ABL1 fusion gene, the resulting protein of 210 (p210; e13a2 or e14a2) or 190 (p190; e1a190; e1a2) kilodalton is formed.
- **Formation of new BCR-ABL1 fusion gene:** Formation of new BCR-ABL fusion gene coding for the BCR-ABL fusion putative oncoprotein has increased receptor tyrosine protein kinase activity, which is presumably involved in the pathogenesis of CML.
  - BCR-ABL fusion putative oncoprotein binds ATP then transfers phosphate groups to receptor tyrosine kinase residues on various substrate proteins forming phosphorylated substrate within cells that activates intracellular signal transduction pathways involved in unrestricted cell proliferation, maturation, and resistance to apoptosis, ultimately resulting in the leukemic phenotype.
  - Administration of receptor tyrosine kinase inhibitor blocks ATP binding to BCR-ABL, so the substrate cannot be phosphorylated, hence proliferation of the leukemic cells cannot occur.
  - Levels of detection of disease include analysis of morphologic features, cytogenetic alterations and

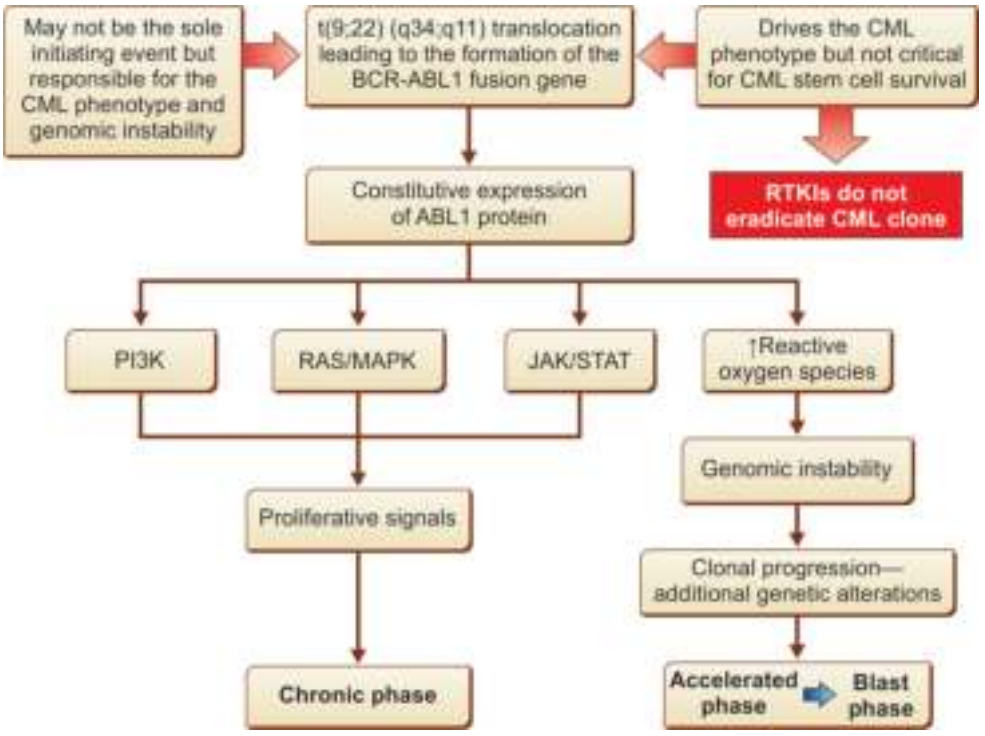


Fig. 9.68: Schematic representation of pathogenesis in chronic myelogenous leukemia (CML), BCR-ABL1 positive.

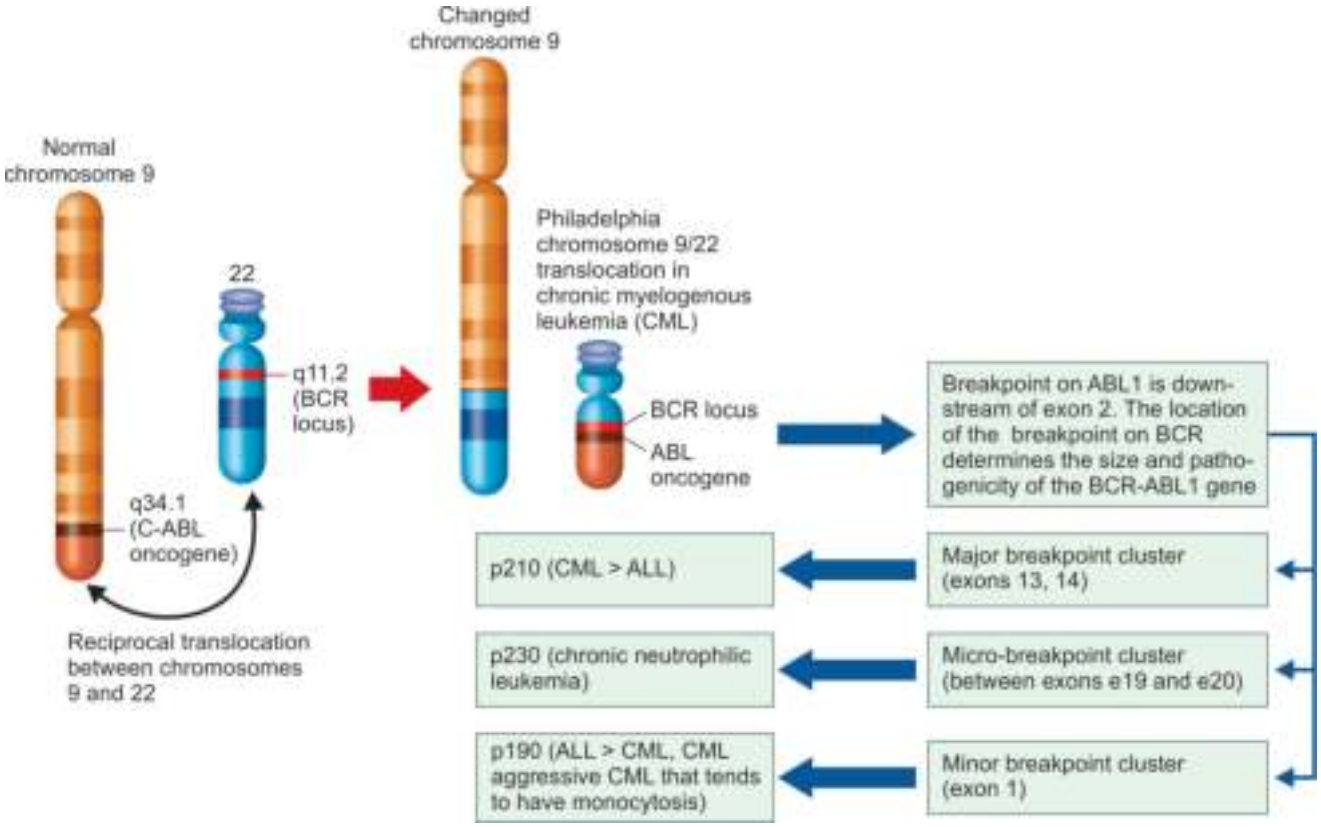


Fig. 9.69: Schematic representation of chronic myelogenous leukemia (CML), BCR-ABL1 positive. It shows balanced reciprocal translocation 9/22 known as Philadelphia chromosome. A reciprocal translocation involves two nonhomologous chromosomes, with exchange of the acentric segment. There is exchange of a segment between two nonhomologous chromosomes. Balanced reciprocal translocation 9/22 known as Philadelphia chromosome is seen in chronic myelogenous leukemia. Positivity of this Philadelphia chromosome indicates good prognosis. In interstitial translocation, a segment of a chromosome is lost and reattaches to nonhomologous chromosome.

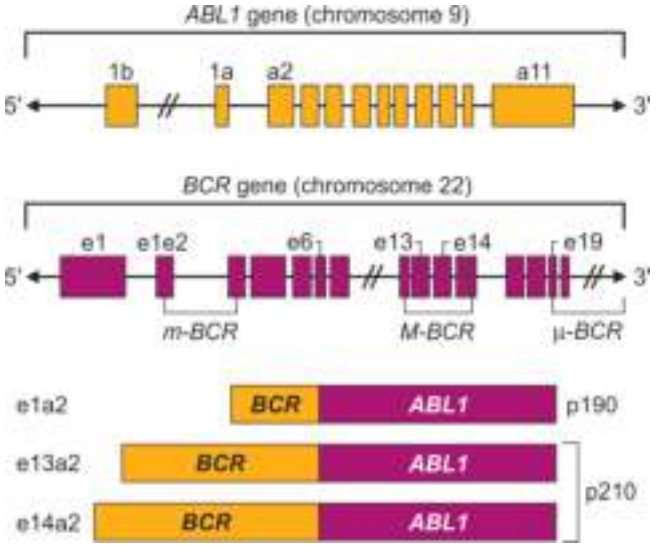


Fig. 9.70: Schematic representation of CML, BCR-ABL1 fusion gene in CML, BCR-ABL1 positive. It depicts exon maps of the normal BCR gene on chromosome 22 and ABL1 on chromosome 9 gene, and the BCR-ABL1 fusion gene formed by the Philadelphia chromosome translocation found in CML and *de novo* acute leukemias. Breakpoints in the ABL1 gene occur upstream of exon 2. Breakpoints in the major breakpoint cluster region (M-BCR) of the BCR gene occur in exon 13 and exon 14 in CML; breakpoints in the minor breakpoint cluster region (m-BCR,3' of exon 1) in acute leukemias. Following BCR-ABL1 fusion gene, the resulting protein of 210 or 190 is formed.

reverse transcriptase (RQ)-PCR following receptor tyrosine kinase inhibitor (RTKI) therapy. Detection of the BCR gene rearrangement has diagnostic and prognostic significance in CML cases.

Additional Chromosomal/Molecular Genetic Alterations in Progression of CML

Untreated cases of chronic myelogenous leukemia (CML) progressing from chronic phase to accelerated phase and blast phase, are accompanied by development of additional chromosomal abnormalities in 80% of patients.

- Frequent additional chromosomal/molecular genetic alterations in blast phase of CML include mutations in RUNX1, ASXL1, IKZF1, TP53, RB1, RAS, Myc, AML-1, EVI-1, CDKN2A, frequent cytogenetic aberrations (Philadelphia chromosome duplication, trisomy 8, isochromosome 17q, loss of Y chromosome), rare cytogenetic alterations (translocation (15;17), translocation (3;21) (p26; q22) and translocation (3;3), inv(3). Thus, repeated chromosome analysis can be useful in predicting progression of CML disease.
- Mutation of TP53 gene is rare in CML chronic phase. Additional molecular genetic alterations in the progression of chronic myelogenous leukemia (CML), BCR-ABL1 positive is given in Table 9.77.

Table 9.77 Additional molecular genetic alteration in the progression of CML, BCR-ABL1 positive

Molecular Genetic Alterations	Examples
Molecular most frequent mutations	TP53, RB1, RAS, Myc, AML-1, EVI-1, CDKN2A
Cytogenetic frequent aberrations	Philadelphia chromosome duplication, trisomy 8, trisomy 19, isochromosome 17q, loss of Y chromosome
Cytogenetic rare aberrations	Translocation (15;17), translocation (3;21) (p26;q22), translocation (3;3)/inv (3)

MOLECULAR GENETIC ANALYSIS

Molecular genetic analysis is the gold standard diagnostic test for CML, BCR-ABL1 positive. Diagnostic and prognostic significance of molecular genetic analysis of BCR/ABL1 gene rearrangement in CML is given in Table 9.78.

- Diagnostic tests for CML include genomic polymerase chain reaction (PCR) and Northern blot analysis that can determine the exact breakpoints of DNA fusion products. Reverse transcriptase PCR (RT-PCR) and Northern blot analysis demonstrate BCR-ABL1 transcription at the RNA level.
- Immunoprecipitation and Western blot analysis demonstrate BCR-ABL1 protein by using antibodies against N-terminal region of BCR and the C-terminal region of ABL1.
- Molecular genetic analysis of gene arrangements involving the BCR-ABL1 genes is clinically useful for confirmation of Philadelphia positive or negative cases of CML, diagnosis of blast phase, monitoring of patients by using receptor tyrosine kinase inhibitor therapy and after for detection of residual disease,

Table 9.78 Diagnostic and prognostic significance of molecular analysis of BCR-ABL1 gene rearrangement in chronic myelogenous leukemia

Differential diagnosis of CML
Diagnosis of CML with negative Philadelphia chromosome
Differential diagnosis of CML in blast phase from *de novo* acute lymphoblastic leukemia (ALL), when Philadelphia chromosome is positive
Establishment of diagnosis of CML when the patient develops blast phase in the course of disease
Monitoring of CML cases on tyrosine kinase inhibitor (TKI) therapy to determine therapeutic response, resistance to (TKI) therapy or relapse
Detection of minimal residual disease (MRD) in post-bone transplant patients

confirmation of remission and early detection of relapse.

CLINICAL FEATURES

Most chronic myelogenous leukemia patients are diagnosed in the chronic phase of the disease. About 50% of patients are asymptomatic and diagnosed during a routine examination. Disease is insidious onset with leukocytosis, anemia, fatigue, weight loss and night sweats.

- Majority of patients have massive splenomegaly with dragging sensation in the left upper quadrant.
- Rarely, patients present with marked thrombocytosis without leukocytosis, mimicking essential thrombocythemia.
- About 5% of patients present with fever, bone pain and bleeding in the accelerated phase of the blast phase.
- Criteria distinguishing CML, BCR-ABL1 positive at different stages of evolution are given in Table 9.79.

Table 9.79 Criteria distinguishing CML, BCR-ABL1 positive at different stages of evolution

Parameters	CML Chronic Phase	CML Accelerated Phase	CML Blast Phase
Approximate length of CML phases			
Duration of CML phase	2–5 years	6–18 months	3–4 months
Blasts and basophils in the bone marrow and peripheral blood			
Blasts in the peripheral blood	<10%	10–19%	≥20% (patients can develop AML-M1, AML-M2, AML-M4, AML-M6, AML-M7, ALL-L1, ALL-L2)
Blasts in the bone marrow	<10%	10–19%	≥20% (AML-M1, AML-M2, AML-M4, AML-M6, AML-M7, ALL-L1, ALL-L2)
Basophils	<20%	>20%	>20%
CML response to first tyrosine kinase inhibitors (TKIs)			
Therapeutic response to tyrosine kinase inhibitors (TKIs)	High response to chemotherapy	Development of resistance to chemotherapy due to failure of response to tyrosine kinase inhibitors	Failed complete hematologic/molecular remission of CML treated with ≥1 tyrosine kinase inhibitor
Two or more BCR-ABL mutations	Absent	Present	Present
Karyotype at diagnosis			
Chromosomal mutations by cytogenetic study	Philadelphia chromosome	▪ Philadelphia chromosome duplication ▪ Trisomy 8 ▪ Trisomy 19 ▪ Isochromosome 17q	▪ Philadelphia chromosome duplication ▪ Trisomy 8 ▪ Trisomy 19 ▪ Isochromosome 17q ▪ Chromosome Y loss
Molecular mutations			
Gene mutations	Absent	RUNX1 (33%), ASXL1 (21%), IKZF1 (20%), TP53, RB1, RAS, Myc, AML-1, EVI-1, CDKN2A	RUNX1 (33%), ASXL1 (21%), IKZF1 (20%), TP53, RB1, RAS, Myc, AML-1, EVI-1, CDKN2A
Bone marrow megakaryocyte's dysplasia			
Megakaryocyte's dysplasia	No dysplasia in megakaryocyte	Atypical megakaryocytes	Atypical megakaryocytes
Bone marrow fibrosis			
Reticulin/collagen	Absent	Reticulin/collagen deposition present	Reticulin/collagen deposition present
White blood cells escape from control			
White blood cells escape from control	Absent	Persistent increase in white blood cells $>10\times10^9$/L	Persistent increase in white blood cells $>10\times10^9$/L
Platelets			
Thrombocytosis	Absent	$>1{,}000\times10^9$/L	$>1{,}000\times10^9$/L
Thrombocytopenia	Absent	$<100\times10^9$/L	$<100\times10^9$/L

Contd...

Table 9.79 Criteria distinguishing CML, BCR-ABL1 positive at different stages of evolution (*Contd...*)

Parameters	CML Chronic Phase	CML Accelerated Phase	CML Blast Phase
Spleen size			
Splenomegaly	Present	Persistent splenomegaly/unresponsive to therapy	Persistent splenomegaly/unresponsive to therapy
Extramedullary sites involved			
Extramedullary blasts	Absent	Absent	Present
Bone marrow trephine biopsy			
Large foci of blasts in the bone marrow	Absent	Absent	Present

Pathology Pearls: Splenomegaly in Chronic Myelogenous Leukemia, BCR-ABL1 Positive

- The gross appearance of the spleen is solid and deep red, although areas of infarction may appear as light-colored regions.
- The red pulp distribution of the leukemic infiltrate usually compresses and obliterates the white pulp. Leukemic cells are present in the splenic cords and sinuses.
- Leukemic cells are shifted towards immature forms. It is essential to assess the maturity of the leukemic cells in the splenectomy specimens, because splenomegaly particularly in the face of receptor tyrosine kinase inhibitor (RTKI), may be associated with progression of the disease.

CML, BCR-ABL1 Positive in Chronic Phase

In CML, BCR-ABL1 positive in chronic phase, the leukemic cells are minimally invasive and mostly confined to the bone marrow, peripheral blood, spleen and liver.

- Patient presents with weakness, malaise, unexplained fever, night sweats, weight loss, sternal tenderness, dragging sensation in the left upper quadrant of abdomen due to massive splenomegaly. Occasionally, patient may have bleeding from mucocutaneous surface (gastrointestinal tract, skin) and retina.
- Clinical examination reveals pallor, tenderness over the lower sternum and massive splenomegaly, and occasionally hepatomegaly.
- Splenomegaly correlates with magnitude of leukocytosis. The gross appearance of spleen is solid and uniformly deep red, although areas of splenic infarcts may appear as light-colored regions. Leukemic cells infiltrate into the red pulp of spleen. The red pulp distribution of the leukemic infiltrate usually compresses and obliterates the white pulp.
- Hepatomegaly also correlates magnitude of leukocytosis. Leukemic cells can also infiltrate into the hepatic sinusoids and portal areas. Any organ can be infiltrated by leukemic cells. However, extramedullary involvement other than spleen and liver is uncommon in CML chronic phase.
- Sternal tenderness over lower sternum is reliable sign of the disease. Presence of petechiae and ecchymoses reflects quantitative or qualitative platelet abnormalities.
- Demonstration of Philadelphia chromosome or molecular BCR-ABL1 fusion gene analysis can establish the diagnosis of chronic myelogenous leukemia associated with bone marrow fibrosis, which differentiates it from primary myelofibrosis.
- Presence of micromegakaryocytes arranged in large clusters or sheets associated with marked reticulin bone marrow fibrosis demonstrated by bone marrow trephine biopsy may be considered presumptive evidence of CML in accelerated phase. Increasing splenomegaly and patient not responding to receptor tyrosine kinase inhibitor (RTKI) also suggest the progression of CML in chronic phase to accelerated phase and finally to blast phase.

Laboratory Diagnosis of Chronic Myelogenous Leukemia BCR-ABL1 Positive in Chronic Phase

Biochemical Investigations

- Granulocytes synthesize cobalamin and haptocorrin resulting in increase in total serum cobalamin and unsaturated cobalamin binding capacity; and haptocorrin levels.
- Uric acid and lactate dehydrogenase are elevated secondarily to increased cell turnover.
- Serum muramidase is within normal range or slightly increased.

Peripheral Blood Smear Examination

Chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase with basophilia in Giemsa-stained peripheral blood smear is shown in Figs 9.71 and 9.72.

White blood cells

- Extreme leukocytosis and neutrophilic cells at various stages of maturation and basophilia are the most striking abnormality. White blood cell count is usually >100 × 10^9/L with a median of 170 × 10^9/L.
- Peripheral blood smear exhibits a shift to the left with all stages of granulocyte maturation.

- The predominant cells are myelocytes and segmented neutrophils (myelocyte bulge 15–30%). Blasts and promyelocytes usually do not exceed 20% of leukocytes in the peripheral blood. Eosinophils and basophils are most often increased in both relative and absolute terms.
- The hallmark of CML is basophilia with basophil count often exceeding 1×10^9/L.
- Monocytes are moderately increased. Signs of myeloid dysplasia including pseudo-Pelger-Hüet anomaly (hyposegmentation of neutrophil nucleus) and deceased neutrophil alkaline phosphatase (NAP) score are frequent.
- Low or absent NAP is characteristic finding in CML but not specific.
- Monocytosis, myeloid dysplasia and micromegakaryocytes are overlapping features in both chronic myelogenous leukemia (CML) and chronic myelomonocytic leukemia (CMML). Philadelphia chromosome is demonstrated in CML but absent in CMML.
- Differential white blood cell count in CML, BCR-A BL1 positive in chronic phase includes myeloblasts (<10%), promyelocytes (2–8%), myelocytes (15–30%), metamyelocytes (15–25%), band forms (5–15%), neutrophils (40–70%), basophils (2–10%), eosinophils (>4% in few cases) and monocytes (2–10%).
- Differential white blood cell counts in chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase are given in Table 9.80.

Red blood cells

- At the time of diagnosis, peripheral blood smear shows mild to moderate normocytic normochromic picture with hemoglobin concentration in the range of 9–13 g/dl.
- Severity of anemia is proportional to the increase in the leukocytes due to suppression of erythroid cells by proliferating leukemic cells in the bone marrow.
- Nucleated red blood cells may be demonstrated. Reticulocyte count is usually within normal range or slightly increased.

Platelets

- Platelet count can exceed 1000×10^9/L with variation in platelet shape in 50% of cases.
- Platelet function is frequently abnormal.
- Megakaryocyte fragments and micromegakaryocytes can be demonstrated in 25% of cases.

Bone Marrow Smear Examination

Chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase in Giemsa-stained bone marrow aspirate smear is shown in Figs 9.73 and 9.74.

- **Cellularity:** Bone marrow is hypercellular with a striking increase in the myeloid to erythroid ratio (10:1 to 50:1) reflective of the myelopoiesis. The active red bone marrow can extend into the long bones. Cortical thinning and erosion of the trabeculae can be demonstrated.
- **Myeloid lineage:** The hematopoietic cells in bone marrow are primarily immature granulocytes with <20% blasts by revised 2024 WHO classification of hematopoietic and lymphoid neoplasms, an important characteristic that distinguishes CML from all forms of acute leukemias. Auer rods can be demonstrated in the myeloblasts in blast phase, but this is an unusual finding.
- **Erythroid lineage:** Bone marrow shows normoblastic erythropoiesis because the increased tyrosine kinase activity does not directly affect erythropoiesis, however, normoblasts can be decreased.
- **Megakaryocytic lineage:** Bone marrow shows increased micromegakaryocytes smaller than normal megakaryocytes (dwarf megakaryocytes) with frequent immature and atypical forms, hyposegmented nuclei in CML. Sometimes, micromegakaryocytes can be demonstrated in accelerated phase of chronic myelogenous leukemia. On the other hand, macromegakaryocytes are demonstrated in myelodysplastic syndrome (MDS).
- **Gaucher-like cells:** Gaucher-like cells are present in bone marrow due to overload of cerebrosides caused by increased cell turnover, and not due to lack of β-glucocerebrosidase enzyme.
- **Bone marrow fibrosis:** The bone marrow can become fibrotic, late in the clinical course of the disease.

Bone Marrow Trephine Biopsy Examination

Bone marrow trephine biopsy is performed only if the findings in the peripheral blood are atypical or if a cellular bone marrow aspirate cannot be obtained.

Neutrophil Alkaline Phosphatase (NAP) Scoring System

- Neutrophil alkaline phosphatase (NAP) is present in specific granules of neutrophils. NAP scoring system is a useful test to differentiate CML from leukemoid reactions.
- NAP score is low in CML but high in leukemoid reactions. Based on the number of granules and intensity of staining of neutrophils are scored 0–4. At least 100 consecutive neutrophils are counted and the sum of their scores is determined.
- Normal range of neutrophil alkaline phosphatase (NAP) score in adults is 25–100, and 150–300 in children. NAP scoring system is given in Table 9.81. NAP scoring system in various hematological disorders is given in Table 9.82.

Cytogenetic and Molecular Genetic Alterations Analysis

Philadelphia chromosome t(9;22) is demonstrated by cytogenetic analysis in CML patients. BCR-ABL1 is demonstrated by molecular technique (RT-PCR) in CML. Molecular genetic monitoring for patients with chronic myelogenous leukemia, BCR-ABL1 positive in chronic phase are given in Table 9.83.

Immunophenotyping

Immunophenotyping has a limited role in the diagnosis of chronic myelogenous leukemia. It is being used in CML cases with blast phase.

- Determination of the cell lineage of blast transformation is clinically important, because the patients with lymphoblast transformation have a better response to chemotherapy and better survival span than those with blast transformation of other lineages.

- Philadelphia chromosome positive immunophenotyping shows positivity for CD33, cCD11, CD36, CD117, CD15 and anti-myeloperoxidase (anti-MPO).

Markers	Expression
▪ CD33	▪ Positive
▪ cCD11	▪ Positive
▪ CD36	▪ Positive
▪ CD117	▪ Positive
▪ CD15	▪ Positive
▪ Anti-myeloperoxidase (anti-MPO)	▪ Positive

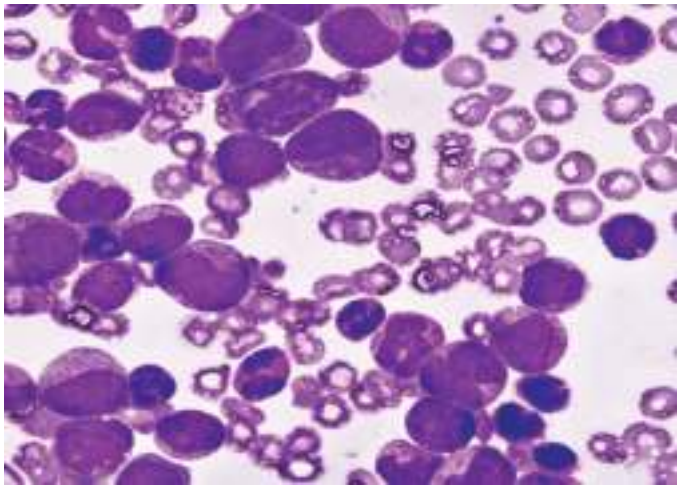


Fig. 9.71: Chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase with basophilia in Giemsa-stained peripheral blood smear. It shows hypercellular bone marrow with a striking increase in the myeloid to erythroid ratio (10:1 to 50:1) reflective of the myelopoiesis. The active red bone marrow can extend into the long bones. Cortical thinning and erosion of the trabeculae can be demonstrated. The hematopoietic bone marrow cells are primarily immature granulocytes with <20% blasts by revised 2024 WHO classification of hematopoietic and lymphoid neoplasms, an important characteristic that distinguishes CML from all forms of acute leukemias. Auer rods can be demonstrated in the myeloblasts in blast phase, but this is an unusual finding (1000X).

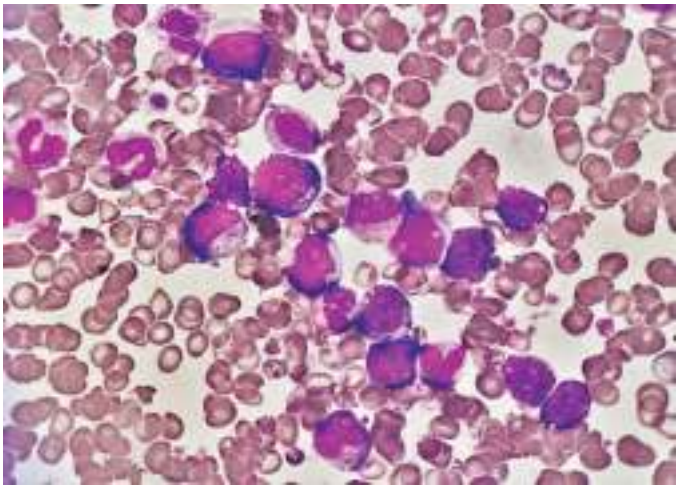


Fig. 9.72: Chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase in Giemsa-stained peripheral blood smear.

Table 9.80 Differential white blood cell counts in chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase

Differential White Blood Cells	Percentage
Myeloblasts	<10%
Promyelocytes	2–8%
Myelocytes	15–30%
Metamyelocytes	15–25%
Band cells (immature form of neutrophils)	5–15%
Neutrophils	40–70%
Basophils	2–10% (often $>1 \times 10^9$/L)
Eosinophils	>4% (few cases)
Monocytes	2–10%

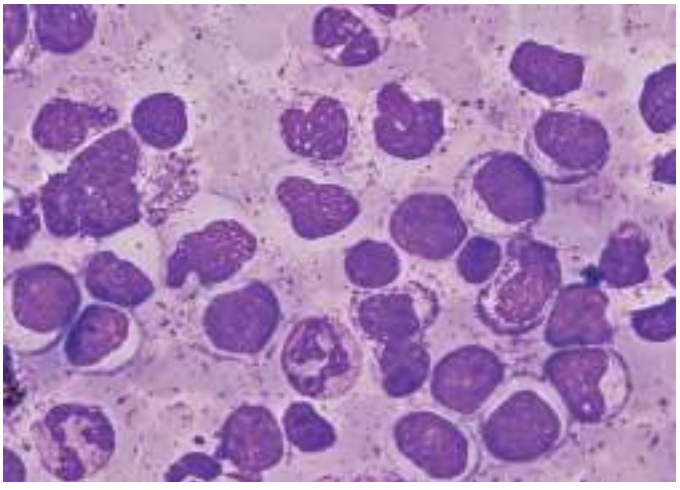


Fig. 9.73: Chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase in Giemsa-stained bone marrow aspirate smear. It shows hypercellular bone marrow with a striking increase in the myeloid to erythroid ratio (10:1 to 50:1) reflective of the myelopoiesis. The active red bone marrow can extend into the long bones. Cortical thinning and erosion of the trabeculae can be demonstrated. The hematopoietic bone marrow cells are primarily immature granulocytes with <20% blasts by revised 2024 WHO classification of hematopoietic and lymphoid neoplasms, an important characteristic that distinguishes CML from all forms of acute leukemias. Auer rods can be demonstrated in the myeloblasts in blast phase, but this is an unusual finding (1000X).

CML, BCR-ABL1 Positive in Accelerated Phase

In untreated patients, CML, BCR-ABL1 positive in chronic phase progresses to the CML accelerated phase in approximately 3–5 years of diagnosis.

- Presence of micromegakaryocytes arranged in large clusters or sheets associated with marked bone marrow fibrosis demonstrated by trephine bone marrow biopsy may be considered presumptive evidence of accelerated phase of CML.

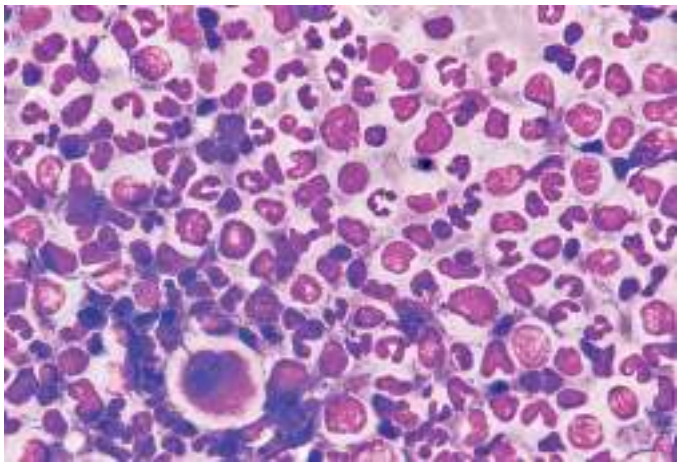


Fig. 9.74: Chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase in Giemsa-stained bone marrow aspirate smear.

Table 9.81 Neutrophil alkaline phosphatase (NAP) scoring system

NAP Score	Individual Neutrophil Cell Staining Scoring
Score 0	Negative staining
Score 1	Diffuse pale cytoplasm with occasional granules
Score 2	Moderate blue granules
Score 3	Cells with uneven distribution of blue granules
Score 4	Cells with uniform blue granules (strong positivity)

Normal range of neutrophil alkaline phosphatase (NAP) score in adults is 25–100, and 150–300 in children.

Table 9.82 Neutrophil alkaline phosphatase (NAP) scoring system in various hematologic disorders

Neutrophil Alkaline Phosphatase (NAP): Low Scores	Neutrophil Alkaline Phosphatase (NAP): High Scores
▪ Chronic myelogenous leukemia, BCR-ABL1 positive	▪ Leukemoid reactions
▪ Atypical myeloid leukemia	▪ Chronic neutrophilic leukemia (CNL)
▪ Juvenile CML	▪ Myelofibrosis
▪ Paroxysmal nocturnal hemoglobinuria (PNH)	▪ Polycythemia vera (PV)
▪ Hereditary hypophosphatasia	▪ Essential thrombocythemia (ET)

Table 9.83 Molecular genetic monitoring for patients with chronic myelogenous leukemia (CML), BCR-ABL1 positive

Period of Treatment	Comments
At the time of diagnosis of CML	▪ Karyotype of bone marrow cell metaphases ▪ Fluorescence *in situ* hybridization to variant, cryptic translocations ▪ Qualitative polymerase chain reaction to identify transcript type
During treatment of CML	Real-time QRT-PCR (real-time quantitative polymerase chain reaction) is done to determine BCR-ABL1 transcript levels, every three months until major molecular response (MMR) and then every six months and/or karyotype of bone marrow cell metaphases at 3, 6, and 12 months until elimination of the Philadelphia chromosome in bone marrow metaphases (CCyR) treated with tyrosine kinase inhibitor (TKI)
At the time of progression of CML disease	Real-time QRT-PCR, mutational analysis, karyotype of bone marrow cell metaphases; immunophenotype

- Patient presents with fever, night sweats, weight loss, weakness, malaise, bone pain and lymphadenopathy.
- Approximately 30% of those patients with CML in accelerated phase die before developing blast phase.
- CML in accelerated phase involves organs outside bone marrow, peripheral blood and spleen. Defining criteria of CML, BCR-ABL1 positive in accelerated phase (AP) are given in Table 9.84.

Laboratory Diagnosis of Chronic Myelogenous Leukemia (CML), BCR/ABL1 Positive in Accelerated Phase

Peripheral Blood Smear Examination

- Peripheral blood smear examination shows anemia, persistent thrombocytopenia (platelet count $<100 \times 10^9$/L), increasing WBCs ($>10 \times 10^9$/L), immature granulocytes (10–19% blasts), absolute eosinophilia and basophilia (basophil count ≥20%). Chronic myelogenous leukemia (CML), BCR-ABL1 positive in accelerated phase in Giemsa-stained peripheral blood smear is shown in Figs 9.75 and 9.76.

Bone Marrow Smear Examination

- Bone marrow aspirate smear examination shows hypercellular marrow and immature granulocytes (>10 × 10⁹/L).
- Myelofibrosis may develop and sideroblasts.

Bone Marrow Trephine Biopsy Examination

- Presence of micromegakaryocytes arranged in large clusters or sheets associated with marked reticulin bone marrow fibrosis demonstrated by bone marrow trephine biopsy may be considered presumptive evidence of CML, BCR-ABL1 positive in accelerated phase.
- Increasing splenomegaly and patient not responding to RTKI therapy also suggest the progression of chronic phase to accelerated phase and finally to blast phase in CML patients.

Molecular Genetic Alterations

- Evolution of the neoplastic clone in CML, BCR-ABL1 positive may be associated with development of new abnormal karyotypes, often an extra chromosome 8 or 9 (trisomy 8 or 9) or isochromosome 17q[i(17q)].
- Diagnosis is confirmed by finding the Philadelphia chromosome in samples examined with cytogenetic or molecular studies.
- The classic Philadelphia chromosome cytogenetic abnormality may be absent in 5% of CML patients, but the use of fluorescence *in situ* hybridization (FISH) or reverse transcription polymerase chain reaction (RT-PCR) can confirm the diagnosis.
- Molecular studies can demonstrate mutations in RUNX1 (33%). ASXL1 (21%), IKZF1 (20%), TP53, RB1, RAS, Myc, AML-1, EVI-1, CDKN2A genes in CML patients.

Frequent chromosomal changes	▪ Philadelphia chromosome duplication + ▪ Trisomy 8+ ▪ Trisomy 19+ ▪ Isochromosome 17q+ ▪ Loss of Y chromosome
Rare chromosomal changes	▪ Translocation (15;17)+ ▪ Translocation (3;21) (p26; q22)+ ▪ Translocation (3;3), inv(3)+
Very rare chromosomal changes	▪ Deletion of chromosome 5 (–5) or deletion of 5q ▪ Deletion of chromosome 7 (–7) or deletion of 7q
Additional molecular genetic mutations (genes involved)	RUNX1 (33%). ASXL1 (21%), IKZF1 (20%), TP53, RB1, RAS, Myc, AML-1, EVI-1, CDKN2A

CML, BCR-ABL1 Positive in Blast Phase

In majority of cases, CML, BCR-ABL1 positive in chronic phase progresses to accelerated phase, that terminates into blast phase. Without intervention of CML, in accelerated phase symptoms worsen over next 3–5 years with onset of blast phase. Clinical features of CML BCR-ABL1 positive in blast phase are similar to those of acute leukemia.

Table 9.84 Defining criteria of CML, BCR-ABL1 positive in accelerated phase

Hematologic Molecular Genetic Alterations: Diagnostic Criteria of CML, BCR-ABL1 Positive in Accelerated Phase
▪ Persistent or increasing high white blood cell count (>10 × 10⁹/L), unresponsive to RTKI therapy ▪ Persistent or increasing splenomegaly unresponsive to RTKI therapy ▪ Persistent thrombocytosis (>1000 × 10⁹/L) unresponsive to RTKI therapy ▪ Persistent thrombocytopenia (<100 × 10⁹/L) ▪ Presence of ≥20% basophils in the peripheral blood ▪ Presence of 10–19% blasts in peripheral blood and/or bone marrow ▪ Additional clonal chromosomal abnormalities in Philadelphia chromosome positive at diagnosis include duplication of Philadelphia chromosome, trisomy 8, trisomy 19, isochromosome 17 and loss of Y chromosome does not present in the CML chronic phase. Molecular mutations include RUNX1(33%). ASXL1(21%), IKZF1(20%), TP53, RB1, RAS, Myc, AML-1, EVI-1, CDKN2A ▪ Any new clonal chromosomal abnormality occurs in Philadelphia chromosome positive cells during tyrosine kinase therapy
Provisional Response to Receptor Tyrosine Kinase Inhibitors Therapy
▪ Hematologic resistance or failure to achieve a complete hematologic response. Any hematologic, cytogenetic, or molecular indications resistance to two sequential RTKIs. Occurrence of two or more mutations in the BCR-ABL1 fusion gene during RTKI therapy ▪ Complete hematological response is defined as total white blood cell count <10 × 10⁹/L, platelet count <450 × 10⁹/L, absence of immature granulocytes in the differential white blood cell count, and spleen not palpable ▪ Observation of lymphoblasts even <10% in the peripheral blood and/or bone marrow indicates lymphoblastic transformation and warrants further clinical, cytogenetic or molecular analysis ▪ Presence of ≥20% blasts in the peripheral blood and/or bone marrow, or infiltrative proliferation of blasts in an extramedullary site is diagnostic of CML blast phase ▪ Large clusters or sheets of small, abnormal megakaryocytes associated with marked reticulin or collagen fibrosis in bone marrow trephine biopsy specimens may be considered presumptive evidence of accelerated phase of chronic myelogenous leukemia, although these findings are usually associated with one or more of the diagnostic criteria listed above

Chronic myelogenous leukemia (CML), BCR-ABL1 positive in accelerated phase is defined by the presence of ≥1 of the following hematological/cytogenetic criteria or provisional criteria concerning response to receptor tyrosine kinase inhibitor (RTKI) therapy.

- Patient presents with refractory leukocytosis, extramedullary masses (chloromas), increasing splenomegaly, sudden onset of bone pain, central nervous system involvement and lymphadenopathy.
- Unusual manifestations include spinal cord compression, visual disturbance, cerebral stroke and priapism

(erection of penis). With the onset of blast phase, response to poor with fatal outcome in <6 months using traditional chemotherapy.

- Extramedullary proliferation of blasts most often occurs in the lymph nodes, skin, bone and central nervous system, and any other sites.
- Formation of tumor mass in these sites is known as 'chloroma'. On fresh incision of the extramedullary masses appear green, presumably due to the presence of the myeloperoxidase (MPO) enzyme in the myeloid cells. Greenish discoloration of tumor mass has been known as '**chloroma**'. The green color of chloroma fades to a dirty yellow color, on exposure of the tumor to surrounding air.

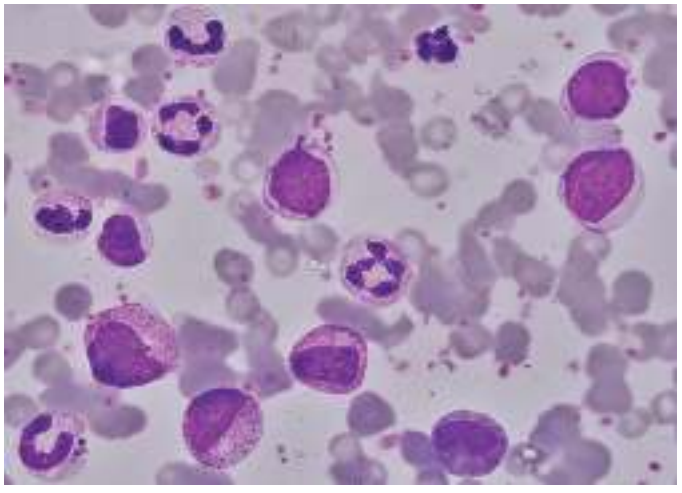


Fig. 9.75: Chronic myelogenous leukemia (CML), BCR-ABL1 positive in accelerated phase in Giemsa-stained peripheral blood smear. CML accelerated phase is defined by the presence of ≥1 of the following hematological/cytogenetic criteria or provisional criteria concerning response to tyrosine kinase inhibitor (TKI) therapy: (a) persistent or increasing high white blood cell count (>10 × 10^9/L), (b) unresponsive to tyrosine kinase inhibitor (TKI) therapy or (c) persistent or increasing splenomegaly unresponsive to tyrosine kinase inhibitor (TKI) therapy, (d) persistent thrombocytosis (>1000 × 10^9/L) unresponsive to tyrosine kinase inhibitor (TKI) therapy, (e) persistent thrombocytopenia (<100 × 10^9/L), (f) presence of ≥20% basophils in the peripheral blood; and (g) presence of 10–19% blasts in peripheral blood and/or bone marrow.

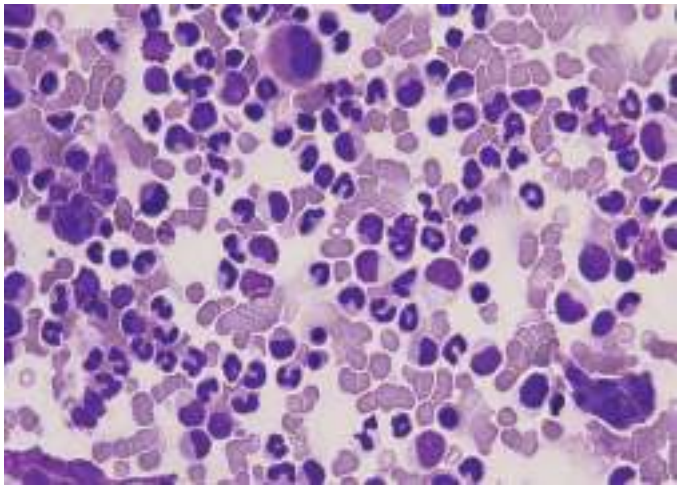


Fig. 9.76: Chronic myelogenous leukemia (CML) in accelerated phase with hemophagocytosis, BCR-ABL1 positive in Giemsa-stained peripheral blood smear (1000X).

Laboratory Diagnosis of Chronic Myelogenous Leukemia (CML), BCR-ABL1 Positive in Blast Phase

- Hematologic diagnostic criteria for CML, BCR-ABL1 positive in blast phase include moderate to marked diffuse bone marrow fibrosis and presence of ≥20% blasts in the bone marrow and peripheral blood of a patient previously diagnosed as suffering from CML.
- Characteristics of chronic myelogenous leukemia, BCR-ABL1 positive in blast phase are given in Table 9.85. Termination of CML, BCR-ABL1 positive in chronic phase via accelerated phase into blast phase and development of acute myelogenous leukemias (AML-M1, AML-M2, AML-M4, AML-M4E0, AML-M6, AML-M7) including their features, cytochemistry and immunophenotyping is given in Table 9.86. Peripheral blood smear CML, BCR-ABL1 positive in blast phase (*see* Fig. 9.31).

Peripheral Blood Smear and Bone Marrow Examination

- Chronic myelogenous leukemia, BCR-ABL1 positive is a clonal pluripotent hematopoietic stem cell disorder. Any type of blast involvement is possible including myeloblasts, lymphoblasts, erythroblasts or megakaryoblasts in CML, BCR-ABL1 positive in blast phase. Bone marrow and blood demonstrate blasts ≥20%.
- Cytochemistry, ultrastructural and immunophenotyping analysis are essential to identify type of blasts.
- About 60–70% of patients with blast phase demonstrate myeloblasts (AML-M1, AML-M2, AML-M4, AML-M6, AML-M7), approximately 25–35% of cases show lymphoblasts (ALL-L1, ALL-L2 of B cell origin) with positivity for CALLA+/CD10+ and elevated terminal deoxynucleotidyl transferase (TdT). Acute megakaryoblastic leukemia (AML-M7) may be associated with bone marrow fibrosis.
- Erythroblastic and megakaryoblastic blast phases are uncommon.
- Acute myelogenous leukemia with expression of lymphoid-related antigens is rare.
- Extramedullary proliferation of blasts forming tumor mass known as 'chloroma' in skin, lymph node, bone, central nervous system, or any organs.
- Cytochemistry and immunophenotyping of blasts are essential to analyze expression of antigens in more than one cell lineages.
- Chronic myelogenous leukemia, BCR-ABL1 positive in blast phase in Giemsa-stained peripheral blood smear is shown in Figs 9.77 to 9.79.
- Chronic myelogenous leukemia, BCR-ABL1 positive in blast phase in Giemsa-stained bone marrow aspirate smear is shown in Fig. 9.80.

Bone Marrow Trephine Biopsy Examination

- Bone marrow trephine biopsy examination shows ≥20% blasts arranged in clusters associated with marked collagen or reticulin fibrosis.
- Chronic myelogenous leukemia, BCR-ABL1 positive in blast phase in hematoxylin and eosin-stained bone marrow trephine biopsy is shown in Fig. 9.81.
- Bone marrow fibrosis grade 2 or 3 demonstrated by reticulin stain in CML, BCR-ABL1 positive in blast phase in bone marrow trephine biopsy section is shown in Fig. 9.82.

Lymph Node Biopsy and Histologic Examination

Chronic myelogenous leukemia, BCR-ABL1 positive in blast phase in hematoxylin and eosin-stained lymph node biopsy section is shown in Fig. 9.83. Myeloperoxidase (MPO) stain positivity in a case of CML blast phase, BCR-ABL1 positive in hematoxylin and eosin-stained lymph node biopsy section is shown in Fig. 9.84.

Additional Chromosomal Alterations in CML, BCR-ABL1 Positive in Blast Phase

Frequent chromosomal changes	▪ Philadelphia chromosome duplication+ ▪ Trisomy 8+ ▪ Trisomy 19+ ▪ Isochromosome 17q+ ▪ Loss of Y chromosome
Rare chromosomal changes	▪ Translocation (15;17)+ ▪ Translocation (3;21) (p26; q22)+ ▪ Translocation (3;3), inv(3)+
Very rare chromosomal changes	▪ Deletion of chromosome 5 (–5) or deletion of 5q ▪ Deletion of chromosome 7 (–7) or deletion of 7q
Genes involved	RUNX1 (33%), ASXL1 (21%), IKZF1 (20%), TP53, RB1, RAS, Myc, AML-1, EVI-1, CDKN2A

Table 9.85 Characteristics of chronic myelogenous leukemia (CML), BCR-ABL1 positive in blast phase

- Blast phase of CML is characterized by the presence of one or more of the following findings:
  - Presence of ≥20% myeloblasts in the bone marrow or nucleated cells in the peripheral blood
  - Extramedullary blast proliferation is known as chloroma.
  - Presence of blasts arranged in larger clusters demonstrated in trephine bone marrow biopsy
  - Additional clonal molecular genetic alterations include duplication of Philadelphia chromosome, trisomy 8, trisomy 19, isochromosome 17, and loss of Y chromosome does not present in the CML chronic phase

CLINICAL COURSE

In the absence of treatment, chronic myelogenous leukemia (CML) has a triphasic or biphasic clinical course as it progresses from chronic phase to an accelerated phase and then to blast phase (acute phase). Sometimes, CML goes from chronic phase to blast phase when blast phase is of lymphoid lineage. Summary of the clinical course of CML, BCR-ABL1 positive in chronic phase is given in Table 9.87. Summary of the clinical course of CML, BCR-ABL1 positive in accelerated phase is given in Table 9.88. Summary of the clinical course of CML, BCR-ABL1 positive in blast phase is given in Table 9.89.

Table 9.86 Termination of chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase via accelerated phase into blast phase and development of acute leukemias (AML-M1, AML-M2, AML-M4, AML-M4EO, AML-M6, AML-M7) including their features, cytochemistry and immunophenotyping

FAB Class	Features	Cytochemistry	Immunophenotype by Flow Cytometry
AML-M1	Acute myeloblastic leukemia without maturation (contains <10% promyelocytes or more mature myeloid forms)	Myeloperoxidase + (>3%)	▪ CD34+ ▪ CD33+ ▪ HLA-DR+ ▪ CD13+
AML-M2	Acute myeloblastic leukemia with maturation	Myeloperoxidase + (>10%)	▪ CD34+ ▪ CD13+ ▪ HLA-DR+ ▪ CD15+ ▪ CD33+
AML-M4	Acute myelomonocytic leukemia (monocytes and promonocytes in the bone marrow exceed 20%)	▪ Myeloperoxidase + ▪ Esterase +	▪ CD34– ▪ CD13+ ▪ HLA-DR+ ▪ CD14+ ▪ CD33+ ▪ CD64+
AML-M4Eo	Acute myelomonocytic leukemia with abnormal eosinophils (>5%)	▪ Myeloperoxidase + ▪ Esterase +	▪ CD34– ▪ CD13+ ▪ HLA-DR+ ▪ CD11+ ▪ CD33+ ▪ CD14+

Contd...

Table 9.86 Termination of chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase via accelerated phase into blast phase and development of acute leukemias (AML-M1, AML-M2, AML-M4, AML-M4E0, AML-M6, AML-M7) including their features, cytochemistry and immunophenotyping (*Contd...*)

FAB Class	Features	Cytochemistry	Immunophenotype by Flow Cytometry
AML-M6	Acute erythroleukemia (shows >50% nucleated bone marrow cells are erythroid, often severely dyserythropoietic)	▪ Erythroblast shows strong positivity with periodic acid–Schiff (PAS) stain ▪ Esterase negative	▪ Glycophorin A+ ▪ Hemoglobin A+
AML-M7	Acute megakaryocytic leukemia may have micromegakaryoblasts	PAS +/–	▪ CD41+ ▪ CD61+ ▪ Platelet peroxidase + ▪ Electron microscopy

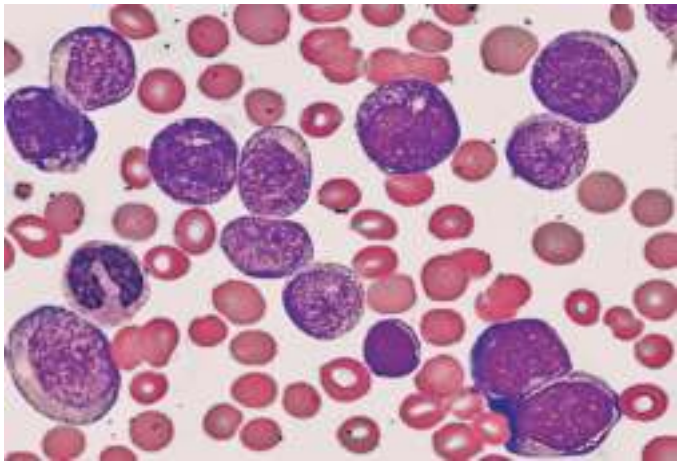


Fig. 9.77: Chronic myelogenous leukemia (CML), BCR-ABL1 positive in blast phase in Giemsa-stained peripheral blood smear. In majority of cases, CML chronic phase terminates in accelerated phase followed by blast phase. Without intervention of CML, symptoms worsen over next 3–5 years with onset of blast phase. Clinical findings in blast phase are similar to those of acute leukemia. Hematologic diagnostic criteria for CML blast phase include moderate to marked diffuse bone marrow fibrosis and ≥20% blasts in the peripheral blood and bone marrow of a patient previously diagnosed as suffering from CML (1000X).

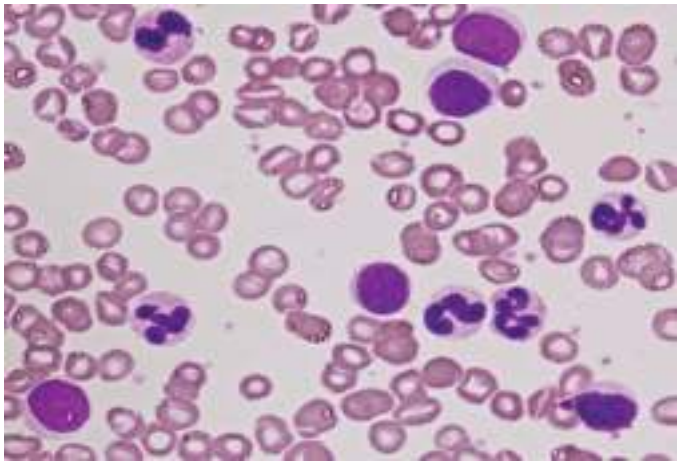


Fig. 9.78: Chronic myelogenous leukemia (CML-M7), BCR-ABL1 positive in blast phase in Giemsa-stained peripheral blood smear (1000X).

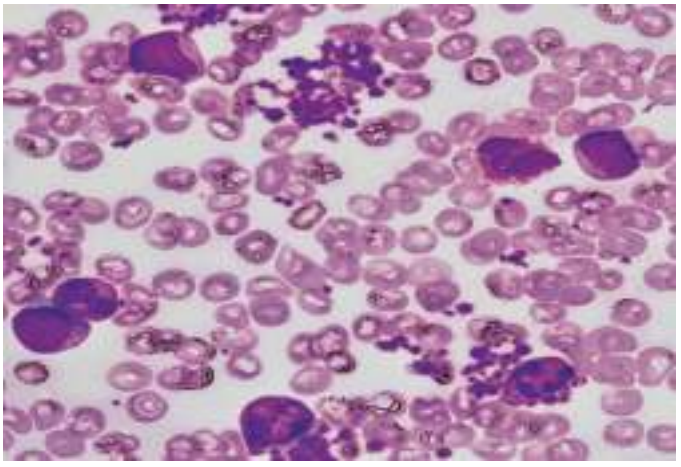


Fig. 9.79: Chronic myelogenous leukemia, BCR-ABL1 positive in blast phase in Giemsa-stained peripheral blood smear shows abundant platelets (1000X).

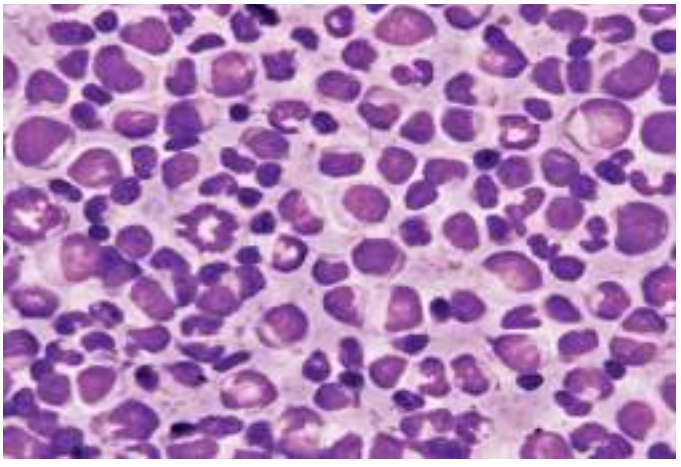


Fig. 9.80: Chronic myelogenous leukemia, BCR-ABL1 positive in blast phase in bone marrow aspirate smear. In majority of cases, CML chronic phase terminates in accelerated phase followed by blast phase. Without intervention of CML, symptoms worsen over next 3–5 years with onset of blast phase. Clinical features in blast phase are similar to those of acute leukemia. Hematologic diagnostic criteria for blast phase include moderate to marked diffuse bone marrow fibrosis and ≥20% blasts in the peripheral blood and bone marrow of a patient previously diagnosed as suffering from CML (1000X).

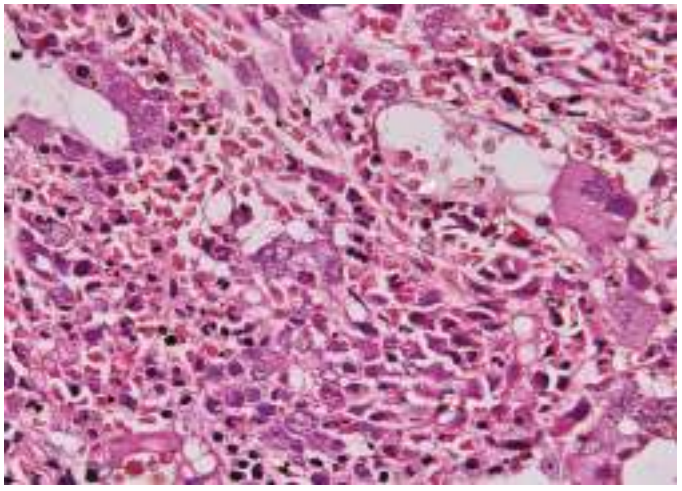


Fig. 9.81: Chronic myelogenous leukemia, BCR-ABL1 positive in blast phase in hematoxylin and eosin-stained bone marrow trephine biopsy. It shows ≥20% blasts arranged in clusters associated with marked collagen or reticulin fibrosis. In majority of cases, CML chronic phase progresses to accelerated phase and terminates into blast phase. Without intervention of CML, symptoms worsen over next 3–5 years with onset of blast phase. Clinical features in blast phase are similar to those of acute leukemia. Hematologic diagnostic criteria for blast phase include moderate to marked diffuse bone marrow fibrosis and ≥20% blasts in the peripheral blood and bone marrow of a patient previously diagnosed as suffering from CML.

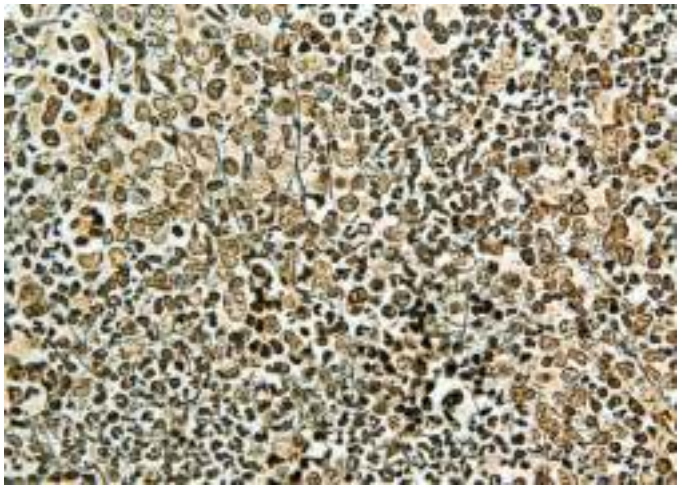


Fig. 9.82: Bone marrow fibrosis grade 2 or 3 demonstrated by reticulin stain in chronic myelogenous leukemia blast phase, BCR-ABL1 positive in bone marrow trephine biopsy section (1000X).

DIFFERENTIAL DIAGNOSIS

Chronic myelogenous leukemia (CML), BCR-ABL1 positive should be differentiated from leukemoid reactions and chronic neutrophilic leukemia. Comparison of CML, BCR-ABL1 positive in chronic phase and leukemoid reaction is given in Table 9.90. Comparison of CML, BCR-ABL1 positive and atypical chronic myelogenous leukemia (aCML), BCR-ABL1 negative is given in Table 9.91. Major characteristics

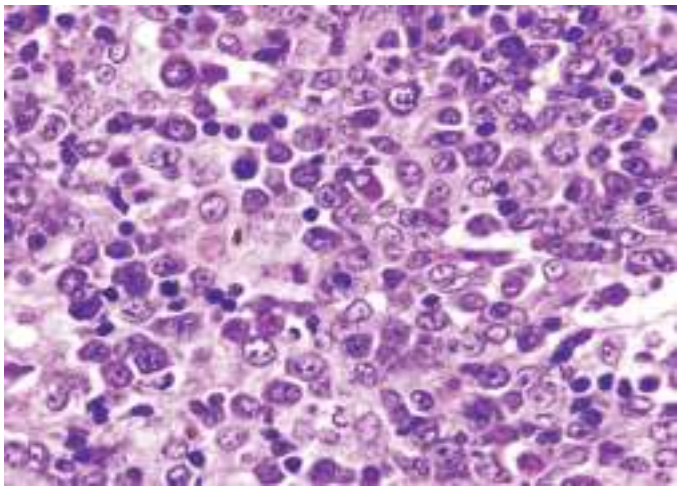


Fig. 9.83: Chronic myelogenous leukemia, BCR-ABL1 positive in blast phase in hematoxylin and eosin-stained lymph node biopsy section (400X).

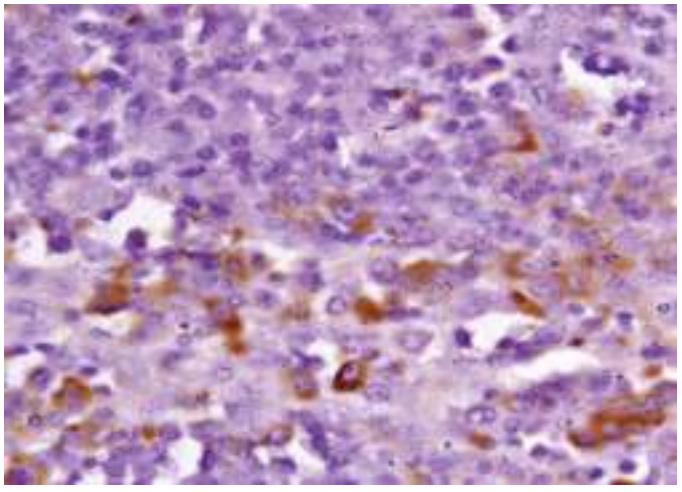


Fig. 9.84: Myeloperoxidase (MPO) stain positivity in a case of chronic myelogenous leukemia (CML) blast phase, BCR-ABL1 positive in hematoxylin and eosin-stained lymph node biopsy section (1000X).

of CML, BCR-ABL1 positive in atypical chronic myelogenous leukemia (aCML), BCR-ABL1 negative and chronic neutrophilic leukemia are given in Table 9.92.

TARGETED THERAPY

Basic aim of CML, BCR-ABL1 positive therapy is to reduce the leukocyte burden, restore bone marrow function, reduce splenomegaly and abolish clinical manifestations.

- Leukapheresis is sometimes performed initially to reduce the leukocyte burden with a purpose in reduction of blood hyperviscosity.
- Supportive measures during therapeutic management include blood transfusions to treat severe anemia and antibiotics to treat infections.

Table 9.87 Summary of the clinical course of chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase

Clinical Features and Laboratory Diagnosis	Hematologic Molecular Genetic Alterations
Clinical features	
Signs and symptoms	Anemia, sternal tenderness, discomfort in left upper quadrant, massive splenomegaly (>90%) and hepatomegaly, weight loss, night sweats, priapism, visual disturbances, gout and headache
Abdominal findings	▪ Massive splenomegaly because of infiltration of mature and immature granulocytes, i.e. leukemia cells in the red pulp of spleen ▪ Hepatomegaly because of infiltration of mature and immature granulocytes, i.e. leukemia cells in the hepatic sinusoids and portal tracts
Progression of CML chronic phase	Increasing splenomegaly and not responding to treatment suggests progression of chronic phase to accelerated and/or blast phase disease
Peripheral blood smear examination	
Total leukocyte count	$>100 \times 10^9$/L with a median of 170×10^9/L
Myeloblasts	<10%
Myelocytes	15–30%
Promyelocytes	2–8%
Metamyelocytes	15–25%
Band forms	5–15%
Neutrophils	40–70%
Basophilia	2–10% (often exceeding 1×10^9/L)
Eosinophilia	>4% (few cases)
Monocytes	<3% except in rare case with the p190 BCR-ABL1 isoform, which often mimics chronic myelomonocytic leukemia (CMML)
Platelet count	Normal or increased $\geq 1000 \times 10^9$/L, or marked thrombocytopenia uncommon
Bone marrow examination	
Cellularity	Hypercellular bone marrow with marked expansion at myelocytic stage
Myeloid precursors	Increased
Erythroid precursors	Significantly decreased
Myeloid to erythroid ratio	Increased
Myeloblasts	<10% (blast count increases as CML progresses to accelerated phase and terminates in blast phase)
Myelocytes	Markedly increased
Basophil count	Increased
Eosinophil count	Increased
Megakaryocytes	▪ Megakaryocyte number may be normal or slightly decreased. But 40–50% of cases exhibit moderate to marked megakaryocytic proliferation ▪ Size of megakaryocytes is smaller than normal and containing hyposegmented nuclei termed as 'dwarf' megakaryocytes
Pseudo-Gaucher cells	Common with foamy cytoplasm and derived from the neoplastic clone
Bone marrow trephine biopsy examination	
Bone marrow trephine biopsy is performed, only if the findings in the peripheral blood are atypical or if aspiration is not obtained due to cellular bone marrow	
Molecular genetic alterations	
Philadelphia chromosome t(9;22)	Cytogenetic alterations
BCR-ABL1 fusion protein	Molecular genetic alterations

Most cases of CML (hematopoietic stem cell disorder) are diagnosed based on peripheral blood findings and/or BCR-ABL1 by cytogenetic and molecular genetic techniques

Table 9.88 Summary of the clinical course of chronic myelogenous leukemia (CML), BCR-ABL1 positive in accelerated phase

Diagnostic criteria of chronic myelogenous leukemia, BCR-ABL1 positive in accelerated phase	
▪ Most cases of CML (pluripotent stem cell disorder) are diagnosed based on peripheral blood findings and/or BCR-ABL1 by cytogenetic and molecular genetic techniques	
▪ Diagnostic criteria of CML, BCR/ABL1 positive in accelerated phase: one or more (≥1) of the following findings: • Persistent increase in white blood cell counts >10 × 10⁹/L • Progressive increasing splenomegaly and unresponsive to tyrosine kinase inhibitor (TKI) therapy • Persistent increase in platelets >1000 × 10⁹/L • Increase in basophil count in peripheral blood >20% • Presence of 10–19% blasts in peripheral blood and/or bone marrow • Presence of additional clonal Philadelphia chromosome, trisomy 8, trisomy 19, isochromosome 17q • Presence of molecular mutations TP53, RB1, RAS, Myc, AML-1, EVI-1, CDKN2A • Bone marrow trephine biopsy is associated with marked collagen or reticulin fibrosis	
Peripheral blood smear examination	
White blood cell count	Persistent increase in white blood cell counts >10× 10⁹/L
Myeloblasts	10–19%
Basophils	>20%
Platelet count	<100 × 10⁹/L unrelated to therapy
Persistent thrombocytosis	>1000 × 10⁹/L unresponsive to therapy
Clonal genetic aberrations	Present in accelerated phase of CML, but not demonstrated in chronic phase
Bone marrow examination	
Cellularity	Hypercellular bone marrow with marked expansion
Myeloid series	Increased
Erythroid series	Significantly decreased
Myeloid to erythroid ratio	Increased
Myeloblasts	10–19% blasts (blast count increases as CML progresses and terminates in blast phase)
Myelocytes	Markedly increased
Additional cytogenetic alterations	
Frequent chromosomal alterations	▪ Duplication of Philadelphia chromosome ▪ Trisomy 8 ▪ Trisomy 19 ▪ Isochrome 17q ▪ Loss of Y chromosome
Rare chromosomal alterations	▪ Translocation (15;17) ▪ Translocation (3;21) (p26; q22) ▪ Translocation (3;3)/inv (3)
Very rare chromosomal alterations	▪ Deletion of chromosome 5 (–5) or the long arm (5q–) ▪ Deletion of chromosome 7 (–7) or the long arm (7q–)
Additional molecular genetic alterations	
Gene mutations	RUNX1 (33%). ASXL1 (21%), IKZF1 (20%), TP53, RB1, RAS, Myc, AML-1, EVI-1, CDKN2A

Table 9.89 Summary of the clinical course of chronic myelogenous leukemia (CML), BCR-ABL1 positive in blast phase

Laboratory Investigations	Hematologic Molecular Genetic Alterations
Peripheral blood smear examination	
Blasts number	≥20% blasts
Type of blasts	Myeloblasts (70–80%) or lymphoblasts (20–30%)
Bone marrow examination	
Blasts number	≥20% blasts

Contd...

Table 9.89 Summary of the clinical course of chronic myelogenous leukemia (CML), BCR-ABL1 positive in blast phase (*Contd...*)

Laboratory Investigations	Hematologic Molecular Genetic Alterations
Myeloblast proliferation	Extramedullary region forming blast chloroma in skin, lymph node, bone, central nervous system or any organ
Type of blast cells in CML blast phase	▪ CML is pluripotent stem cell disorder capable of differentiating along myeloid, lymphoid and megakaryocytes ▪ Myeloblasts are demonstrated in 70–80% of cases ▪ Lymphoblasts of B cell origin are demonstrated in 20–30% of cases
Bone marrow trephine biopsy examination	
Myeloblasts or lymphoblasts are arranged in clusters	Presence of large number of myeloblasts ≥20% in clusters in the bone marrow trephine biopsy is associated with marked collagen or reticulin fibrosis
Additional chromosomal alterations	
Frequent chromosomal changes	▪ Duplication of Philadelphia chromosome ▪ Trisomy 8 ▪ Trisomy 19 ▪ Isochrome 17q ▪ Loss of Y chromosome
Rare chromosomal changes	▪ Translocation (15;17) ▪ Translocation (3;21) (p26; q22) ▪ Translocation (3;3), inv(3)
Very rare chromosomal changes	▪ Deletion of chromosome 5 (–5) or deletion of 5q ▪ Deletion of chromosome 7 (–7) or deletion of 7q
Additional molecular genetic alterations	
Gene mutations	RUNX1 (33%). ASXL1 (21%), IKZF1 (20%), TP53, RB1, RAS, Myc, AML-1, EVI-1, CDKN2A

Most cases of CML (pluripotent stem cell disorder) are diagnosed based on peripheral blood findings and/or BCR-ABL1 by cytogenetic and molecular genetic techniques
Loss of response to tyrosine kinase inhibitor (TKI) therapy is characterized by loss of a complete hematologic response within 3 months, loss of a major cytogenetic response within 12 months (>37% Philadelphia metaphases present) and increasing levels of BCR-ABL as assessed by real-time PCR.

Table 9.90 Comparison of chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase and leukemoid reaction

Parameters	CML, BCR-ABL1 Positive in Chronic Phase	Leukemoid Reaction
Age group, etiology and clinical features		
Age group	Peak 35–45 years	Any age group
Etiology	Clonal disorder of pluripotent stem cell	Infections
Clinical features	Patient presents with weight loss, night sweats, itching, left hypochondrial pain, massive splenomegaly (>90%) gout, priapism, visual disturbances and headache	Patient presents with fever without splenomegaly
Peripheral blood smear examination		
Total leukocyte count	TLC >100 × 10^9/L with a median of 170 × 10^9/L	TLC <50 × 10^9/L (moderate increase)
Differential WBC count	Myeloblasts (<10%), myelocytes (15–30%), promyelocytes (2–8%), metamyelocyte (15–25%), band form (5–15%), neutrophils (40–70%)	Proportion of immature myeloid cells in small to moderate in number, myeloblasts seldom exceed 5% and myelocytes seldom exceed 15–30%
Basophils	2–10% (often exceeding 1 × 10^9/L)	Basophil count within normal range

Contd...

Table 9.90 Comparison of chronic myelogenous leukemia (CML), BCR-ABL1 positive in chronic phase and leukemoid reaction (*Contd...*)

Parameters	CML, BCR-ABL1 Positive in Chronic Phase	Leukemoid Reaction
Toxic granules in neutrophils	Absent	Present
Neutrophil alkaline phosphatase (NAP) score	Low score	High score
RBC morphology	Normocytic normochromic picture, anemia (30%)	Normocytic normochromic picture
Platelets number	Normal or increased $\geq 1000 \times 10^9$/L or marked thrombocytopenia uncommon	Platelet count normal
Dohle bodies (cytoplasmic inclusions in neutrophils, thought to be remnant of rough endoplasmic reticulum)	Absent	Present
Bone marrow smear examination		
Cellularity	Hypercellular bone marrow	Slightly increased cellularity in bone marrow
Myeloid cell lineage	Myeloid precursor cells at different stages of maturation	Normal
Erythroid cell lineage	Suppressed showing normoblastic erythropoiesis	Normal
Myeloid to erythroid ratio	10:1 to 50:1	Normal to slightly increased
Megakaryocyte morphology	Micromegakaryocytes (dwarf megakaryocytes) with hyposegmented nuclei	Megakaryocytes morphology normal
Molecular genetic alterations		
Philadelphia chromosome (balanced translocation 9;22)	Positive	Absent
BCR-ABL1 fusion transcript	Positive	Absent
Biochemical alterations		
Serum cobalamin synthesized by granulocytes	Increased	Normal
Serum haptocorrin synthesized by granulocytes	Increased	Normal
Total serum cobalamin	Increased	Normal
Serum unsaturated cobalamin binding capacity	Increased	Normal
Serum uric acid	Increased secondarily to increased cell turnover	Normal
Serum lactic dehydrogenase	Increased secondarily to increased cell turnover	Normal
Management and clinical course		
Tyrosine kinase inhibitor (TKI) imatinib drug	Indicated with good hematologic and cytogenetic response	Underlying cause treated (TKI never indicated)
Allogeneic hematopoietic stem cell transplantation	Indicated with good clinical course	Never done
Clinical course	Progressive from chronic to accelerated and blast phase	Transient

Table 9.91 Comparison of chronic myelogenous leukemia (CML), BCR-ABL1 positive and atypical chronic myelogenous leukemia (aCML), BCR-ABL1 negative

Features	Chronic Myelogenous Leukemia (CML), BCR-ABL1 Positive in Chronic Phase	Atypical Chronic Myelogenous Leukemia (aCML), BCR-ABL1 Negative
Cytogenetic and molecular genetic alterations		
Philadelphia chromosome t(9;22)	Positive	Negative
Other cytogenetic abnormalities	Absent	Trisomy 8, t(5;12), t(5;10)
BCR-AML1 fusion transcript	Positive	Negative
Age group and clinical findings		
Age group	Elderly persons (mean 55 years)	Elderly persons (65–72 years)
Anemia, thrombocytopenia	Less common	More common
Peripheral blood smear examination		
Total leukocyte count	$>100\times10^9$/L with a median of 170×10^9/L	$\geq13\times10^9$/L
Blasts	<10%	<10%
Immature granulocytes	>20%	10–20%
Basophils	2–10% (often exceeding 1×10^9/L)	<2%
Eosinophils	>4% (few cases)	<4%
Monocytes	<3% except in rare case with the p190 BCR-ABL1 isoform, which often mimics chronic myelomonocytic leukemia (CMML)	>3%
Platelet	Normal or increased $\geq1000\times10^9$/L, or marked thrombocytopenia uncommon	$\geq450\times10^9$/L
Bone marrow smear examination		
Cellularity	Hypercellular bone marrow	Hypercellular bone marrow
Myeloid to erythroid ratio	>10:1	<10:1
Blasts	<10%	<10%
Megakaryocytes	Normal or increased	Decreased
Dysplasia	Absent	Present

Both chronic myelogenous leukemia (CML) and atypical chronic myelogenous leukemia (aCML) have similar clinical features and progress to accelerated phase to blast phase. Neutrophil alkaline phosphatase (NAP) score is low in both disorders.

Table 9.92 Major characteristics of chronic myelogenous leukemia (CML), BCR-ABL1 positive in atypical chronic myelogenous leukemia (aCML), BCR-ABL1 negative and chronic neutrophilic leukemia

Specimen Tested	Observation	CML, BCR-AML1 Positive in Chronic Phase	aCML, BCR-AML1 Negative	Chronic Neutrophilic Leukemia
Peripheral blood smear examination	▪ Total leukocyte count ▪ Myeloblasts ▪ Promyelocytes, myelocytes, metamyelocytes ▪ Neutrophils and band forms ▪ Basophilia	▪ Not determined ▪ <2% ▪ ≥10% ▪ Not determined ▪ Present	▪ $>13\times10^9$/L ▪ <20% ▪ ≥10% ▪ Not determined ▪ Minimal or <2% of leukocytes	▪ $>25\times10^9$/L ▪ <1% ▪ <10% ▪ >80% ▪ Not determined
Bone marrow examination	▪ Granulocytic hyperplasia ▪ Granulocytic dysplasia ▪ Megakaryocytic dysplasia	▪ Present ▪ Minimal or absent ▪ Usually, present	▪ Present ▪ Prominent ▪ May or may not be present	▪ Present ▪ Minimal or absent ▪ Minimal or absent
Molecular studies	BCR-ABL or variant transcripts	Present	Absent	Absent

Table 9.93 Commonly used drugs to treat chronic myelogenous leukemia (CML), BCR-ABL1 positive

Indication	Therapeutic Agents
Intervention during clinical presentation	
Cytoreduction at clinical presentation	Hydroxyurea
White blood cells 100×10^9/L	Allopurinol or alternative control of high white blood cell count by busulfan
CML, chronic phase treated by first-line of tyrosine kinase inhibitors	
Tyrosine kinase inhibitor (TKI)	Imatinib, dasatinib, nilotinib
CML, chronic phase resistant to first-line or second-line of tyrosine kinase inhibitors	
Tyrosine kinase inhibitors (TKIs)	Bosutinib
Tyrosine kinase inhibitors (TKIs) in patients with T3151 mutation	Ponatinib

Interferon-α is used in clinical trials with tyrosine kinase inhibitor (TKI).

- Prior to the discovery of molecular targeted therapy, Interferon-α (IFN-α) plus cytarabine have been considered standard therapy for CML patients, who have not been candidates for an allogeneic hematopoietic stem cell (HSC) transplantation.
- Interferon-α (IFN-α), a glycoprotein, has a myelosuppressive effect by inhibiting myeloid progenitor cells, which induces a remission in 55–75% of CML cases. In some cases, interferon-α (IFN-α) eliminates the Philadelphia chromosome. CML patients treated in the early stage has favorable cytogenetic response.

Therapeutic Drugs and their Genetic Target

Currently, tyrosine kinase inhibitor (TKI) imatinib mesylate (**Gleevec**) is considered the first line of treatment option except in pregnant CML patients.

- Imatinib competitively binds to the ATP binding site of receptor tyrosine kinase of ABL1 (BCR-ABL1 and TEL/ABL1) stem cell receptor (c-KIT), and platelet-derived growth factor receptor resulting in inhibiting receptor tyrosine kinase activity and prevention of cell signal transduction. CML patients tolerate well imatinib with side effects of gastrointestinal tract and skin.
- Approximately, 85% of CML patients achieve complete hematologic response by 12 months. Imatinib therapy also improves molecular genetic alterations and delays progression of the disease to blast phase in CML patients.
- Some patients may develop primary resistance to imatinib therapy. Patients initially responsive to imatinib treatment can develop secondary resistance related to development of additional mutations in the tyrosine kinase domain of BCR-ABL1. A specific ABL1 protein mutant T3151, in which threonine is mutated to isoleucine at the gatekeeper position of receptor tyrosine kinase domain in 15% of CML patients.
- Prior to initiating imatinib therapy, baseline analysis should be performed by real-time polymerase chain reaction (RT-PCR) to evaluate bone marrow morphologic analysis and cytogenetic study of BCR-ABL1 transcripts. It is essential to perform real-time polymerase chain reaction (RT-PCR) at least every three months to analyze BCR-ABL1 transcripts. A complete cytogenetic response with absence of Philadelphia chromosome and BCR-ABL1 transcripts indicate good response in CML cases.
- Commonly used drugs to treat CML, BCR-ABL1 positive are given in Table 9.93. Therapeutic drugs, their genetic target in chronic myelogenous leukemia, BCR-ABL1, positive are given in Table 9.94. Hematologic, cytogenic and molecular responses in CML, BCR-ABL1 positive are given in Table 9.95. Development of resistance to imatinib (tyrosine kinase inhibitor) in CML, BCR-ABL1 positive patients is given in Table 9.96.

Table 9.94 Therapeutic drugs, their genetic target in chronic myelogenous leukemia (CML), BCR-ABL1 positive

Generic Name	Trade Name	Genetic Target
Imatinib	Gleevec	▪ BCR-ABL1 ▪ PDGFRA ▪ c-KIT
Nilotinib	Tasigna	▪ BCR-ABL1 ▪ PDGFRA ▪ c-KIT
Laptinib	Tykerb	▪ HER2/neu ▪ EFGR
Soratinib	Nexavar	▪ EGFR ▪ VEGFR ▪ PDGFRA ▪ BRAF

Table 9.95 Hematologic, cytogenetic and molecular responses in chronic myelogenous leukemia (CML), BCR-ABL1 positive

Response	Characteristics
Hematologic response	
Peripheral blood smear and clinical examination	▪ TLC 10×10^9/L ▪ Basophils <5% ▪ Absence of myelocytes, promyelocytes and blasts ▪ Platelet count $<450 \times 10^9$/L ▪ Nonpalpable spleen
Cytogenetic response	
Major cytogenetic response	▪ Complete response: absence of Philadelphia positive metaphases or <1% BCR-ABL positive nuclei of ≥200 cells demonstrated by fluorescence *in situ* hybridization (FISH) ▪ Partial response: 1–35% Philadelphia positive metaphases
Minor cytogenetic response	36–65% Philadelphia positive metaphases
Minimal cytogenetic response	66–95% Philadelphia positive metaphases
Cytogenetic response none	>95% Philadelphia positive metaphases
Molecular response	
Molecular response 4, 5	Ratio of BCR-ABL1 to ABL1 (or other housekeeping gene) ≤0.0032% (≥4.4 log reduction) on international scale
Molecular response 4	Ratio of BCR-ABL1 to ABL1 (or other housekeeping gene) ≤0.01% (≥4 log reduction) on international scale
Molecular response 3	Ratio of BCR-ABL1 to ABL1 (or other housekeeping gene) ≤0.1% (≥43 log reduction) on international scale
Molecular response 2 (corresponds to complete cytogenetic response)	Ratio of BCR-ABL1 to ABL1 (or other housekeeping gene) ≤0.1% (≥2 log reduction) on international scale

Table 9.96 Development of resistance to imatinib (tyrosine kinase inhibitor) in chronic myelogenous leukemia (CML), BCR-ABL1 positive patients

Primary Resistance to Imatinib (Tyrosine Kinase Inhibitor) in CML
Complete hematologic response is not achieved by 3–6 months
Cytogenetic response is not achieved by 6 months in >95% of patients and persistence of Philadelphia chromosome metaphases
Partial cytogenetic response is not achieved by 12 months in >56% of patients and persistence of Philadelphia chromosome metaphases
Secondary Resistance to Imatinib (Tyrosine Kinase Inhibitor) in CML
Absence of complete hematologic response
Loss of a partial or complete cytogenetic response
Increasing levels of BCR-ABL1 as analyzed by real-time polymerase chain reaction (RT-PCR)

ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

Allogeneic hematopoietic stem cell (HSC) transplantation can be performed in CML, BCR-ABL1 positive patients, who meet the bone marrow transplant criteria.

- Limitations of allogeneic hematopoietic stem cell transplantation are based on availability of a compatible (HSC) donor and age of the CML patients <65 years.
- Prior to allogeneic HSC transplantation, high-dose chemotherapy or imatinib drug should be administered.
- Allogeneic HSC transplantation is most successful, when performed during the chronic phase of CML. About 85% of CML patients receiving HLA-matched hematopoietic stem cells, have survival rate more than three years.
- Indications for allogeneic HSC transplantation in CML, BCR-ABL1 positive are given in Table 9.97.

Table 9.97 Indications for allogeneic hematopoietic stem cell (HSC) transplantation in chronic myelogenous leukemia (CML), BCR-ABL1 positive

CML Phase	Indications for Allogeneic HSC Transplantation
CML chronic phase	▪ Intolerance to tyrosine kinase inhibitor therapy ▪ After first-line failure of tyrosine kinase inhibitor therapy and the T3151 mutation in Philadelphia chromosome ▪ After second-line failure of tyrosine kinase inhibitor therapy
CML accelerated phase	▪ After failure of tyrosine kinase inhibitor therapy in newly diagnosed case ▪ Development of accelerated phase while on tyrosine kinase inhibitor therapy ▪ Development of accelerated phase while on tyrosine kinase inhibitor therapy
CML blast phase	Indication of allogeneic bone marrow transplantation in patients with blast phase

Best outcome of allogeneic HSC transplantation is seen in CML in the chronic phase.
Chronic phase CML patients should always be started on tyrosine kinase inhibitor and should not be considered for allogeneic HSC transplantation.

CHRONIC NEUTROPHILIC LEUKEMIA

Chronic neutrophilic leukemia (CNL) is a rare myeloproliferative neoplasm, that always involves bone marrow, peripheral blood, spleen and liver. CNL is predominantly seen in older patients with median age 65 years at diagnosis with slight male predominance.

- Chronic neutrophilic leukemia is characterized by persistent neutrophilia with a slight shift to the left in peripheral blood without Philadelphia chromosomal abnormality and BCR-ABL1 fusion transcript, hypercellular bone marrow and hepatosplenomegaly. Basophilia and monocytosis are absent.
- Bone marrow becomes hypercellular due to increased proliferation of neutrophilic granulocyte without dysplastic changes. It is essential to rule out etiology of reactive neutrophilia, CML, BCR-ABL1 positive, aCML, polycythemia vera, primary myelofibrosis or myelodysplastic/myeloproliferative neoplasm.
- Chronic neutrophilic leukemia must be distinguished from reactive leukemoid reactions that can be seen in association with infections or inflammatory processes or as a paraneoplastic phenomenon secondary to plasma cell neoplasms, lymphomas and some carcinomas.
- Revised 2024 WHO diagnostic criteria for chronic neutrophilic leukemia are given in Table 9.98.

Table 9.98 Revised 2024 WHO diagnostic criteria for chronic neutrophilic leukemia (CNL)

Peripheral Blood Smear
Peripheral blood shows leukocytosis with total WBCs count $\geq 25 \times 10^9$/L
Band and segmented neutrophils ≥80% white blood cell count
Myeloid precursors such as promyelocytes, myelocytes, and metamyelocytes constitute <10% of white blood cells
Myeloblasts constitute <1% of WBCs
Monocyte count $<1 \times 10^9$/L
Absence of dysgranulopoiesis
Hypercellular Bone Marrow
Increased neutrophils in percentage and number
Normal neutrophil maturation
Myeloblasts constitute <5% of nucleated cells
Not Meeting WHO Criteria for Following Myeloproliferative Neoplasms
Chronic myelogenous leukemia (CML), BCR-ABL1 positive
Polycythemia vera (PV)
Essential thrombocythemia (ET)
Primary myelofibrosis (PMF)
Absence of the Following Gene Mutations
PDGFRA
PDFGRB
FGFR1
PCM1-JAK2
Presence of the Following Molecular Mutations
CSF3R T6181 mutation common
Other activating CSF3R mutation
Or in the absence of a CSF3R mutation, persistent neutrophilia (≥3 months), splenomegaly, and/or, if present, demonstration of clonally myeloid cells by cytogenetic or molecular studies
CNL lacks BCR-ABL1 gene arrangement, which distinguishes CNL from CML, BCR-ABL1 positive

Hepatosplenomegaly is demonstrated in chronic neutrophilic anemia. Adapted from revised 2024 WHO classification of hematopoietic neoplasms.

PATHOPHYSIOLOGY

In chronic neutrophilic leukemia, neutrophilic proliferation may be related to abnormal cytokine production, which most probably a clonal bone marrow HSC with limited myeloid potential.

- In chronic neutrophilic leukemia, normal apoptotic signal may be disrupted. Cytogenetic study is normal

in 25% of patients. However, about 25% of patients can demonstrate abnormalities of chromosome 20q–, 21+, 11q–, 6 and 9.
- Philadelphia chromosome and BCR-ABL1 fusion transcript is not demonstrated in chronic neutrophilic leukemia. If a plasma cell proliferation is demonstrated, clonality of the neutrophils should be established by cytogenetic and molecular studies for diagnosing chronic neutrophilic leukemia.

CLINICAL FEATURES

Majority of chronic neutrophilic leukemia patients are symptomatic at the time of diagnosis.
- Patient presents with fatigue, weight loss, night sweats, easy bruising and bone pain.
- Clinical examination reveals hepatosplenomegaly as a result of leukemic infiltration. However, leukemic infiltration can be demonstrated in any tissue/organ.

LABORATORY DIAGNOSIS

The most notable feature of chronic neutrophilic leukemia is neutrophilia (25×10^9/L). Mature segmented forms and band forms of neutrophils predominate.
- Peripheral blood smear examination shows more immature cells such as promyelocytes, myelocytes, and metamyelocytes that constitute <10% of white blood cells.
  - Neutrophils can have toxic granules, however, lack dysplastic features. Both the red blood cell and platelet have normal morphology.
  - Platelet count is usually within normal range, but thrombocytopenia can develop as the disease progresses and the spleen enlarges. Neutrophilic alkaline phosphatase score is usually increased.
- Bone marrow smear examination shows hypercellular bone marrow, because of granulocytic hyperplasia with myeloid to erythroid ratio 20:1 or even higher. There is no increase in the number of myeloblasts. Auer rods are absent in myeloblasts. Bone marrow examination demonstrates excessive proliferation of erythroid cells and megakaryocytes.

Laboratory Diagnosis of Chronic Neutrophilic Leukemia

Peripheral Blood Smear Examination

- The most notable feature of chronic neutrophilic leukemia is neutrophilia ($\geq 25 \times 10^9$/L).
- Band and segmented neutrophils constitute >80% white blood cell count.
- Promyelocytes, myelocytes, and metamyelocytes constitute <10% of WBCs.
- Myeloblasts constitute <1% of WBCs.
- Monocyte count is $<1 \times 10^9$/L.
- Absence of dysgranulopoiesis is observed
- Neutrophils can have toxic granules with absence of dysplastic features.
- Morphology of red blood cell and platelet morphology is normal.
- Platelet count is usually within normal range, but thrombocytopenia can develop as the CNL disease progresses associated with increased in size of spleen.
- Chronic neutrophilic leukemia in Giemsa-stained peripheral blood smear is shown in Fig. 9.85.

Neutrophilic Alkaline Phosphatase Score

Neutrophilic alkaline phosphatase score is usually increased. Neutrophil alkaline phosphatase (NAP) stain positivity in chronic neutrophilic leukemia in Giemsa-stained peripheral blood smear is shown in Fig. 9.86.

Bone Marrow Smear Examination

- Bone marrow becomes hypercellular as a result of granulocytic hyperplasia with myeloid to erythroid ratio 20:1 or higher. There is no increase in the number of myeloblasts. Auer rods are absent in myeloblasts.
- Increased neutrophils in percentage and number
- Normal neutrophil maturation is observed
- Myeloblasts constitute <5% of nucleated cells in bone marrow
- Bone marrow demonstrates excessive proliferation of erythroid cells and megakaryocytes.
- Chronic neutrophilic leukemia in Giemsa-stained bone marrow smear is shown in Fig. 9.87.

Bone Marrow Trephine Biopsy Examination

- Chronic neutrophilic leukemia (CNL) in hematoxylin and eosin-stained bone marrow trephine biopsy section is shown in Fig. 9.88.

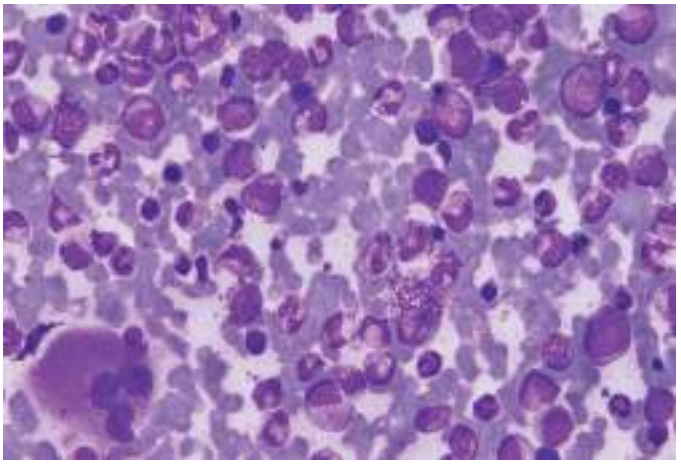


Fig. 9.85: Chronic neutrophilic leukemia in Giemsa-stained peripheral blood smear. The most notable feature of CNL is neutrophilia ($\geq 25 \times 10^9$/L). Band and segmented neutrophils constitute >80% white blood cell count. Neutrophils can have toxic granules, however, lack dysplastic features. Promyelocytes, myelocytes, and metamyelocytes constitute <10% of white blood cells. Myeloblasts are <1% of WBC. Monocyte count is $<1 \times 10^9$/L and absence of dysgranulopoiesis. Both the red blood cell and platelet have normal morphology (1000X).

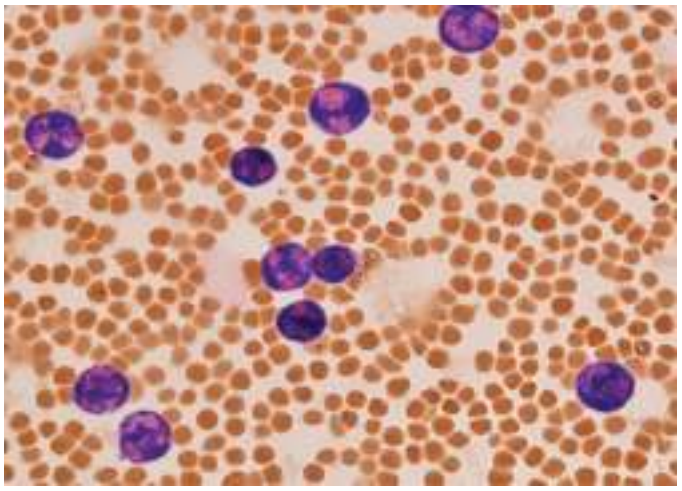


Fig. 9.86: Neutrophil alkaline phosphatase stain positivity in chronic neutrophilic leukemia in Giemsa-stained peripheral blood smear (1000X).

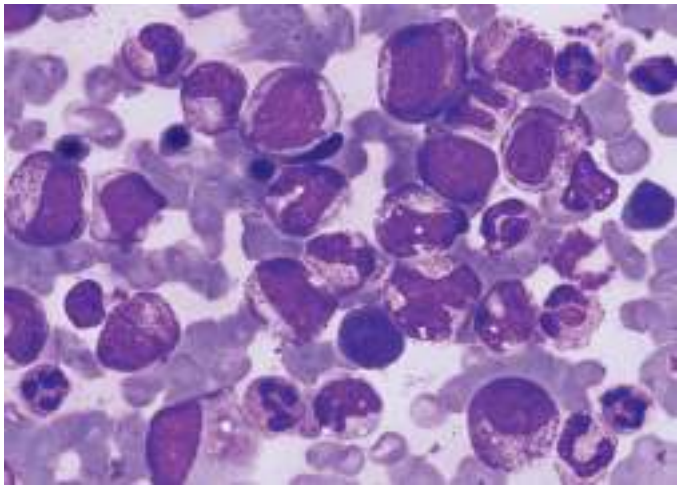


Fig. 9.87: Bone marrow aspirate smear examination shows hypercellular marrow, because of granulocytic hyperplasia with myeloid to erythroid ratio 20:1 or even higher. There is no increase in the number of myeloblasts. Auer rods are absent in myeloblasts. Bone marrow demonstrates excessive proliferation of erythroid cells and megakaryocytes (1000X).

TARGETED THERAPY

Hydroxyurea is the first-line targeted therapy for chronic neutrophilic leukemia. Hematologic response lasts for one year. Interferon-α (IFN-α) is the second line of therapy. Allogeneic HSC transplantation is potentially curative treatment for those eligible cases.

CLINICAL COURSE

Median survival of chronic neutrophilic leukemia is 2.5 years. The patients can develop accelerated phase in CML characterized by progressive neutrophilia unresponsive to treatment, anemia, thrombocytopenia, and splenomegaly. Peripheral blood smear examination can demonstrate myeloblasts and other immature myeloid cells.

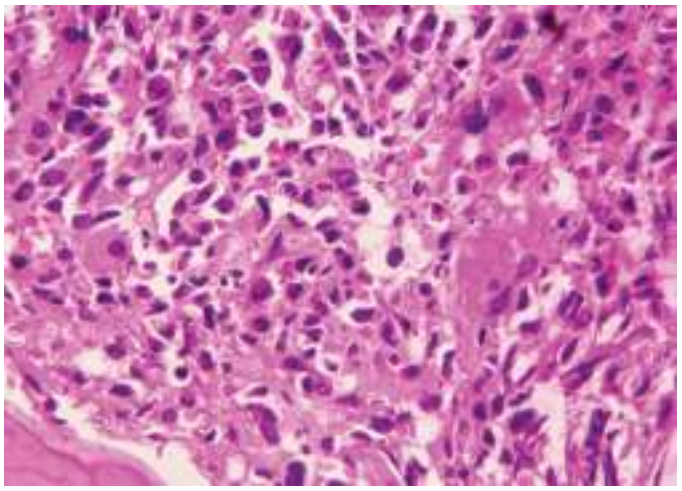


Fig. 9.88: Chronic neutrophilic leukemia in hematoxylin and eosin-stained bone marrow trephine biopsy section (400X).

DIFFERENTIAL DIAGNOSIS

Chronic neutrophilic leukemia (CNL) must be differentiated from CML, BCR-ABL1 positive and reactive neutrophilia due to infection or inflammation. Differential diagnosis from other myeloid neoplasms requires an absence of circulating blast cells, absolute monocytosis, eosinophilia and basophilia.

ESSENTIAL THROMBOCYTHEMIA

Essential thrombocythemia (ET) is a myeloproliferative neoplasm affecting primarily megakaryocytic lineage, which demonstrates sustained proliferation of megakaryocytes in the bone marrow and extreme thrombocytosis in the peripheral blood with qualitative disorder of platelets (thrombocytopathy).

- Thrombocytosis is a component of CML, primary myelofibrosis and polycythemia vera. However, it is now well established that essential thrombocythemia is a hematologic neoplasm with distinct clinical manifestations and complications.
- Most essential thrombocythemia (ET) patients have somatic mutation in Janus kinase 2 (JAK2), calretinin (CALR) or myeloproliferative leukemia (MPL) oncogene with subsequent dysregulation of the JAK2/STAT5 pathway.
- About 65% of ET patients demonstrate JAK2 (V617F) mutation associated with increased risk for thrombosis.
- Approximately 20–30% of ET patients demonstrate **CALR exon 9 mutation** associated with bone marrow fibrosis.
- About 3–5% of patients demonstrate **MPL exon 10 mutation** associated short survival.

Table 9.99 Diagnostic criteria for essential thrombocythemia must meet all three major and both minor criteria

Major Diagnostic Criteria of Essential Thrombocythemia (must Fulfill all Three Criteria)
Persistent elevation of platelets (≥450 × 10⁹/L) in the peripheral blood
Presence of acquired JAK2 (V617F), CALR, or MPL gene mutations (DNA testing)
Trephine bone marrow biopsy shows proliferation of megakaryocytic lineage with increased in number of mature large megakaryocytes with hyperlobulated nuclei, left shift in neutrophil granulopoiesis or erythropoiesis. Increase in reticulin fibrosis (grade I) may be rarely demonstrated.
Not meeting WHO diagnostic criteria for CML, polycythemia vera, primary myelofibrosis, myeloproliferative dysplasia or other myeloproliferative neoplasms
No evidence of reactive thrombocytosis and normal iron stores
Minor Diagnostic Criteria (must Fulfill Both Criteria)
Demonstration of clonal hematopoietic stem cell disorder
No evidence for reactive thrombocytosis such as inflammation

Diagnosis of essential thrombocythemia requires meeting all major and two minor criteria.

- About 5–10% of patients with triple negative mutations, i.e. JAK2 (V617F), CALR exon 9, and MPL have longest survival rate.
- Synonyms of ET include primary thrombocythemia, primary thrombocytosis, hemorrhagic thrombocythemia and idiopathic thrombocytosis. Diagnostic criteria for essential thrombocythemia are given in Table 9.99.

PATHOPHYSIOLOGY

Essential thrombocythemia (ET) is a clonal disorder of pluripotent HSC affecting all three lineages, but in some ET cases involving only the megakaryocytic lineage.

- Clonal population of HSC are hypersensitive to cytokines such as IL-3 and IL-6, and not to GM-CSF. Sensitivity to inhibitory effects of TGF-β to clonal population of cells is decreased, minimizing inhibition of thrombopoiesis.
- Thus, a combination of increased sensitivity to IL-3 and IL-6, and decreased sensitivity to negative regulators promote unrestricted proliferation of megakaryocytes resulting in increased production of platelets. Serum levels of thrombopoietin are normal or slightly elevated.

JAK2 (V617F) Gene Mutations

Janus Kinase 2 (JAK2) (V617F) is located on chromosome band 9p24 encodes JAK2 protein, i.e. receptor tyrosine kinase activator of cell signal transducers. JAK2 protein is cytoplasmic domain of receptor tyrosine kinase closely associated with cytokine receptors and distributed near the cell membrane.

- When a receptor tyrosine kinase binds cytokine, JAK2 protein gets transphosphorylated and activated. In turn, JAK2 phosphorylates signal transducers and activators of transcription STAT5 proteins. JAK2/STAT5 activation pathways are suppressed by negative regulators.
- Gain-of-function mutation in the JAK2 (V617F) tyrosine kinase gene is most often demonstrated in essential thrombocythemia, which results in autonomous cell signal transduction leading to unrestricted proliferation of the megakaryocytic lineage and production of platelets. Presence of the JAK2 (V617F) mutation is associated with thrombosis in these patients.

MPL Gene Mutations

Essential thrombocythemia (ET) is caused due to myeloproliferative leukemia (MPL) gene mutation that results in the replacement of the amino acid serine with the amino acid asparagine at position 505 in the protein (written as Ser505Asn or S505N).

- MPL (myeloproliferative leukemia) gene encodes thrombopoietin receptor protein also known as CD110, that participates in maintenance of hematopoietic stem cells, cell growth and proliferation of megakaryocytes leading to formation of platelets. Binding of thrombopoietin-to-thrombopoietin receptor leads to 'turning on' the thrombopoietin receptor, activation of JAK/STAT signaling pathway, resulting in maintenance of HSC and regulates megakaryocytes cell growth and proliferation with production of platelets.
- MPL gene mutations (MPLW5151 and MPLW515K) have been demonstrated in 4% of essential thrombocythemia patients. MPL gene mutation leads to cytokine-independent growth and constitutive downstream signaling pathways.
- MPL gene (exon 10) mutation is associated with short survival in 3–5% of ET patients.

Pathology Pearls: Essential Thrombocythemia

- About 65% of patients demonstrate mutation in JAK2 (V617F) associated with increased risk of thrombosis.
- Approximately 20–30% of patients demonstrate mutation CALR exon 9 and associated with increased rate of bone marrow fibrosis.
- About 3–5% of patients demonstrate mutation in MPL exon 10 and associated with short survival.
- About 5–10% of patients with triple negative mutations, i.e. JAK2 (V617F), CALR exon 9, and MPL associated with longest survival rate.

CLINICAL FEATURES

Essential thrombocythemia most often affects 50–60 years of age. It may also affect 20–30 years of age. Clinical manifestations of ET are variable. Extreme thrombocytosis is frequently demonstrated in these patients. Symptomatic patients present with symptoms related to thrombi formation in the microvasculature.

- Patient develops neurological manifestations such as headache and paresthesia of extremities due to platelet-mediated ischemia and thrombi formation in microvasculature; and painful toes and fingers due to circulatory insufficiency related to thrombi formed in the microvasculature. Hemorrhagic episodes primarily affect skin and mucous membranes lining gastrointestinal tract, oral cavity, and urinary bladder.
- Slightly palpable spleen is observed in 50% of cases. Splenic atrophy or silent splenic infarction may occur. Platelet coagulation activity (i.e. platelet aggregation) may be increased due to loss of prostaglandin E_2 (PGE_2) receptor. Storage pool defects in platelets promote hemorrhagic manifestations.

LABORATORY DIAGNOSIS

Diagnosis of ET is established by examination of peripheral blood, bone marrow aspiration, tests for hemostasis (prothrombin time, activated partial thromboplastin time, platelet aggregation tests), molecular genetic analysis of JAK2 (V617F), CALR exon 9, MPL mutations, and other tests, i.e. elevated levels of serum cobalamin, unsaturated cobalamin binding capacity, serum uric acid, lactic dehydrogenase and acid phosphatase.

Laboratory Diagnosis of Essential Thrombocythemia

Biochemical Investigations

Biochemical laboratory tests demonstrate elevated levels of serum cobalamin, unsaturated cobalamin binding capacity, serum uric acid, lactic dehydrogenase and acid phosphatase.

Hemostasis Tests

- Laboratory tests alone are not reliable in predicting bleeding and thrombotic manifestations in essential thrombocythemia.
- Prothrombin time–INR (PT–INR) and activated partial thromboplastin time (APTT) are most often within normal range.
- Platelet aggregation studies are abnormal with defective platelet aggregation with ADP, epinephrine, and collagen.
- Other platelet abnormalities in essential thrombocythemia include decreased or mutate MPL receptors for thrombopoietin, shortened platelet survival, increased plasma β-thromboglobulin, acquired storage pool deficiency due to abnormal *in vivo* platelet activation and decreased ATP secretion.

Peripheral Blood Smear Examination

Red blood cells

- Development of anemia depends on severity of hemorrhagic episodes. Severe hemorrhagic episodes result in microcytic hypochromic picture.
- Nucleated red blood cells are demonstrated in 25% of patients. In some patients, erythrocytosis is demonstrated. It should be differentiated from polycythemia vera.
- Aggregated platelets can lead to an erroneous increase in the erythrocyte count on automated cell counters.

White blood cells

- Leukocytosis is almost always present. WBC count is often in the range of **22×10^9/L to 40×10^9/L.** Occasional myelocyte and metamyelocyte can be demonstrated.
- Mild basophilia and eosinophilia may be present.
- Neutrophilic alkaline phosphatase (NAP) score is variable.

Platelets

- Most striking finding in the peripheral blood smear is the presence of extreme and constant thrombocytosis.
- Platelet counts are >450×10^9/L and often in the range of 1000×10^9/L to 5000×10^9/L. Giant bizarre platelets and aggregates of platelets can be demonstrated.
- Megakaryocytes and megakaryocyte fragments can also be observed. However, platelet morphology may appear normal in many patients.
- Abnormalities in the platelet aggregation and adhesion defects suggest defects in the platelet function. Platelet abnormalities demonstrated in essential thrombocythemia are given in Table 9.100.
- Essential thrombocythemia in Giemsa-stained peripheral blood smear is shown in Fig. 9.89.

Bone Marrow Smear Examination

- Bone marrow smear examination demonstrates marked cellular hyperplasia with a striking increase in number of functional megakaryocytes either dispersed throughout smear or arranged in clusters along the sinusoidal borders.
  - The background of stained bone marrow smears demonstrates numerous platelets.
  - Megakaryocytes are large sized with abundant mature cytoplasm and frequently increased lobulation of nuclei. Mitotic figures are increased.
- Erythroid and myeloid hyperplasia are evident.
- Significant bone marrow fibrosis is generally absent.

Bone Marrow Trephine Biopsy Examination

- Essential thrombocythemia in hematoxylin and eosin-stained bone marrow trephine biopsy section examination demonstrates marked bone marrow hyperplasia with a striking increase in number of functional megakaryocytes either dispersed throughout smear or arranged in clusters along the sinusoidal borders.

- The background of stained sections demonstrates numerous platelets. Megakaryocytes are large sized with abundant mature cytoplasm and frequently increased lobulation of nuclei. Mitotic figures are increased.
- Essential thrombocythemia in hematoxylin and eosin-stained bone marrow trephine biopsy section is shown in Fig. 9.90.

Molecular Genetic Alterations Analysis

- About 65% of patients demonstrate JAK2 (V617F) mutation associated with increased risk of thrombosis.
- Approximately 20–30% of patients demonstrate CALR exon 9 mutation associated with increased rate of bone marrow fibrosis.
- About 3–5% of patients demonstrate MPL exon 10 mutation associated with short survival rate.
- About 5–10% of patients with triple negative mutations, i.e. JAK2 (V617F), CALR exon 9, MPL have longest survival rate.

MANAGEMENT

Essential thrombocythemia is treated by administration of hydroxyurea to achieve cytoreduction of platelets and leukocytes. Decreasing platelet and leukocyte count lower the risk of thrombosis in these patients.

- Although anagrelide, an inhibitor of megakaryocytic differentiation can be administered to reduce platelets, yet not tolerated by the patients due to side effects.
- Therapeutic trials of interferon-β (IFN-β) demonstrate improvement in both hematologic parameters and clinical manifestations on nearly all patients.

DIFFERENTIAL DIAGNOSIS

Essential thrombocythemia (ET) is diagnosed by exclusion of other myeloproliferative neoplasms and secondary or reactive thrombocytosis associated with many acute and chronic infections, inflammatory diseases, Hodgkin's disease and carcinoma.

- Platelet count in essential thrombocythemia often exceeds 1000×10^9/L, that remains persistent over a period of months or years.

Table 9.100 Platelet abnormalities demonstrated in essential thrombocythemia

Decreased or mutated MPL receptors on platelets for thrombopoietin (TPO)
Life span of platelets shortened
Increased plasma β-thromboglobulin (β-TG)
Acquired urinary thromboxane B_2 (vasoconstrictor and platelet aggregating agent)
Defective epinephrine, collagen, ADP-induced platelet aggregation
Decreased ATP secretion
Development of acquired von Willebrand disease and storage pool deficiency

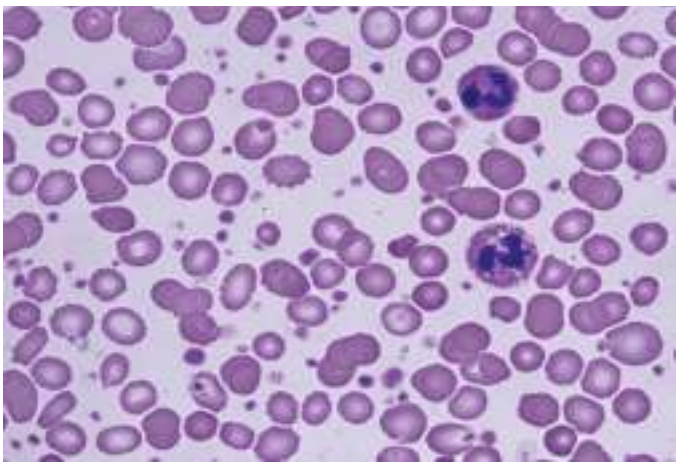


Fig. 9.89: Essential thrombocythemia in Giemsa-stained peripheral blood smear. Most striking finding in the peripheral blood is the presence of extreme and constant thrombocytosis. Platelet counts are $>450 \times 10^9$/L and often in the range of 1000×10^9/L to 5000×10^9/L. Giant bizarre platelets and aggregates of platelets can be demonstrated. Megakaryocytes and megakaryocyte fragments can also be observed. However, platelet morphology may appear normal in many patients. Abnormalities in the platelet aggregation and adhesion defects suggest defects in the platelet function (1000X).

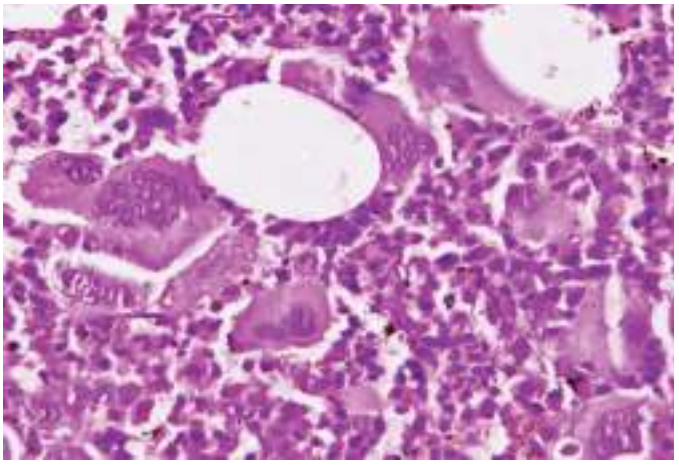


Fig. 9.90: Essential thrombocythemia in hematoxylin and eosin-stained bone marrow trephine biopsy section. It demonstrates marked hyperplasia with a striking increase in number of functional megakaryocytes either dispersed throughout smear or arranged in clusters along the sinusoidal borders. The background of stained smears demonstrates numerous platelets. Megakaryocytes are large sized with abundant mature cytoplasm and frequently increased lobulation of nuclei. Mitotic figures are increased (1000X).

- Platelet count in secondary or reactive thrombocytosis rarely reaches 1000×10^9/L, that is transitory. Platelet functions are normal in secondary or reactive thrombocytosis and absence of splenomegaly.
- Disorders associated with thrombocytosis are given in Table 9.101. Comparison of bone marrow morphologic findings in myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis

Table 9.101 Disorders associated with thrombocytosis

Essential thrombocythemia (ET)
Chronic myelogenous leukemia (CML)
Primary myelofibrosis (PML)
Secondary or reactive thrombocytosis ▪ Acute and chronic inflammatory conditions ▪ Hemolytic anemia ▪ Metastatic carcinoma ▪ Hodgkin disease ▪ Non-Hodgkin lymphoma ▪ Postsplenectomy ▪ Postoperative cases ▪ Iron deficiency anemia

(MDS/MPN with ring sideroblasts–thrombocytosis) and essential thrombocythemia is given in Table 9.102. Clinicomorphologic features in distinguishing essential thrombocythemia and secondary thrombocytosis are given in Table 9.103.

POLYCYTHEMIA VERA

Polycythemia vera (PV) is a myeloproliferative neoplasm characterized by unregulated proliferation of primarily, the erythroid lineage in the bone marrow and increased red blood cells in the peripheral blood. Blood that flows more slowly can reduce amount of oxygen to vital organs.

- Increased concentration of red blood cells can clump together and form clots inside the blood vessels and interrupt blood flow, that can cause cerebral stroke associated with fatal outcome.
- Polycythemia vera most often affects 40–60 years of age group. Males are more affected than females. Disease rarely affects children.
- Diagnostic criteria for polycythemia vera are given in Table 9.104. To clarify the pathogenesis of polycythemia vera, it is classified into three different groups: polycythemia vera, secondary thrombocytosis and relative thrombocytosis. Classification of polycythemia and associated conditions is given in Table 9.105. Comparison of polycythemia vera and other types of polycythemias is given in Table 9.106.

PATHOPHYSIOLOGY

Polycythemia vera (PV) is a clonal hematopoietic stem cell disorder characterized by increased

Table 9.102 Comparison of bone marrow morphologic findings in myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis (MDS/MPN with RS-T) and essential thrombocythemia

Bone Marrow Morphologic Findings	MDS/MPN with RS-T	Essential Thrombocythemia
Cellularity (age-matched)	Increased	Normal
Myeloid hyperplasia	Absent	Absent
Megakaryocyte proliferation	Present	Present
Megakaryocyte morphology	Hypolobated megakaryocyte	Staghorn hyperlobated nuclei
Megakaryocyte size	Small (micromegakaryocytes)	Large (macromegakaryocyte)
Megakaryocyte clusters	Absent	Present, arranged in loose clusters
Bone marrow fibrosis	MF-0 or MF-1	MF-0
Ring sideroblasts on iron stain	Present ≥15%	Absent

Table 9.103 Clinicomorphologic features in distinguishing essential thrombocythemia (ET) and secondary thrombocytosis

Parameters	Essential Thrombocythemia (ET)	Secondary Thrombocytosis
Platelet increase status	Persistent	Transient
History of thrombotic/bleeding episode/vasomotor symptoms	Present	Present (1–2%)
Hepatosplenomegaly	Present	Absent
History of infections/ inflammatory stimuli or nonmyeloid malignancy	Absent	Present
Increased phase reactants (e.g. CRP)	Absent	Present
Bone marrow megakaryocyte	Large megakaryocytes including staghorn appearance and showing clustering	Megakaryocytes with normal morphology or increased in size without clustering
Clonal molecular abnormality	Usually present [JAK2 (V617F), MPLW515K/L, or CALR mutations]	Absent

Table 9.104 Diagnostic criteria for polycythemia vera require two major criteria (1 and 2); and one minor criterion; or one major criterion and two minor criteria

Major Diagnostic Criteria
Absolute erythrocytosis in the range of 6×10^{12}/L to 10×10^{12}/L, hemoglobin >18.5 g/dl in males, 16.5 g/dl in females, or other evidence of increased red blood cells volume hematocrit >52% in males and >48% in females
Presence of JAK2 (V617F) or other functionally similar mutations, e.g. JAK2 exon 12 mutation
Minor Diagnostic Criteria
Bone marrow trephine biopsy shows panmyelosis with proliferation of myeloid elements (erythroid, myeloid and megakaryocytic cells)
Subnormal serum erythropoietin levels below the level of <4 mU/ml
Demonstration of endogenous erythroid colony formation *in vitro*

erythropoiesis and other cell lineages in bone marrow (erythropoietin level within normal range) and increased red blood cells in blood circulation. Polycythemia vera is an erythropoietin-independent unrestricted proliferation of erythroid lineage cells, which are hypersensitive to erythropoietin, insulin-like growth factor 1 and IL-3. Erythroid lineage cell escapes apoptosis. The maturation of erythroid lineage cells is morphologically normal, and the functions of red blood cells have normal life span.

JAK2 (V617F) Gene Mutation

JAK2 (V617F) is located on chromosome band 9p24, which encodes JAK2 protein. JAK2 protein is a

Table 9.105 Classification of polycythemia and associated conditions

Polycythemia Vera (Primary Polycythemia)—Erythropoietin-independent
Normal or increased erythropoietin levels
Autonomous proliferation of erythroid lineage
Secondary Polycythemia (Erythropoietin Driven)
High altitude
Chronic obstructive pulmonary disease
Congenital heart disease
Heavy tobacco smokers
Pickwickian syndrome (clinically known as obesity hypoventilation syndrome)
Familial erythrocytosis
Hemoglobin with high oxygen affinity
Congenital decrease in erythrocyte 2,3-bisphosphoglycerate (2,3-BPG)
Inappropriate increased erythropoietin production in various disorders: ▪ Renal cell carcinoma ▪ Hepatocellular carcinoma ▪ Cerebellar hemangioma ▪ Pheochromocytoma ▪ Adrenal adenoma with Cushing's syndrome ▪ Uterine leiomyoma ▪ Adult polycystic disease of kidney ▪ Administration of androgens ▪ Renal ischemia
Relative Polycythemia
Gaisbock's syndrome (stress polycythemia, spurious polycythemia, pseudopolycythemia)
Dehydration is present

Table 9.106 Comparison of polycythemia vera and other types of polycythemias

Parameters	Polycythemia Vera	Secondary and Relative Polycythemias
Total blood volume	Increased	Normal or increased
Total leukocyte count	Increased	Normal
Immature red blood cells	Occasional	None
Platelets	Increased	Normal
NAP score	Increased	Normal
Erythrocyte sedimentation rate (ESR)	Decreased	Normal
Serum iron	Decreased	Normal or increased
Erythropoietin	Decreased or absent	Normal or increased
Blood histamine	Increased	Normal
Unsaturated vitamin B_{12}-binding capacity	Increased	Normal
Basophil count	Increased	Normal
Hyperuricemia	Present or absent	Normal
Hyperuricosuria	Present or absent	Normal
Chromosome studies	Abnormal >90% JAK2 (V617F) positive	Negative

cytoplasmic protein, i.e. receptor tyrosine kinase closely associated with cytokine receptors and distributed near the cell membrane. When a cytokine receptor binds cytokine, JAK2 protein gets transphosphorylated and activated. In turn, JAK2 phosphorylates signal transducers and activators of transcription STAT5 proteins. JAK2 /STAT5 activation pathways are suppressed by negative regulators.

- Gain-of-function mutation in the JAK2 (V617F) receptor tyrosine kinase gene is demonstrated in 95–100% of polycythemia vera patients. JAK2 (V617F) mutation results in autonomous cell signal transduction leading to excessive proliferation of erythroid lineage cells.
- In addition, about 5% of patients carry other mutations in exon 12 of JAK2 that disrupts normal JAK2 function. It has been proposed that JAK2 (V617F) mutation triggers ET phenotype. Secondly, when a second JAK2 (V617F) mutation occurs, JAK2 protein triggers the erythropoietin (Epo) receptors converting the disease phenotype to polycythemia vera.
- JAK2 (V617F) mutation is demonstrated in polycythemia vera (95–100%), essential thrombocythemia (50%) and primary myelofibrosis (50%).

TET2 Gene Mutation

Ten-eleven translocation 2 (TET2) mutation is also demonstrated in some cases of polycythemia vera, that is not a disease initiating event, rather accelerating erythropoiesis and contributing to development of polycythemia vera.

MPL, LNK and BCL-X Gene Mutations

In the absence of mutations in JAK2 (V617F) gene, other mutations in genes coding for aberrant MPL, LNK (lymphocyte adaptor protein) and antiapoptotic protein members of the BCL-2 family (BCL-X) disrupt the normal JAK/STAT signaling pathway resulting in proliferation of clone in polycythemia vera. The defect in apoptosis creates accumulation of altered hypersensitive hematopoietic stem cell and erythroid lineage cells. Thrombopoietin receptor hyperresponsiveness and resistance to programmed cell death (apoptosis) are demonstrated in megakaryocytic lineage, resulting in thrombocytosis in polycythemia vera.

Epigenetic Alterations

Gene encodes isocitrate dehydrogenase 1 (IDH-1), that functions in citric acid cycle and converts isocitrate to α-ketoglycerate with the associated production of NADPH. Somatic mutation in the gene for the isocitrate dehydrogenase 1 manifests epigenetic modifications of citrate cycle, that contributes to development of polycythemia vera.

CLINICAL FEATURES

Polycythemia vera has been reported in the several members of the same family suggesting a familial predisposition can exist. It is gradual onset with history of mild symptoms for several years. Symptoms are related to increase in red blood cell mass and cardiovascular diseases due to hyperviscosity of blood.

- Patient presents with headache, weakness, pruritus, dizziness, diaphoresis, visual disturbances, weight loss, paresthesia, dyspnea, joint pain and epigastric discomfort.
- Clinical examination reveals splenomegaly due to expansion of red pulp and obliteration of white pulp, skin plethora (excess of blood and marked by turgescence and reddish complexion), conjunctival plethora (excess of blood and marked by turgescence and reddish complexion), retinal blood vessels engorged, hepatomegaly and hypertension.
- About 33% of the patients' experience hemorrhagic or thrombotic phenomenon linked to myocardial infarction, retinal vein thrombosis, thrombophlebitis, and cerebral stroke.
- After a span of 2–10 years, patient may develop bone marrow failure accompanied by moderate increase in spleen size, bleeding, and anemia.
- Acute myelogenous leukemia (AML) develops as an abrupt transition in 5–10% of patients due to administration of myelosuppressive drugs.
- Overall median survival of polycythemia vera exceeds 10–20 years. Clinical features and physical examination findings in polycythemia vera are given in Table 9.107.

Laboratory Diagnosis of Polycythemia Vera

Laboratory investigations of polycythemia vera (PV) include peripheral blood examination, bone marrow examination and bone marrow trephine biopsy.

Erythrocyte Sedimentation Rate

Erythrocyte sedimentation rate does not exceed 2–3 mm/hour.

Peripheral Blood Smear Examination

Peripheral blood smear examination in polycythemia vera shows following findings in RBCs, WBCs and platelets.

Red blood cells (RBCs)

- Most striking in the peripheral blood findings is an absolute erythrocytosis in the range of 6×10^{12}/L to 10×10^{12}/L, hemoglobin >18.5 g/dl in males, 16.5 g/dl in females, or other evidence of increased red blood cells volume, and hematocrit >52% in males and >48% in females.
- Absolute erythrocytosis is in the range of 6×10^{12}/L to 10×10^{12}/L, hemoglobin >18.5 g/dl in males and 16.5 g/dl in females, or other evidence of increased red blood cells volume, and hematocrit >49% in males and >48% in females.

- In early polycythemia vera disease, peripheral blood smear shows normocytic normochromic picture.
  - Repeated therapeutic phlebotomy, iron deficient erythropoiesis can result in iron deficiency anemia simulating a thalassemia.
  - Nucleated red blood cells may be demonstrated.
  - Red blood cells appear crowded even at the featured edge of peripheral blood smear.
- In advanced polycythemia vera disease, peripheral blood smear demonstrates thrombocytosis and large platelets, reactive lymphocytes and segmented neutrophils. Peripheral blood smear also shows leukoerythroblastic anemia, poikilocytosis, dacrocytes and thrombocytopenia resembling findings like myelofibrosis. In polycythemia vera, patients develop acute myelogenous leukemia. Peripheral blood smear examination shows numerous myeloblasts, marked anisopoikilocytosis of red blood cells and thrombocytopenia.

White blood cells

- Leukocytosis occurs in the range of 12×10^9/L to 20×10^9/L in 66% of patients.
- In the early polycythemia vera disease, there can be relative increase in granulocytes with normal white blood cell count.
- A shift to the left (presence of myelocytes and metamyelocytes) can be demonstrated. Demonstration of myeloblasts and promyelocytes is unusual.
- Relative and absolute basophilia is commonly demonstrated.

Platelets

- Megakaryocytic hyperplasia in the bone marrow is accompanied by increase in platelet count in peripheral blood in 50% of cases.
- Platelet count $>400 \times 10^9$/L is demonstrated in 20% of patients. In occasional case, platelet count occasionally exceeds 100×10^9/L. Giant platelets can be present.
- Qualitative abnormalities are reflected by abnormal platelet aggregation to epinephrine (most common), collagen, ADP or thrombin.
- Abnormal multimeric forms of von Willebrand factor (vWF) are demonstrated in 50% of patients, which acquire von Willebrand disease (vWD).

Reticulocyte Count

Reticulocyte count is normal or slightly elevated.

Neutrophil Alkaline Phosphatase (NAP) Score

Neutrophil alkaline phosphatase score is most often higher than 100.

Bone Marrow Smear Examination

- Bone marrow is moderate to marked hypercellular in majority of patients.
- Myeloid and erythroid hyperplasia are most often present.
- Myeloid to erythroid ratio is normal.
- Megakaryocytic hyperplasia is present.
- Megakaryocytes are large, but not bizarre arranged in clusters.
- Iron is absent in 95% of cases.
- Bone marrow fibrosis is absent in initial polycythemia vera disease.

Bone Marrow Trephine Biopsy Examination

Polycythemia vera, so-called masked/proliferation presentation stage

- In polycythemia vera, so-called masked/proliferation presentation stage, bone marrow trephine biopsy section shows mildly hypercellular bone marrow showing predominance of large and giant hypersegmented megakaryocytes.
- There is slight to marked increase in reticulin fibers directly proportion to the degree of bone marrow cellularity.
- Iron stores are depleted due to diversion of iron from storage sites to the numerous developing erythroblasts.

Postpolycythemic myelofibrosis overt stage

- In the postpolycythemic myelofibrosis overt stage, bone marrow trephine biopsy section shows reticulin and collagen fibrosis and shows prominent megakaryocytes arranged in clusters. There is decrease in erythropoiesis and granulopoiesis with shift to the left. Less than 10% blasts are present. Dysplasia is unusual.
- Megakaryocytes revealing different sizes are always prominent feature, which are more easily evaluated by using the periodic acid–Schiff (PAS) staining reaction.
- Immunostaining for CD61 demonstrates the atypia in the megakaryocytic population, including a population of small immature megakaryocytes. Overt reticulin fibrosis that is invariably present in postpolycythemic vera myelofibrosis with myeloid metaplasia in a span of 2–10 years.
- Trilineage proliferation is demonstrated by the naphthol-ASD-chloroacetate (CAE) stain, which is accurately identifying the granulocytic cells (red reaction product) assists in the assessment of the relative proportion of the major bone marrow lineages (erythropoietic and granulopoietic). At this stage megakaryocytes show increased pleomorphism, with significant differences in size but no relevant maturation defects such as cytoplasmic or nuclear differentiation.

PRIMARY MYELOFIBROSIS

Primary myelofibrosis (PMF) is a clonal hematopoietic stem cell (HSC) disorder characterized by proliferation of predominantly megakaryocytes and granulocytes in the bone marrow, which in fully developed disease is associated with gradual increase in reactive connective tissue (i.e. reticulin and collagen fibers) in bone marrow secondary to underlying disorder, and extensive extramedullary hematopoiesis in spleen (splenomegaly), liver and lymph nodes. PMF shows a stepwise evolution from an initial prefibrotic/early stage to overt fibrotic stage, which most often affects persons over 50 years of age with equal sex predilection and gradual onset disease.

- Primary myelofibrosis presents either as *de novo* or as an evolutionary consequence of essential thrombocythemia or polycythemia vera.

Table 9.107 Clinical presentation and physical examination findings and percentage in polycythemia vera (PV)

Clinical Features	
Headache	48%
Weakness	48%
Pruritus	43%
Dizziness	43%
Diaphoresis (sweating to an unusual degree)	33%
Visual disturbances	31%
Weight loss	29%
Paresthesia	29%
Dyspnea	26%
Joint symptoms	26%
Epigastric discomfort	24%
Physical Examination	
Splenomegaly	70%
Skin plethora (excess of blood and marked by turgescence and reddish complexion)	67%
Conjunctival plethora (excess of blood and marked by turgescence and reddish complexion)	60%
Retinal blood vessels engorged	46%
Hepatomegaly	40%
Systolic blood pressure >140 mm Hg	72%
Diastolic blood pressure >90 mm Hg	32%

- Extensive primary myelofibrosis inhibits normal hematopoiesis resulting in bone marrow hypoplasia and extensive extramedullary hematopoiesis in liver (hepatomegally), spleen (splenomegaly) and sometimes in lymph nodes (lymphadenopathy) as a result of islands of proliferating erythroid, myeloid and megakaryocytic lineage cells.
- Synonyms of primary myelofibrosis include agnogenic myeloid metaplasia, leukoerythroblastic anemia, myelofibrosis with myeloid metaplasia, chronic idiopathic myelofibrosis, aleukemic myelosis, myelosclerosis, and splenomegaly myelophthisic.

PATHOPHYSIOLOGY

Primary myelofibrosis (PMF) evolves from prefibrotic stage with minimal reticulin fibrosis to overt fibrotic stage with marked reticulin or collagen fibrosis of grades 2 and 3.

- Primary myelofibrosis is a reactive process secondary to clonal proliferation of hematopoietic stem cell (HSC) having chromosomal abnormalities. Fibroblasts do not demonstrate chromosomal abnormalities.
- Myelofibrotic component is composed of excessive collagen, fibroblasts, cytoadhesive molecules (fibronectin and vitronectin) and laminin (glycoprotein that supports adhesion and growth of cells).

Cytokines Role in Pathogenesis of Primary Myelofibrosis

Monoclonal megakaryocytes play an important role in the pathogenesis of primary myelofibrosis.

- Stromal reaction (fibroblasts proliferation, collagen deposition, necrosis) is demonstrated in the areas of megakaryocytes.
  - Stromal reaction is mediated by cytokines such as platelet-derived growth factor (**PDGF**), transforming growth factor-β (**TGF-β**) and epidermal growth factor (EGF) derived from platelets.
  - Reduced platelet concentration of PDGF and elevated serum PDGF are characteristics of primary myelofibrosis due to abnormal release. TGF-β stimulates increased expression of genes for collagen and fibronectin synthesis; and inhibits synthesis of collagenase enzymes. Thus, the net result is the accumulation of stromal elements in the bone marrow.
- Prominent angiogenesis in the bone marrow and spleen occurs due to increased serum levels of vascular endothelial growth factor (VEGF).
- Teardrop red blood cells and leukoerythroblastosis in peripheral blood are indicative of extramedullary hematopoiesis.

JAK2 (V617F) Gene Mutation

JAK2 (V617F) gene is located on chromosome band 9p24, that encodes JAK2 protein. JAK2 is a cytoplasmic protein, i.e. receptor tyrosine kinase closely associated with cytokine receptors and distributed near the cell membrane. When a receptor tyrosine kinase binds cytokine, the JAK2 protein gets transphosphorylated and activated. In turn, JAK2 phosphorylates signal transducers and activators of transcription STAT5 proteins. JAK2/STAT5 activation pathways are suppressed by negative regulators.

- Gain-of-function mutation in the JAK2 (V617F) receptor tyrosine kinase gene results in autonomous cell signal transduction leading to unrestricted proliferation of erythroid lineage cells. In addition, about 5% of patients carry other mutations in exon 12 of JAK2 that disrupts normal JAK2 function.
- Gain-of-function mutation in JAK2 (V617F) gene is also demonstrated in about 95–100% of polycythemia vera patients.
- JAK2 (V617F) allele burden has been associated with transformation and progression of primary myelofibrosis and increased cardiovascular symptoms.

Table 9.108 Clinical findings at diagnosis among patients with primary myelofibrosis (PMF)

Clinical Findings (>50% Cases)	Clinical Findings (10–50% Cases)	Clinical Findings (<10% Cases)
Splenomegaly (90%)	Weight loss	Jaundice
Hepatomegaly (50%)	Night sweats	Lymphadenopathy
Weakness	Bleeding manifestations	Peripheral edema
Anemia	Pain in the left hypochondrial region at splenic site	Gout
Leukocytosis	Leukocytopenia	
Thrombocytosis	Thrombocytosis or thrombocytopenia	

CLINICAL FEATURES

Patient with primary myelofibrosis presents with anemia, splenomegaly (90%) and hepatomegaly (50%), weakness, weight loss, anorexia, night sweats, low-grade fever, pruritus, bone pain and pain in extremities, Bleeding occasionally may be the presenting symptoms. Leukocytosis or marked thrombocytosis is common in primary myelofibrosis. Marked thrombocytosis may lead to thrombosis or hemorrhagic phenomenon.

- Clinical examination reveals splenomegaly (90%), hepatomegaly (50%), pallor and petechiae.
- Myeloid metaplasia occurs in spleen, liver, kidneys, adrenal glands, lymph nodes, peritoneal and extra-peritoneal surfaces and spinal cord. Clinical findings at diagnosis among patients with primary myelofibrosis (PMF) are given in Table 9.108.

Table 9.109 Revised 2024 WHO diagnostic criteria for prefibrotic/early primary myelofibrosis

Diagnostic criteria of primary myelofibrosis require all major criteria and at least one minor criterion
Major Diagnostic Criteria (must Fulfill all Four Criteria)
Bone marrow is hypercellular showing megakaryocyte proliferation and atypia, increased age-adjusted bone marrow cellularity, granulocytic proliferation
Bone marrow without evidence of reticulin fibrosis
Not meeting the criteria for other myeloproliferative neoplasms (MPNs), i.e. CML, polycythemia vera and essential thrombocythemia
Evidence of JAK2 (V617F), CALR, or MPL mutations; or in the absence of these mutations, presence of clonal marker or absence of minor reactive reticulin fibrosis
Minor Diagnostic Criteria (must Fulfill at least One of the Criteria)
Leukocytosis ≥11 × 10^9/L
Anemia
Palpable splenomegaly (infrequent)
Increased serum lactic dehydrogenase (LDH) above reference range

- Atypical acute form of primary myelofibrosis progresses rapidly within few months to one year is associated with development of anemia and leukopenia. Bone marrow trephine biopsy demonstrates proliferation of reticulin and collagen fibers.
- Patients with systemic lupus erythematosus (SLE) have cytopenia like primary myelofibrosis, who can develop autoimmune myelofibrosis but without splenomegaly. It is essential to perform antinuclear antibody (ANA) test to rule out systemic lupus erythematosus.
- Revised 2024 WHO diagnostic criteria for prefibrotic/early primary myelofibrosis and overt primary myelofibrosis are given in Tables 9.109 and 9.110.

Table 9.110 Revised 2024 WHO diagnostic criteria for overt primary myelofibrosis (PMF)

Diagnostic criteria of primary myelofibrosis require all major criteria and at least one minor criterion
Major Diagnostic Criteria (must Fulfill all Four Criteria)
Presence of megakaryocyte proliferation with abnormal morphology (atypia), reticulin and/or collagen bone marrow fibrosis >1 grade, accompanied by increased age adjusted bone marrow cellularity, granulocytic differentiation and often decreased erythropoiesis.
Bone Marrow Fibrosis Grade ≥MF2 (Grades 2 and 3)
Not meeting the criteria for other myeloproliferative neoplasms (MPNs), i.e. CML, polycythemia vera and essential thrombocythemia
Evidence of JAK2 (V617F), CALR, or MPL mutations; or in the absence of these mutations, presence of clonal marker or absence of minor reactive reticulin fibrosis
Minor Diagnostic Criteria (must Fulfill at least One of the Criteria)
Leukocytosis ≥11 × 10^9/L
Anemia
Palpable splenomegaly ≥5 cm
Increased serum lactic dehydrogenase (LDH) above reference range

Adapted from revised 2024 World Health Organization (WHO) classification of hematopoietic neoplasms.

Laboratory Diagnosis of Primary Myelofibrosis

Various laboratory tests performed in establishing diagnosis of primary myelofibrosis include peripheral blood smear, bone marrow, molecular genetics and elevated serum uric acid lactic dehydrogenase (LDH). Serum cobalamin level can be elevated or within normal range.

Peripheral Blood Smear Examination

- Peripheral blood smear findings in primary myelofibrosis demonstrate both qualitative and quantitative cellular abnormalities. Primary myelosclerosis in Giemsa-stained peripheral blood smear is shown in Fig. 9.91.
- Anemia, leukocytosis with shift to the left and thrombocytosis are demonstrated in the initial stage at diagnosis.
- At a later stage of disease, pancytopenia and leukoerythroblastosis with anisocytosis and poikilocytosis are seen. In 35–55% of patients, hemoglobin is <10 g/dl.

Red blood cells

- Peripheral blood smear shows normocytic normochromic. Utilization of folic acid by neoplastic clone results in macrocytic anemia.
- Anemia correlates directly with the extent of bone marrow fibrosis and the effectiveness of extramedullary hematopoiesis.
- Anemia becomes more severe with the progression of the disease due to sequestration of red blood cells and expanded blood volume.
- In primary myelofibrosis, most important abnormality in red blood cells is presence of teardrop cells (dacrocytes). However, ovalocytes (elliptocytes) may be demonstrated.
- Numerous or few normoblasts are seen. Basophilic stippling is commonly demonstrated.
- Reticulocyte count is increased to 2–15%.

White blood cells

- In the initial stage of disease, total leukocyte is usually elevated ranging from 15×10^9/L to 30×10^9/L. There is presence of immature granulocytes with <5% blasts.
- Other findings demonstrated in peripheral blood smear include basophilia, eosinophilia and pseudo-Pelger-Hüet anomaly. Philadelphia chromosome is not demonstrated.

Platelets

- In the initial stage of disease, platelet count may be high or normal or low. Thrombocytopenia occurs as a result of sequestration of platelets in enlarged spleen.
- The platelets demonstrate dysplastic change, giant and bizarre forms with hypogranularity.
- Circulating megakaryocyte fragments, naked megakaryocytes and mononuclear micromegakaryocytes are also demonstrated.
- Micromegakaryocytes should be differentiated from small lymphocytes by differentiating features such as demarcation in membranes, cytoplasmic blebbing with bull's eye granules in the cytoplasm.
- Qualitative platelet abnormalities include abnormal platelet aggregation, adhesion and defective procoagulant activity on exposure to collagen.

Bone Marrow Smear Examination

The bone marrow aspiration is usually difficult, hence 'dry tap' obtained. In case, bone marrow is successful, bone marrow smears do not demonstrate abnormality. Hence, bone marrow trephine biopsy is essential to demonstrate myelofibrosis and establish diagnosis.

Bone Marrow Trephine Biopsy Examination

- Bone marrow trephine biopsy section is hypercellular with varying degrees of diffuse fibrosis and focal aggregates of megakaryocytes.
- Three histologic patterns of bone marrow trephine biopsy have been described:
  - Bone marrow trephine biopsy demonstrates panhyperplasia with absence of myelofibrosis but a slight increase in connective tissue reticulin. It shows myeloid atrophy with fibrosis with prominent collagen and reticulin fibers and <30% cellularity.
  - Bone marrow demonstrates myelofibrosis and myelosclerosis with bony trabeculae occupying 30% of the bone marrow biopsy with extensive fibrosis.
- Primary myelofibrosis (PMF) in hematoxylin and eosin-stained bone marrow trephine biopsy is shown in Fig. 9.92. Primary myelofibrosis in reticulin stained bone marrow trephine biopsy section is shown in Fig. 9.93. Comparison of bone marrow morphologic findings in prefibrotic primary myelofibrosis and essential thrombocytosis/thrombocythemia is given in Table 9.111. World Health Organization grading of primary myelofibrosis is given in Table 9.112. Diagnostic findings in primary myelofibrosis are given in Table 9.113.

Molecular Genetic Alterations

- Molecular genetic analysis is essential to differentiate primary myelofibrosis from chronic myelogenous leukemia (positive Philadelphia chromosome), as both demonstrate some degree of fibrosis. Philadelphia chromosome is not demonstrated in myelofibrosis.
- JAK2 (V617F) mutation is demonstrated in 50% of patients.
- Trisomy or deletion of chromosome 6–12 abnormalities are associated with myelofibrosis. Complete or partial loss of chromosomes 5, 7 and 20 is associated with primary myelofibrosis treated with chemotherapy.

Clinical Course

- About 15% of patients can develop major intravascular hemolytic episodes during disease with hemosiderinuria and decreased haptoglobin.
- Hypersplenism, paroxysmal nocturnal hemoglobinuria-like defective red blood cells and anti-red blood cell antibodies contribute to intravascular hemolysis.
- Combination of thrombocytopenia and platelet abnormality result in bleeding diathesis ranging from petechiae and ecchymosis.
- Defective platelet aggregation is a common finding.Serum uric acid and lactic dehydrogenase are elevated in these patients.

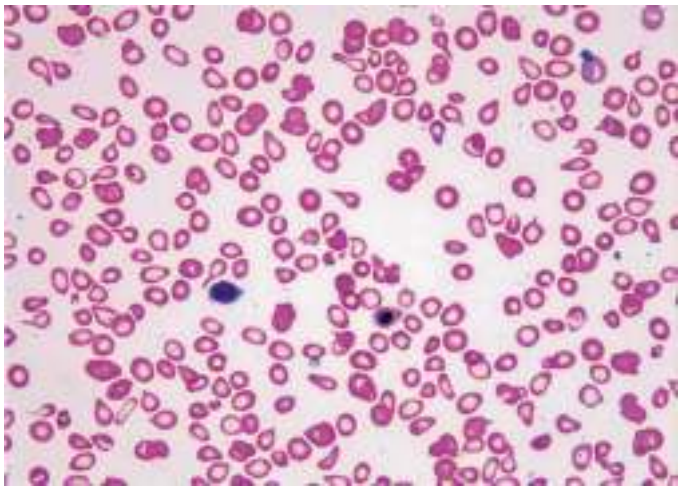


Fig. 9.91: Primary myelosclerosis in Giemsa-stained peripheral blood smear. It shows many teardrop cells (dacrocytes) and normoblasts. Basophilic stippling is commonly demonstrated. Reticulocyte count is increased to 2–15%. The majority of patients have absolute reticulocyte count >60 × 10^9/L. Combination of thrombocytopenia and platelet abnormality results in bleeding diathesis ranging from petechiae and ecchymosis. Defective platelet aggregation is a common finding. Anemia becomes more severe with the progression of the disease due to sequestration of red blood cells and expanded blood volume. Anemia correlates directly with the extent of bone marrow fibrosis and the effectiveness of extramedullary hematopoiesis (1000X).

PROGNOSIS AND TREATMENT

The average survival rate after the diagnosis of primary myelofibrosis is 4–5 years. The main causes of fatal outcome include recurrent infections, hemorrhage, thrombosis and cardiac failure. About 10–15% of patients with primary myelofibrosis can develop acute myelogenous leukemia (**AML**) in majority of patients and acute lymphoblastic leukemia (**ALL**) in some cases.

- Purpose of therapy in primary myelofibrosis is to improve cytopenia and reduce splenomegaly. Recombinant erythropoietin, androgens and corticosteroids are administered to stimulate hematopoiesis to treat anemia.

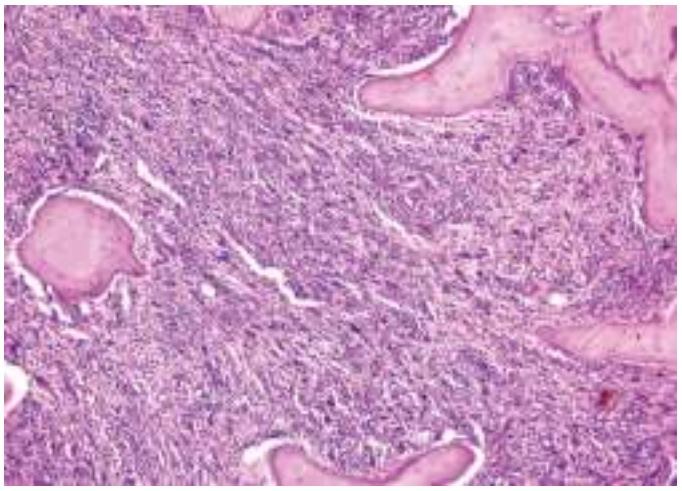


Fig. 9.92: Primary myelofibrosis (PMF) in hematoxylin and eosin-stained bone marrow trephine biopsy. Bone marrow trephine biopsy section is hypercellular with varying degrees of diffuse fibrosis and focal aggregates of megakaryocytes (400X).

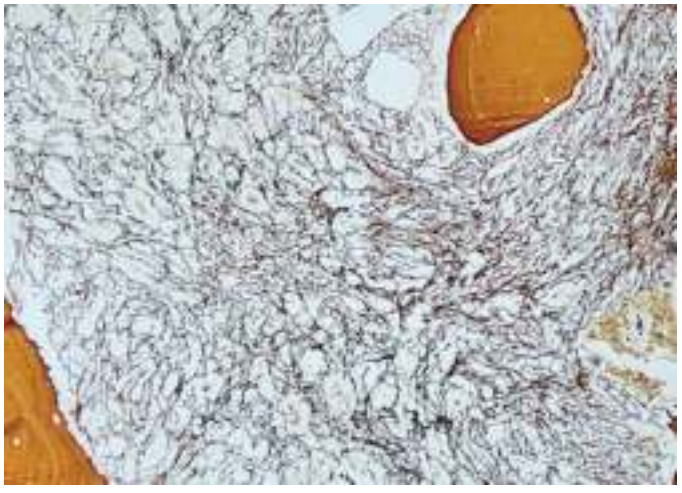


Fig. 9.93: Primary myelofibrosis (PMF) in reticulin stained bone marrow trephine biopsy section. The bone marrow aspiration is usually difficult, hence 'dry tap' obtained. In case, bone marrow is successful, bone marrow smears do not demonstrate abnormality. Hence, trephine bone marrow biopsy is essential to demonstrate myelofibrosis and establish diagnosis (1000X).

Table 9.111 Comparison of bone marrow morphologic findings in prefibrotic primary myelofibrosis and essential thrombocytosis/thrombocythemia (ET)

Bone Marrow Morphologic Features	Early/Prefibrotic Myelofibrosis	Essential Thrombocythemia
Bone marrow cellularity (age matched)	Increased	Normal
Myeloid hyperplasia	Present	Present
Megakaryocyte morphology	Present	Present
Megakaryocyte size	Bulbous, cloud-like, naked eye	Staghorn hyperlobated nuclei
Megakaryocyte clusters	Small and large	Large
Myelofibrosis (MF)	MF-0, MF-1	MF-0

Table 9.112 Diagnostic findings in primary myelofibrosis

Parameters	Clinical and Laboratory Findings
Primary myelofibrosis (PMF) prefibrotic stage	
Peripheral blood smear findings	▪ Anemia absent or mild ▪ Leukocytosis absent or slight ▪ Thrombocythemia invariable ▪ Absent or slight anisopoikilocytosis including teardrop red blood cells
Bone marrow	▪ Cellular bone marrow with mild increase in granulopoiesis, increased clustered dysmorphic megakaryocytes ▪ Slight increase in reticular fibers on silver stain
Cytogenetic and molecular alterations	▪ BCR-ABL1 fusion gene absent ▪ Presence of JAK2, CALR, or MPL mutations indicative of diagnosis MDS, one of these gene mutations is demonstrated in 90% of cases
Palpable splenomegaly	▪ Infrequent
Primary myelofibrosis (PMF) fully developed fibrotic stage	
Peripheral blood smear	▪ Anisopoikilocytosis with teardrop (dacrocytes) red blood cells in virtually every oil immersion field ▪ Nucleated red blood cells ▪ Increased CD34+ cells
Bone marrow	▪ Bone marrow reticulin fibrosis plus or minus collagen fibrosis present ▪ Bone marrow hypercellular but invariably has increased megakaryocytes, clusters of highly dysmorphic megakaryocytes, and megakaryocytes bare nuclei regardless of overall bone marrow cellularity
Cytogenetic and molecular alterations	▪ BCR-ABL1 fusion gene absent ▪ Presence of JAK2, CALR, or MPL mutations indicative of diagnosis MDS, one of these gene mutations is demonstrated in 90% of cases
Abdominal finding	Frequent palpable splenomegaly

Table 9.113 World Health Organization grading of myelofibrosis

WHO Grade of Myelofibrosis	Description
Myelofibrosis 0 (MF-0)	Scattered linear reticulum with no intersections (crossovers) corresponding to normal bone marrow
Myelofibrosis 1 (MF-1)	Loose network of reticulin with many interactions, especially in perivascular areas
Myelofibrosis 2 (MF-2)	Diffuse and dense increase in reticulin with extensive intersections, occasionally with focal bundles of collagen and /or focal osteosclerosis
Myelofibrosis 3 (MF-3)	Diffuse and dense reticulin with extensive interactions and collagen, often associated with osteosclerosis

Fiber density should be assessed in hematopoietic (cellular) areas.
In grades MF-2 and MF-3, an additional Masson trichrome stain is recommended.

- The patients may require frequent blood transfusion. Splenectomy is indicated in uncontrolled anemia.
- Irradiation helps in the reduction in the size of enlarged spleen and decrease red blood cells destruction.
- Chemotherapeutic agents such as hydroxyurea, thalidomide, lenalidomide and 2-chlorodeoxyadenosine (2-CdA or Leustatin) can be administered to reduce size of enlarged spleen.
- JAK protein receptor tyrosine kinase inhibitors can be administered in patients with advanced myelofibrosis, however, with variable results. Poor prognostic features in primary myelofibrosis are given in Table 9.114.

DIFFERENTIAL DIAGNOSIS

Differentiation of primary myelofibrosis from other conditions associated with myelofibrosis is most important to ensure appropriate therapeutic treatment. Disorders associated with bone marrow fibrosis are given in Table 9.115.

- Both primary myelofibrosis and chronic myelogenous leukemia (CML) demonstrate significant findings such as splenomegaly, anemia and leukoerythroblastic blood picture. Total leukocyte count in primary myelofibrosis is $<50\times10^9$/L and $>100\times10^9$/L (median of 170×10^9/L) in chronic myelogenous leukemia.

Table 9.114 Poor prognostic features in primary myelofibrosis

Hb <10 g/dl
WBC <4 or >30 × 10^9/L
Bone marrow chromosomal abnormalities
Advanced patient age
Raised number of CD34+ cells in the blood

Table 9.115 Disorders associated with bone marrow fibrosis

Primary myelofibrosis (PMF)
Atypical chronic myelogenous leukemia (CML)
Polycythemia vera (PV)
Essential thrombocythemia
Hairy cell leukemia
Acute megakaryocytic leukemia
Hodgkin disease
Non-Hodgkin lymphoma
Metastatic carcinoma
Miliary tuberculosis
Granulomatous diseases
Fungal infections
Radiation
Chemical agents

- Primary myelofibrosis demonstrates prominent poikilocytosis and less shift to the left in blood and bone marrow fibrosis and numerous megakaryocytes.
- Chronic myelogenous leukemia demonstrates some bone marrow fibrosis and prominent myeloid metaplasia. Differentiating features of primary myelofibrosis and chronic myelogenous leukemia (CML), BCR-ABL1 positive are given in Table 9.116.
- It is difficult to differentiate primary myelofibrosis from polycythemia vera. Bone marrow trephine biopsy aids in establishing diagnosis of primary myelofibrosis by demonstration of bone marrow fibrosis.

CLONAL HYPEREOSINOPHILIA

Eosinophils are derived from pluripotent hematopoietic stem cell (HSC) and lineage-specific committed hematopoietic progenitor cells in the bone marrow, which generate granulocytes, erythroblasts, megakaryocytes and macrophages.

- The production of eosinophils is tightly regulated by a network of transcription factors (GATA-1, PU.1, c/EBPs), growth factors and growth-inhibitory cytokines. These growth regulators are produced and secreted by activated T cells, mast cells and eosinophils.
- Major growth factors for eosinophils are GM-CSF, IL-3 and IL-5. IL-5 cytokine is relatively specific for eosinophils. The mobilization of eosinophils from the bone marrow into the blood is regulated by IL-5 and eotaxin.
- The eosinophils are released into the peripheral blood and rapidly migrate to tissues to perform physiologic functions.

Table 9.116 Differentiating features of primary myelofibrosis and chronic myelogenous leukemia (CML), BCR-ABL1 positive

Parameters	Primary Myelofibrosis	Chronic myelogenous leukemia (CML), BCR-ABL1 Positive
Splenomegaly	Present	Present
Anemia	Present	Present
Leukoerythroblastic picture	Present	Present
Total leukocyte count	<50 × 10^9/L	>100 × 10^9/L (median of 170 × 10^9/L)
Granulocyte left shift	Less pronounced	More pronounced
Poikilocytosis	More pronounced	Less pronounced
Bone marrow	Bone marrow fibrosis more pronounced with numerous megakaryocytes	Bone marrow fibrosis less pronounced with marked myeloid hyperplasia
Serum cobalamin level	Less elevated	More elevated
Neutrophil alkaline phosphatase (NAP)	Variable	More elevated
Philadelphia chromosome	Absent	Present

- In clonal hypereosinophilia, the eosinophils infiltrate and cause potential damage to the organs resulting in subsequent release of cytokines, enzymes, and other proteins. It is essential to diagnose the disorders associated with eosinophilia.
- The term 'hypereosinophilic syndrome' (**HES**) describes a group of disorders, that demonstrate an absolute eosinophil count $>1.5\times10^9$/L that persist for >4 weeks. Several mechanisms proposed in the pathogenesis of hypereosinophilic syndrome include overproduction of eosinophilopoietic cytokines or defects in the normal suppressive regulation of eosinophilopoiesis.
- Organ damage in hypereosinophilic syndrome occurs due to eosinophilic infiltration to the tissues such as myocardium, which may result in myocardial fibrosis, progressive heart failure and death.
- It is important to perform serological tests to rule out infection with *Strongyloides stercoralis*, because the patients treated with corticosteroids can experience dissemination of the disease associated with fatal outcome.
- Clonal hypereosinophilia pluripotent hematopoietic stem cell and lineage committed hematopoietic progenitor cells, which occurs in the settings of myeloid and lymphoid neoplasms with eosinophilia and abnormalities of platelet-derived growth factor receptor α (PDGFRA) and β (PDGFRB), or fibroblast growth factor receptor 1 (FGFR1) and chronic eosinophilic leukemia, not otherwise specified (CEL, NOS).
- Primary and secondary causes of eosinophilia are given in Table 9.117. Hypereosinophilic syndrome in Giemsa-stained peripheral blood smear is shown in Fig. 9.94. Hypereosinophilic syndrome in Giemsa-stained bone marrow aspirate smear is shown in Fig. 9.95.

MYELOID AND LYMPHOID NEOPLASMS ASSOCIATED WITH EOSINOPHILIA AND PDGFRA, PDGFRB, OR FGFR1

Myeloid and lymphoid neoplasms associated with eosinophilia are caused by gene mutations encoding the α or β chains of the protein kinases platelet-derived growth factor receptor α and β (PDGFRA, PDGFRB) or fibroblast growth factor receptor 1 (FGFR1). Mutations in these genes result in activation of receptor tyrosine kinase in chronic myeloproliferative disorders.

- Less frequently, these patients can present as acute myelogenous leukemia (AML) or precursor T cell lymphoblastic leukemia/lymphoma.

Table 9.117 Primary and secondary causes of eosinophilia

Primary (Clonal) Eosinophilia
Myeloid neoplasms
• Chronic myelogenous leukemia, BCR-ABL1 positive
• Acute myelogenous leukemia with inv(16)(p13.1;q22) or t(16;16)(p13.1;q22)
• Acute myelogenous leukemia with t(8;21) (p22;q22.1)
• Chronic myelomonocytic leukemia (CMML)
Myeloid and lymphoid neoplasms associated with PDGFRA, PDGFRB, or FGFR1 rearrangements or PCM1-JAK2, ETV6-JAK2, or BCR-JAK2 fusion genes
• Myeloid and lymphoid neoplasms associated with eosinophilia and abnormalities of PDGFRA, PDGFRB or FGFR1
• Myeloid and lymphoid neoplasms associated with PDGFRA rearrangement
• Myeloid and lymphoid neoplasms associated with PDGFRB rearrangement
• Myeloid and lymphoid neoplasms associated with FGFR1 abnormalities
• Acute myelogenous leukemia (AML)
Lymphoid neoplasms
• T cell non-Hodgkin lymphoma
• Hodgkin disease
• Acute lymphoblastic leukemia (ALL)
• Lymphocyte-variant hypereosinophilia caused by an aberrant population of lymphocytes
Mastocytosis
• Cutaneous mastocytosis
• Indolent systemic mastocytosis
• Systemic mastocytosis associated with other clonal, non-mast cell lineage disease
• Mast cell leukemia
• Mast cell sarcoma
• Extracutaneous mastocytoma
Secondary Eosinophilia
Infections: Parasites, specific fungal function
Collagen vascular diseases: Churg-Strauss disease, Wegener granulomatosis, systemic lupus erythematosus
Pulmonary diseases: Idiopathic acute or chronic eosinophilic pneumonia, tropical pulmonary eosinophilia, allergic bronchopulmonary aspergillosis
Gastroenteritis
Adrenal insufficiency
Immunologic conditions
Allergic state
Drug reactions

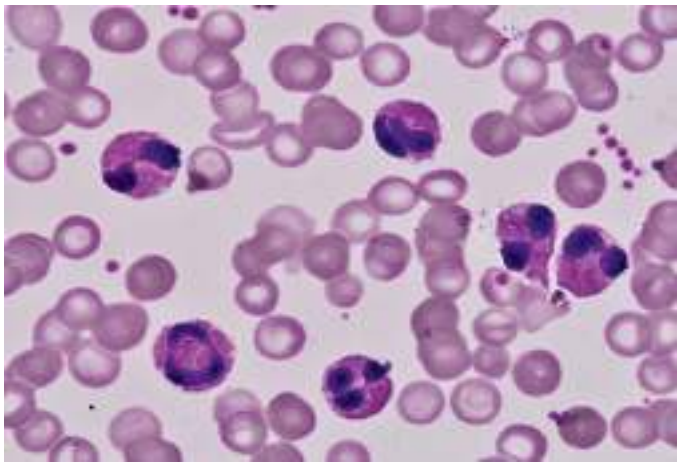


Fig. 9.94: Hypereosinophilic syndrome (HES) in Giemsa-stained peripheral blood smear. The term HES describes a group of disorders, that demonstrate an absolute eosinophil count >1.5×10⁹/L that persist for >4 weeks. Peripheral blood smear shows ring eosinophils (1000X).

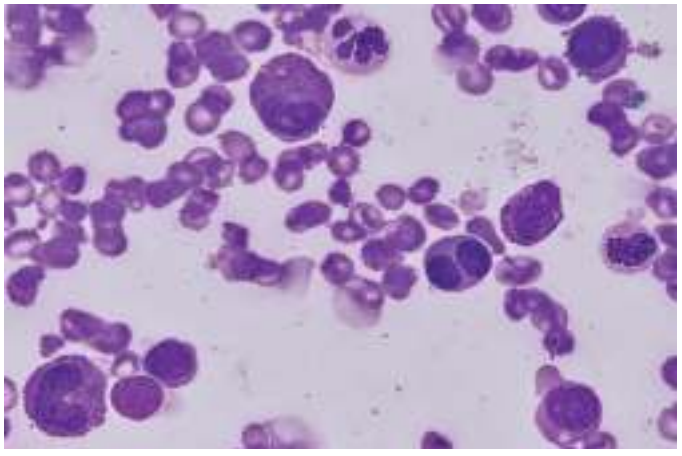


Fig. 9.95: Hypereosinophilic syndrome (HES) in Giemsa-stained bone marrow aspirate smear. The term HES describes a group of disorders, that demonstrate an absolute eosinophil count >1.5×10⁹/L that persist for >4 weeks (1000X).

- Eosinophilia is variable but present in majority of cases. These patients respond to treatment with receptor tyrosine kinase inhibitors (**RTKIs**) such as imatinib or other RTKIs.

Myeloid and Lymphoid Neoplasms with PDGFRA Rearrangement

A gain-of-function of receptor tyrosine kinase (RTK) fusion gene has been demonstrated in hypereosinophilic syndrome patients, who are unresponsive to cortico-steroid therapy, but responsive to receptor tyrosine kinase inhibitors (RTKIs).

- FIP1L1-PDGFRA fusion gene has been described in patients with eosinophilia-associated myeloid and lymphoid neoplasms.
- The mutation results from a small interstitial deletion in chromosome 4 (q12q12) leading to formation of FIP1L1/PDGFRA fusion gene and constitutively activation of receptor tyrosine kinase protein.
- Majority of the cases involving FIP1L1-PDGFRA fusion gene mutation affects 25–55 years of patient with male to female ratio (17:1), which develop eosinophil-induced tissue damage and tissue fibrosis associated with splenomegaly.
- Patients harboring FIP1L1-PDGFRA fusion gene mutation respond to imatinib first generation receptor tyrosine kinase inhibitor (RTKI) therapy.
- Patients developing resistance to imatinib first-line receptor tyrosine kinase inhibitors are treated by second-generation (nilotinib) and third-generation (ponatinib) receptor tyrosine kinase inhibitors (RTKIs) therapy.

Laboratory Diagnosis of Myeloid and Lymphoid Neoplasms with PDGFRA Rearrangement in Clonal Hypereosinophilia

Peripheral Blood Smear Examination

- **White blood cells:** Hypereosinophilia is the most important laboratory finding in myeloid and lymphoid neoplasms associated with FIP1L1-PDGFRA fusion gene mutation.
  - Mature eosinophils with few promyelocytes or myelocytes are present.
  - Eosinophils contain sparse granules, vacuoles and hyper-segmentation or hyposegmentation of nuclei.
  - Presence of <20% blasts in the peripheral blood and/or bone marrow can be demonstrated in few cases.
- **Red blood cells:** Peripheral blood smear shows anemic picture.
- **Platelets:** Platelet count is decreased (thrombocytopenia).

Bone Marrow Smear Examination

- Bone marrow is hypercellular with increased eosinophil promyelocytes and myelocytes.
- Charcot-Leyden crystals composed of eosinophil protein galectin 10 may be demonstrated.
- In majority of cases, bone marrow examination shows an increase in spindle-shaped atypical mast cells with a CD25+ immunophenotype. These findings are also demonstrated in systemic mastocytosis.
- Presence of <20% blasts in the peripheral blood and/or bone marrow can be demonstrated in few cases.

Molecular Genetic Alterations

FIP1L1-PDGFRA fusion gene mutation can be demonstrated by reverse transcriptase-polymerase chain reaction (RT-PCR) or fluorescence *in situ* hybridization (FISH) techniques.

Myeloid and Lymphoid Neoplasms with PDGFRB Rearrangement

Myeloid and lymphoid neoplasms with PDGFRB rearrangement are characterized by formation of ETV6-PDGFRB fusion gene as a result of t(5;12)(q33;p13) rearrangement (most common).

- Myeloid neoplasms originate from pluripotent hematopoietic stem cell (**HSC**), that is capable to differentiate into neutrophils, eosinophils, monocytes, and probably mast cells.
- Myeloid neoplasms with PDGFRB rearrangement can present as chronic myelomonocytic leukemia (CMML) with eosinophilia, atypical chronic myelogenous leukemia (aCML), or MPN with eosinophilia. These patients may develop blast phase.
- Myeloid neoplasms with PDGFRB affects in the age group of 8–72 years with male predilection. Patients are treated by imatinib receptor tyrosine kinase inhibitor (RTKI).

Laboratory Diagnosis of Myeloid and Lymphoid Neoplasms with PDGFRB Rearrangement in Clonal Hypereosinophilia

Peripheral Blood Smear Examination

- **White blood cells:** Total leukocyte count is increased. There is presence of variable increase in the neutrophils, eosinophils, monocytes and precursor cells. The peripheral blood and bone marrow smears demonstrate <20% blasts.
- **Red blood cells:** Peripheral blood smear examination shows anemic picture.
- **Platelets:** Platelet count is decreased (thrombocytopenia).

Bone Marrow Smear Examination

- **Cellularity:** Bone marrow is hypercellular with increase in spindle-shaped mast cells. Reticulin can be demonstrated.
- **Myeloid cells:** The bone marrow and peripheral blood demonstrate <20% blasts.

Molecular Genetic Alterations

- Myeloid neoplasms with PDGFRB rearrangement are diagnosed by molecular genetic analysis by using primers for all known breakpoints to confirm ETV6-PDGFRB.
- Cytogenetic analysis demonstrates the t(5;12).

Myeloid and Lymphoid Neoplasms with FGFR1 Abnormalities

Myeloid and lymphoid neoplasms with FGFR1 abnormalities belong to subgroup of eosinophilic neoplasms, which are characterized by 8p11 chromosome breakpoint resulting in a variety of fusion genes that incorporate part of the FGFR1 gene. All fusion gene products activate tyrosine kinase activity. These neoplasms probably originate from pluripotent hematopoietic stem cell (**HSC**) and affect 3–84 years with male predilection.

- Most of the cases are associated with genetic abnormalities: (a) t(8;13)(p11;q12)—the fusion gene being ZMYM2-FGFR1, (b) t(8;9)(p11;q33)—the fusion gene being CNTRL-FGFR1; and (c) t(6;8)(q27;p11-12)—the fusion gene being FGFR10P1-FGFR1.
- Myeloid and lymphoid neoplasms with FGFR1 abnormalities present with hypereosinophilia. These cases are transformed to acute myelogenous leukemia (AML) or T cell- or B cell-derived acute lymphoblastic leukemia/lymphoma or as mixed phenotype of acute leukemias. Progression of acute myelogenous leukemia to chronic myelogenous leukemia that demonstrates eosinophilia, neutrophilia and occasionally monocytosis.
- The patients do not respond to first-line imatinib receptor tyrosine kinase inhibitor (RTKI) therapy. However, these patients can respond to second-line or third-line RTKIs therapy. Bone marrow hematopoietic stem cell (HSC) transplant can be indicated in chronic phase of leukemia.

IDIOPATHIC HYPEREOSINOPHILIC SYNDROME

Idiopathic hypereosinophilic syndrome (I-HES) represents a heterogenous group of disorders with the common features of prolonged eosinophilia (eosinophil count 1.5×10^9/L for at least 6 months duration not meeting diagnostic criteria of chronic eosinophilic leukemia or familial eosinophilia) of an undetectable etiology and organ system dysfunction.

- There is no evidence of clonality or T cell abnormality. First-line therapy for most cases with idiopathic hypereosinophilic syndrome is the use of corticosteroids.
- Proper follow-up of these patients is essential to assess transformation to leukemia.

CHRONIC EOSINOPHILIC LEUKEMIA, NOS

Chronic eosinophilic leukemia, not otherwise specified (CEL, NOS) is a clonal chronic myeloproliferative disorder in which unregulated clonal proliferation of eosinophilic precursors results in persistently elevated number of eosinophils in the blood, bone marrow or peripheral tissues leading to organ damage and mortality. CEL, NOS most often affects middle-aged persons (male to female ratio 9:1).

- Chronic eosinophilic leukemia, not otherwise specified (CEL, NOS) excludes cases with BCR-ABL1 fusion gene and rearrangement of PDGFRA, PDGFRB or FGFR1, or PCM1-JAK2 or BCR-JAK2 fusion gene. In addition, it is essential to rule out causes of reactive eosinophilia, presence of aberrant T cells and disorders associated with abnormal release of cytokines (GM-CSF, IL-2, IL-3, IL-5).

- Chronic eosinophilic leukemia, not otherwise specified (CEL, NOS) may arise from hematopoietic stem cell (HSC), multipotent progenitor (MPP) cells, or a committed eosinophilic progenitor cell (CFU-E0).
  - In some cases, cytogenetic abnormality such as trisomy 8 can be demonstrated. It is important to distinguish CEL, NOS from idiopathic hypereosinophilic syndrome, that is characterized by eosinophil count 15×10^9/L persistent for 6 months without underlying cause.
  - Diagnostic criteria for chronic eosinophilic leukemia, not otherwise specified (CEL, NOS) are given in Table 9.118.

Clinical Features

Patient presents with fever, significant weight loss, hepatosplenomegaly, congestive heart failure, pulmonary fibrosis, central nervous system abnormalities and occasionally lymphadenopathy. Release of excessive eosinophilic granules into the blood circulation induces fibrosis of vascular endothelial cells resulting in peripheral vasculitis, gangrene of digits, organ damage such as heart and lungs.

Table 9.118 Diagnostic criteria for chronic eosinophilic leukemia, not otherwise specified (CEL, NOS)

Persistent peripheral blood eosinophilia $\geq 1.5 \times 10^9$/L
Presence of <20% blasts in the peripheral blood and bone marrow; and no inv(16) (p13.1q22), t(16;16) (p13.1;q22), t(8;21) (q22;q22.1) and other features of AML
There is presence of clonal cytogenetic or molecular genetic abnormality or blast cells are ≥2% in the peripheral blood or ≥5% in the bone marrow
Exclude eosinophilic disorders that bear PDGFRA, PDFGRB or FGFR1 mutations as well as PCM1-JAK2 or BCR-JAK2 fusion
No BCR-ABL1 fusion gene; and exclusion of polycythemia vera, essential thrombocythemia, primary myelofibrosis, chronic neutrophil leukemia, chronic myelomonocytic leukemia or atypical chronic myelogenous leukemia
Exclusion of all secondary causes of eosinophilia
Exclusion of neoplastic disorders with secondary eosinophilia
Exclusion of neoplastic disorders when eosinophils are a part of neoplastic clone such as AML
Exclude the presence of an aberrant phenotypic T cell population

(a) If the patient presents with persistent eosinophilia but does not meet these diagnostic criteria, the diagnosis can be reactive eosinophilia, idiopathic hypereosinophilia, or idiopathic hypereosinophilic syndrome. (b) Adapted from revised 2024 World Health Organization (WHO) classification of hematopoietic neoplasms.

Laboratory Diagnosis of Chronic Eosinophilic Leukemia, NOS (CEL, NOS)

- Chronic eosinophilic leukemia, not otherwise specified (CEL, NOS) is characterized by persistent eosinophilia $\geq 1.5 \times 10^9$/L.
- There is evidence of clonality and/or demonstration of >2% blasts in the peripheral blood or 5–19% blasts in the bone marrow.

Biochemical Investigations

Serum cobalamin, uric acid and muramidase levels are most often elevated.

Peripheral Blood Smear Examination

Presence of clonal eosinophil blasts ≥2% in the peripheral blood and 10–15% in the bone marrow supports the diagnosis of chronic eosinophilic leukemia, not otherwise specified (CEL, NOS). Anemia and thrombocytopenia may present. Chronic eosinophilic leukemia (CEL) in Giemsa-stained peripheral blood smear is shown in Fig. 9.96.

- **White blood cells:** Total leukocyte count is usually $>30 \times 10^9$/L with 30–70% eosinophils. CEL, NOS is characterized by persistent eosinophilia $\geq 1.5 \times 10^9$/L. There are presence mature eosinophils and some eosinophilic myelocytes. Eosinophil abnormalities are demonstrated such as sparse granularity, nuclear hypersegmentation and hyposegmentation. Clonal blasts >2% are demonstrated in the peripheral blood.
- **Red blood cells:** Peripheral blood smear examination shows anemia.
- **Platelets:** Platelet count is decreased.

Neutrophil Alkaline Phosphatase

Neutrophil alkaline phosphatase (NAP) score is within normal limit.

Bone Marrow Smear Examination

Presence of clonal eosinophil blasts ≥2% in the peripheral blood and 10–15% in the bone marrow supports the diagnosis.

- **Cellularity:** Bone marrow is hypercellular with increased myeloid to erythroid cells.
- **Myeloid lineage:** Bone marrow examination shows a shift to left with many mature eosinophils and eosinophilic myelocytes.
- **Erythroid lineage:** Bone marrow demonstrates normal erythropoiesis.
- **Megakaryocytic linegae:** Bone marrow shows normal megakaryopoiesis.
- **Charcot-Leyden crystals:** Charcot-Leyden crystals are demonstrated in the bone marrow.

Bone Marrow Trephine Biopsy Examination

- Bone marrow trephine biopsy examination shows evident bone marrow fibrosis.
- Charcot-Leyden crystals are most often present.

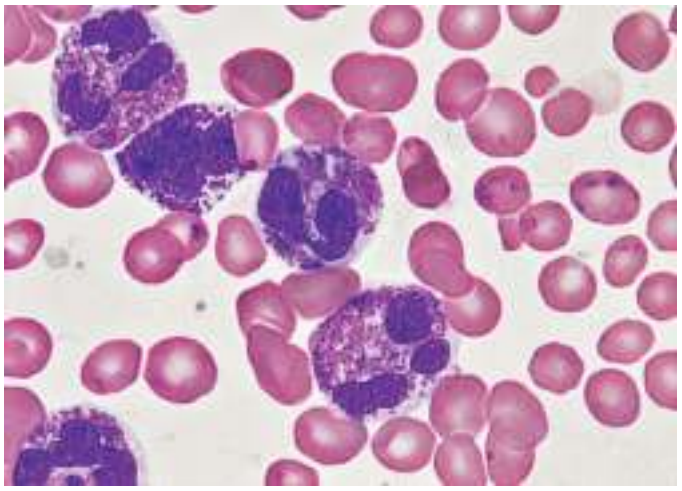


Fig. 9.96: Chronic eosinophilic leukemia (CEL) in Giemsa-stained peripheral blood smear. CEL is a chronic myeloproliferative neoplasm characterized by a clonal proliferation of eosinophil precursors that leads to increased eosinophilia in the peripheral blood, the bone marrow and possibly peripheral tissues. An eosinophil count in blood of 1.5×10^9/L or higher that lasts over time. There should not be parasitic infestation or allergic disorders or other causes of eosinophilia (1000X).

Treatment and Prognosis

In chronic eosinophilic leukemia, not otherwise specified (CEL, NOS), persistent eosinophilia invading heart causes severe cardiac injury resulting in congestive heart failure and fatal outcome within one year after diagnosis. Only a few patients survive beyond one year.

Differential Diagnosis

Chronic eosinophilic leukemia, not otherwise specified (CEL, NOS) should be differentiated from reactive eosinophilia and clonal eosinophilia found in hematologic disorders.

MASTOCYTOSIS (MAST CELL DISEASE)

Mastocytosis is a clonal disorder of bone marrow characterized by clonal proliferation of mast cells resulting in invasion of single organ (skin) or multiple organs.

- Cutaneous mastocytosis demonstrates mast cells in the skin associated with clinical signs without systemic involvement, which usually affects children.
- Systemic mastocytosis involves multiple organs and bone marrow. There can be anemia, leukocytosis or leukopenia, thrombocytopenia and marked eosinophilia. Mast cells share a common progenitor cell with myelomonocytic cells.
  - Other subgroup of systemic mastocytosis is mast cell leukemia (MCL). The diagnostic criteria for MCL must fulfill major and minor criteria for systemic mastocytosis; and bone marrow trephine biopsy demonstrates diffuse infiltration by atypical immature mast cells, bone marrow aspirate demonstrates 20% mast cells and peripheral blood shows 10% mast cells.
  - The aleukemic mast cell leukemia variant has <10% mast cells in the peripheral blood and bone marrow smears along with immature blast-like cells with bilobed or multilobated nuclei. The mast cells are tryptase positive with express c-KIT. Serum tryptase levels are elevated >20 ng/ml. In acute form of aleukemic mast cell leukemia, organ damage is present. Pancytopenia occurs in the later stage of disease, when bone marrow failure occurs in the patients.
- Molecular genetic analysis demonstrates that systemic mastocytosis is a clonal disorder characterized by a somatic point mutation of c-KIT proto-oncogene in mast cells.
  - Stem cell factor (SCF) and its receptor (SCFR), a member of the c-KIT gene critically regulates hematopoiesis and mast cell development.
  - When c-KIT gene is mutated, the SCFR receptor is activated resulting in unrestricted proliferation of clonal mast cells. Serum tryptase level persistently exceeds 20 ng/ml.
- Mast cell sarcoma is a rare, localized mast cell tumor, that demonstrates immature mast cells having sarcoma-like morphology. Mast cell sarcoma transforms to mast cell leukemia in a short span of time.
- Revised 2024 WHO classification of mastocytosis is given in Table 9.119. Diagnostic criteria for systemic mastocytosis and mast cell leukemia according to revised 2024 World Health Organization are given in Table 9.120.

CLINICAL FEATURES

Most patients of mastocytosis are adults with symptoms such as flushing, diarrhea, hypotension and headache related to release of chemical mediators and proteins from mast cells. Patients present with fever, weight

Table 9.119 Revised 2024 WHO classification of mastocytosis

Cutaneous mastocytosis
Indolent systemic mastocytosis
Systemic mastocytosis associated with other clonal, non-mast cell lineage disease
Mast cell leukemia
Mast cell sarcoma
Extracutaneous mastocytoma

Table 9.120 Diagnostic criteria for systemic mastocytosis and mast cell leukemia according to revised 2024 World Health Organization

Systemic Mastocytosis: Diagnostic Criteria
Major diagnostic criterion Bone marrow or other organs demonstrate multifocal, dense infiltrates of tryptase positive mast cells (>15 mast cells aggregate)
Minor diagnostic criteria ▪ Bone marrow trephine biopsy or other organs demonstrate spindle-shaped or atypical morphology in >25% of mast cells, or bone marrow shows >25% immature or atypical mast cells counting all bone marrow cells ▪ Presence of somatic point mutation at codon 816 of c-KIT proto-oncogene in the mast cells from bone marrow or other organs. ▪ Mast cells in peripheral blood, bone marrow, or other tissue coexpress CD2 and/or CD25, in addition to normal mast cell markers ▪ Serum tryptase level >20 ng/ml in the absence of associated clonal myeloid disorder
Mast Cell Leukemia: Diagnostic Criteria
▪ Diagnostic major and minor criteria for systemic mastocytosis must fulfill ▪ Bone marrow trephine biopsy demonstrates diffuse infiltration by atypical immature mast cells ▪ Bone marrow aspirate demonstrates ≥20% mast cells ▪ Peripheral blood shows ≥10% mast cells

Diagnosis of systemic mastocytosis can be made when the major criterion and one minor criterion is present; or when three minor criteria are present.

loss, sweats, cutaneous red brown macules or papules on shoulders or trunk, bone pain, organomegaly in the later stage of disease. Patients developing urticaria when scratched is known as Darier's sign.

Laboratory Diagnosis of Mastocytosis (Mast Cell Disease)

Diagnosis of mast cell disease is based on clinical manifestations, bone marrow examination, bone marrow trephine biopsy examination, histopathologic examination of skin and organs involved, molecular genetics, serum tryptase level, histochemical examination and immunohistochemistry.

Bone Marrow Smear Examination

- Bone marrow smear examination reveals scant cellularity due to marrow fibrosis.
- Aggregates of atypical mast cells admixed with eosinophils some hypogranular mast cells are demonstrated.

Histochemical Staining

- Mast cells are demonstrated by toluidine blue metachromatic stain.
- Mast cells also strongly react with naphthol-ASD-chloroacetate esterase (CAE).

Immunohistochemistry Technique

Mast cells show positivity for tryptase, CD2, CD25 and CD117.

Markers	Expression
▪ CD2	▪ Positive
▪ CD25	▪ Positive
▪ CD117	▪ Positive

PROGNOSIS AND TREATMENT

No standard therapy or long-term cure exists for mast cell disease. Patients are administered aspirin and antihistaminic drugs to relieve symptoms. Experience with chemotherapy and bone marrow hematopoietic stem cell transplantation (HSC) is lacking. Prognosis is worst in mast cell leukemia.

DIFFERENTIAL DIAGNOSIS

Immunophenotype plays important role in differentiating mastocytosis (CD2, CD25, CD117) from hairy cell leukemia (positive for CD25, CD103, CD123, Annexin A1) and Langerhans cell histiocytosis (positive for CD1, S-100 protein). Prognosis is worst in mast cell leukemia.

CHRONIC LYMPHOPROLIFERATIVE NEOPLASMS

Chronic lymphoproliferative disorders are morphologically, immunologically and clinically heterogenous, which include T cell, B cell or natural killer cell immunophenotypes and terminal deoxynucleotidyl transferase (TdT) negativity.

- B cell-derived chronic lymphoproliferative disorders include chronic lymphocytic leukemia (CLL), prolymphocytic leukemia, non-Hodgkin lymphoma (including mantle cell lymphoma) in leukemic phase, hairy cell leukemia and splenic lymphoma with villous lymphocytes.
- T cell-derived chronic lymphoproliferative disorders include T cell prolymphocytic leukemia, adult T cell leukemia/lymphoma, large granulated

lymphocytic leukemia and Sézary syndrome. Occasionally, lymphocytic proliferation is encountered that does not fulfill the morphologic and immunophenotyping criteria for any of the above-mentioned categories. These disorders are best left unclassified.

CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia (CLL) is a monoclonal B cell disorder characterized by increased production of monomorphic, small, and mature but dysfunctional B cells in the blood (5×10^9/L) and bone marrow with or without nodal (i.e. lymph nodes) or extranodal manifestation (e.g. spleen).

- As CLL disease progresses the patient can present with lymphadenopathy, splenomegaly, and hepatomegaly. Due to widespread disease, clinical staging is used in CLL patients. Most cases are detected when a routine blood test is performed.
- CLL accounts for 25% of all leukemias, which is the most common leukemia in Western world. Incidence increases with age and peak is in age group of 60–80 years affecting both men and women.
- Anemia, thrombocytopenia, and neutropenia are related symptoms frequently occur in CLL patients.
- Autoimmune hemolytic anemia is common in CLL patients, which occurs due to autoimmune hemolysis and leukemic cells infiltration into bone marrow.
- Extramedullary involvement of CLL may occur in liver, skin, gastrointestinal tract mucosa and kidneys. Hypogammaglobulinemia and cellular dysfunction induce immunosuppression.
- Diagnosis of CLL is usually established by immunophenotypic analysis of peripheral blood, which reveals a clonal population of CD5+ and CD23+ B cells.
- Patients are treated by use of chemotherapy and an anti-CD20 monoclonal antibody to achieve remission without a complete cure. Best guide to prognosis is the stage of the disease.
- CLL disease, that has acquired somatic mutations in the immunoglobulin genes, is associated with relatively good prognosis as compared to unmutated immunoglobulin gene cases. Molecular genetic analysis also provides prognostic information.
- Approximately 50% of CLL patients demonstrate abnormal karyotype **trisomy chromosome 12**, chromosomal abnormalities of 13q, 14 and 16. Patients showing trisomy 12 have poor prognosis.
- Activation of BCL-2 proto-oncogene leads to decreased apoptosis resulting in increased accumulation of monoclonal B cells.

PATHOGENESIS

Chronic lymphocytic leukemia (CLL) is a disease of clonal B cells that have low proliferative rate and defect in the apoptosis of leukemic B cells. CLL leukemic B cells appear mature but are actually arrested during early development resulting in less capable of differentiating into antibody-synthesizing plasma cells.

- CLL leukemic B cells express B cell receptor (BCR) and antigenic stimulation of the B cell receptor appears to be major pathogenic driver. Recent advances highlight the importance of leukemic B cell proliferation that occurs primarily in the tissue environment of the bone marrow and lymph nodes.
- Key signaling pathways promoting proliferation of leukemic B cell in CLL and their survival in the bone marrow and lymph nodes include activation of B cell receptor (BCR) and NF-κB pathways.
- Positive family history of CLL increases 2–8 times risk for developing CLL. The role of environmental factors and viral infections in the pathogenesis of CLL remains ill-defined.

CLINICAL FEATURES

Patient with chronic lymphocytic leukemia is usually asymptomatic for many years. It is most often diagnosed following the incidental lymphocytosis on routine blood tests or asymptomatic lymphadenopathy.

- CLL patient presents with generalized lymphadenopathy (80%), abdominal fullness, hepatomegaly due to leukemic infiltration in 50–60%, splenomegaly (less marked), pallor, weakness, fatigue, and dyspnea.
- As the CLL disease advances, patient may have recurrent infections, weight loss, anemia and thromboytopenia (purpura and hemorrhagic manifestations). CLL patients can present with night sweats, fever and weight loss; similar to non-Hodgkin lymphoma (NHL) and Hodgkin disease.
- Leukemic B cells of CLL infiltrate lymph nodes, liver, and spleen leading to generalized nontender lymphadenopathy in cervical, axillary, inguinal and abdominal regions, not attached to skin, hepatomegaly, and splenomegaly respectively. Leukemic B cells infiltration may occur in skin, lungs, pleura, kidneys, and gastrointestinal tract. CNS involvement is unusual in CLL patients.

Clinical Pearls: Chronic Lymphocytic Leukemia—Clinical Features

Symptoms of CLL

- Asymptomatic cases detected on routine blood tests
- Susceptibility to infection (pneumonia, herpes simplex and herpes zoster)
- Generalized lymphadenopathy due to leukemic infiltration
- Abdominal discomfort from hepatosplenomegaly from leukemic infiltration
- Bleeding or petechiae in skin or mucous membranes from thrombocytopenia
- Tiredness and fatigue from anemia including autoimmune hemolytic anemia.

Signs of CLL

- Generalized or local lymphadenopathy
- Splenomegaly
- Hepatomegaly
- Petechiae
- Pallor
- Tonsillar enlargement
- Involvement of the lacrimal and salivary glands (Mikulicz's syndrome)
- Richter syndrome [CLL transformation to diffuse large B cell lymphoma (DLBCL)]

Recurrent Infections

Patient with CLL presents with recurrent infections in early course of the disease (respiratory tract, sinuses, ears, skin, and kidney) due to absence of immunoglobulin (IgG), neutropenia and corticosteroid therapy. Herpes zoster infection of skin results in pruritus, vesiculobullous and popular eruptions.

Autoimmune Hemolytic Anemia

Chronic lymphocytic leukemia (CLL) is a malignant neoplasm due to clonal expansion of CD5 expressing B cells. CD5 expressing B cells have been postulated to produce autoantibodies in 10–20% of CLL cases resulting in autoimmune hemolytic anemia. Therefore, direct Coombs test should be performed in CLL cases. Anemia may be accompanied by immune-mediated thrombocytopenic purpura.

Richter Syndrome

Richter syndrome is defined as the transformation of CLL into aggressive lymphoma especially diffuse large B cell lymphoma (**DLBCL**) in 3–5% of cases. Richter syndrome patient presents with rapid onset of fever, abdominal pain, and progressive lymphadenopathy and hepatosplenomegaly associated with aggressive clinical course. Patients are refractory to therapy and have a mean survival of 2 months.

Second Malignancies

The second malignancies that are observed most frequently in CLL patients may be due to specific risk factors, protumorigenic microenvironment and chemotherapy-related immunosuppression.

- Immunodeficiency in CLL may also predispose to the increased risk for developing other malignancies.
- Examples of second malignancies in CLL patients are Hodgkin's disease, B cell prolymphocytic leukemia, multiple myeloma, acute lymphoblastic leukemia, and cancers of breast, gastrointestinal tract, lung, skin, prostate, kidney, urinary bladder, and head and neck.

Gastrointestinal Tract Involvement

Gastrointestinal manifestations have very rarely been described in patients with chronic lymphocytic leukemia (CLL). Clonal B lymphocytic infiltration seems to depend on tumor burden and proliferative activity.

- Conventional staging according to Rai and Benet may not accurately reflect the whole extent of disease. It is not known whether CLL patients should undergo endoscopic evaluation.
- Normal mucosal appearance of GIT does not exclude infiltration of leukemic cells in CLL cases. Histologic examination establishes leukemic infiltration. Patient presents with anorexia and occasionally intestinal obstruction.

Mikulicz Syndrome

Patient with CLL may develop tonsillar enlargement along with Mikulicz syndrome characterized by chronic swelling of the lacrimal and salivary glands usually associated with significantly decreased or lack of lacrimation (dry eyes) and xerostomia (dry mouth), accompanied by lymphocytic infiltration.

LABORATORY DIAGNOSIS

Diagnosis of chronic CLL requires clonal lymphocytosis ($>5 \times 10^9$/L clonal B cells) in the peripheral blood sustained for three months with typical immunophenotype in the peripheral blood demonstrated by flow cytometry.

- Recently revised 2024 WHO classification of hematolymphoid neoplasms describes CLL being distinguishable from small lymphocytic lymphoma (SLL) by its leukemic appearance.
- Diagnostic criteria for SLL include lymphadenopathy and/or splenomegaly (defined by physical examination and CT scan), with $>5 \times 10^9$/L circulating clonal B cells.

- Lymph node biopsy is not generally required unless to establish diagnosis of Richter syndrome.
- **Lymph node biopsy** is the standard diagnostic test **for SLL**, however, presence of circulating clonal B cells with typical immunophenotype is sufficient in patients with an indolent presentation.
- Immunophenotyping is essential flow cytometry of peripheral blood or bone marrow or immunohistochemistry of biopsy material to diagnose chronic lymphocytic leukemia (CLL) cases.

Imaging Techniques

Computed tomography (CT) is not required for routine evaluation of CLL patients. But CT scan can capture the extent of lymph node involvement in CLL cases and response to treatment.

- Lymph node involvement in CLL has low metabolic activity on positron emission tomography (PET) scan.
- PET scan is not essential for routine evaluation of CLL patients, but it can add valuable information in advanced disease or relapsed disease especially when CLL transforms into high-grade lymphoma.

Laboratory Diagnosis of Chronic Lymphocytic Leukemia

Biochemical Investigations

- **Direct antiglobulin test (DAT):** Direct antiglobulin test should be performed before treatment in patient with anemia. Conversion of the DAT negative to DAT positive may herald the onset of autoimmune hemolytic anemia (AIHA).
- **Serum immunoglobulins:** Serum immunoglobulins decrease with the duration of disease. Immunoglobulin M (IgM) small spike is consistent in CLL cases.
- **Serum β_2-microglobulin:** Serum β_2-microglobulin may be elevated. Its level often increases with high tumor burden. High serum β_2-microglobulin more than 3 mg/L has been associated with poor response to treatment and survival.

Peripheral Blood Smear Examination

In chronic lymphocytic leukemia (CLL), monoclonal proliferation of B cells accumulates and replace bone marrow cells over a few years resulting in anemia and thrombocytopenia with advancing disease. Peripheral blood smear findings in CLL are shown in Fig. 9.97.

White blood cells

- About 50% of CLL cases demonstrate leukocytosis in the range of 20×10^9/L to 50×10^9/L. Some patients have total leukocyte count (TLC) >50 $\times 10^9$/L in the range of 100 $\times 10^9$/L even up to 500×10^9/L.
- The diagnosis of CLL requires the presence of $>5 \times 10^9$/L clonal B cells with typical immunophenotype in the peripheral blood, sustained for least three months.
- The clonality of B cells needs to be confirmed by demonstrating light chain restriction using flow cytometry.
- Clonal B cells are small- to medium-sized mature appearing lymphocytes with round nuclei, clumped fragile chromatin, and scanty cytoplasm exhibiting monotonous appearance. Clonal B cells in CLL appear to be more fragile than normal lymphocytes and are susceptible to mechanical disruption during preparing of blood smear producing smudge cells. Smudge cells, bare nuclei appear squashed, are classic features of CLL.
- However, smudge cells can be present in reactive lymphocytosis and other neoplasms. Therefore, presence of smudge cells should not be used to diagnose CLL. The number of smudge cells can be reduced by mixing drop of albumin with drop of blood prior to preparation of peripheral blood smear.
- Prolymphocytes number increase in proportion in rapidly progressive CLL disease. Prolymphocytes are medium- to large-sized cells with clumped chromatin and prominent vesicular nucleolus and abundant cytoplasm constituting <10% of the lymphocytes in typical CLL cases. Presence of >55% of prolymphocytes in the peripheral blood is recognized as a distinct known as 'prolymphocytic leukemia'.
- In advanced CLL disease, anemia and thrombocytopenia are common, most often as a result of replacement of bone marrow by clonal B cells, hypersplenism and immune-mediated hemolytic anemia and thrombocytopenia.

Red blood cells

- Hemoglobin is markedly reduced to <10 g/dl in the advanced disease.
- Peripheral blood smear examination shows normocytic normochromic picture due to bone marrow infiltration by neoplastic cells with a possible contribution to hypersplenism and autoimmune mechanism.

Platelets

In the early stage of disease, platelet count may be normal. But in the later stage, patient develops thrombocytopenia with platelet count $<100 \times 10^9$/L as a result of leukemic cells infiltration in bone marrow with possible hypersplenism and immune-mediated thrombocytopenia.

Bone Marrow Trephine Biopsy Examination

- Bone marrow is always involved in chronic lymphocytic leukemia and most cases of small lymphocytic lymphoma.
- Bone marrow trephine biopsy should be performed in those cases posing diagnostic difficulties or cases with low peripheral blood counts.
- Distinct patterns of bone marrow infiltration by clonal B cells, which have some prognostic value, are observed: nodular, interstitial, diffuse or mixed patterns.
- Advanced disease is most often associated diffuse pattern of bone marrow involvement.
- Reticulin stain demonstrates marked bone marrow fibrosis. There is moderate to marked decrease in hematopoiesis involving erythroid, myeloid and megakaryocytic series.

Bone Marrow Smear Examination

Bone marrow findings in chronic lymphocytic leukemia are shown in Fig. 9.98.

- **Cellularity:** Bone marrow is hypercellular due to monoclonal proliferation of lymphocytes in the bone marrow. Lymphocytes constitute >30% of nucleated cells in the marrow. There is gradual replacement of bone marrow erythroid, myeloid and megakaryocyte by clonal B lymphocytes over a few years. Therefore, the patient develops anemia, neutropenia and thrombocytopenia (Fig. 9.99).
- **Myeloid to erythroid cells ratio:** Myeloid to erythroid ratio is increased due to clonal proliferation of B cells and decreased erythroid cells.
- **Myeloid lineage:** Myeloid cells are normal in number and maturation in the initial stage of disorder.
- **Erythroid lineage:** Bone marrow shows normoblastic erythropoiesis. Patient developing autoimmune hemolytic anemia may show erythroid hyperplasia. Due to proliferation of neoplastic lymphocytes, erythroid precursors are suppressed.
- **Megakaryocytic lineage:** Megakaryopoiesis is normal in the initial stage of disorder. In the later stage, megakaryopoiesis is suppressed resulting in thrombocytopenia.

Immunophenotyping

Flow cytometry is the single most informative diagnostic analysis in CLL patients. Clonal B cells are positive for CD19, CD5 and CD23. These cells typically have weak expression of CD20, CD22 and surface immunoglobulin IgM and IgD. Clonal B cells are negative for FMC7, CD10 and CD103.

Markers	Expression
▪ CD19	▪ Positive
▪ CD5	▪ Positive
▪ CD23	▪ Positive
▪ Clonal Ig gene rearrangements	▪ Positive
▪ CD20	▪ Positive (weak expression)
▪ CD22	▪ Positive (weak expression)
▪ Surface immunoglobulin (IgM and IgD)	▪ Positive (weak expression)
▪ CD103	▪ Negative

CD200 is positive in atypical CLL. CD79a is pan B cell marker.

Surgical Pathology: Lymph Node Biopsy/Splenectomy in Chronic Lymphocytic Leukemia

Gross Morphology

Lymph nodes are enlarged. Cut surface if homogenous and fleshy. Spleen is enlarged and often showing military pattern of white pulp, homogenous infiltration.

Light Microscopy

- Lymph node biopsy is not generally required unless to establish diagnosis of Richter syndrome. Diagnosis of CLL is usually made by looking at peripheral blood smear, bone marrow aspirate and bone marrow trephine biopsy.
- Majority of clonal B cells in CLL are well-differentiated lymphocytes in the peripheral blood, bone marrow and lymph nodes. Many CLL patients develop swollen lymph nodes.
- Occasionally, a lymph node biopsy is performed to determine that a lymph node is swollen due to CLL and not some other condition.
- The architecture of lymph node in CLL is effaced by numerous small neoplastic lymphocytes with clumped chromatin. Mitotic activity is typically low.

Differential Diagnosis

Morphologic features, flow cytometry, immunohistochemistry and cytogenetic analysis are best diagnostic yield in differential diagnosis of chronic lymphocytic leukemia. Distinguishing features of CLL and closely resembling non-Hodgkin lymphomas are given in Table 9.121.

CLINICAL HISTORY AND STAGING SYSTEM

Clinical history of chronic lymphocytic leukemia (CLL) is quite variable, ranging from an indolent disease to incurable disease progressing from asymptomatic lymphocytosis to treatment-refractory disease within few years. Two staging systems have been used to

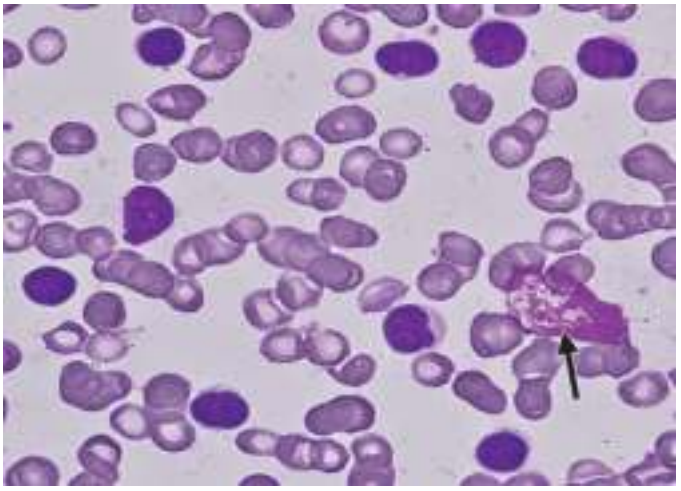


Fig. 9.97: Chronic lymphocytic leukemia (CLL) in Giemsa-stained peripheral blood smear. Peripheral blood smear examination shows round lymphocytes with clumped chromatin and numerous smudge cells (arrow). CLL and small lymphocytic lymphoma (SLL) seem to represent different clinical manifestations of single disease entity (CLL/SLL). Patient presents with peripheral blood lymphocytosis ($<5 \times 10^9$/L) but often develops lymphadenopathy (1000X).

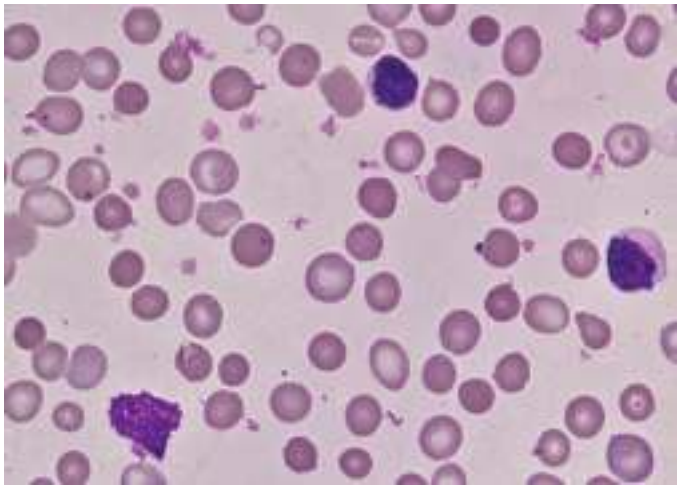


Fig. 9.98: Chronic lymphocytic leukemia (CLL) with autoimmune hemolytic anemia in Giemsa-stained peripheral blood smear. Anemia occurs because of autoimmune hemolysis and leukemic cells infiltration into bone marrow (1000X).

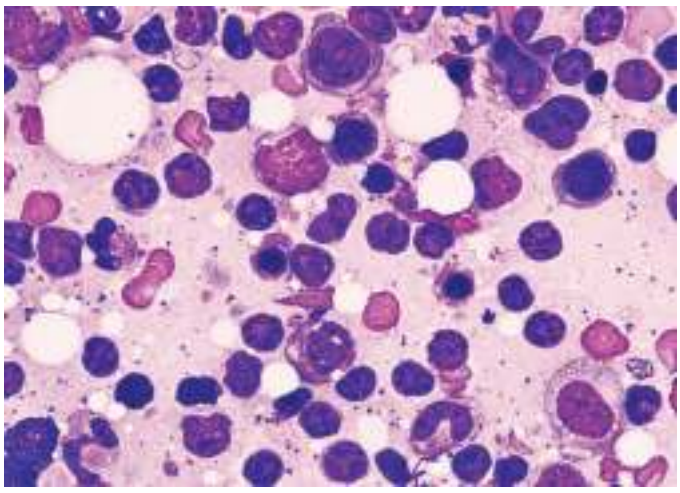


Fig. 9.99: Chronic lymphocytic leukemia (CLL) in Giemsa-stained bone marrow aspirate smear. It shows hypercellular bone marrow due to monoclonal proliferation of B lymphocytes gradually replacing erythroid, myeloid and megakaryocytic cell lines over a few years resulting in anemia, neutropenia and thrombocytopenia. Lymphocytes constitute >30% of nucleated cells in the marrow. Clonal B cells are small- to medium-sized mature appearing lymphocytes with round nuclei, clumped fragile chromatin, and scanty cytoplasm exhibiting monotonous appearance (1000X).

risk-stratify CLL. Rai clinical staging system in chronic lymphocytic leukemia (CLL) used in North America is given in Table 9.122. Binet staging system in chronic

Table 9.121 Morphologic features, flow cytometry, immunohistochemistry and cytogenetic analysis are best diagnostic yield. Distinguishing features of CLL and closely resembling non-Hodgkin lymphomas

Disease Entity	Surface Immunoglobulin (sIg)	CD5	CD10	CD19	CD20	CD23	Karyotype/ FISH
B chronic lymphocytic leukemia (B-CLL)	Weak expression	++	–	++	+	++	13q–, 11q–, 17p, trisomy 12
B cell prolymphocytic leukemia (B-PLL) with larger cells, eccentric nuclei and punched out nucleus	++	+/–	–	++	++	–	t(11;18) in occasional case
Follicular lymphoma (FL) leukemic phase	++	–	+	++	++	+/–	t(14;18), BCL-2a on chromosome 11
Mantle cell lymphoma (MCL)	++	++	–	++	++	–	t(11;14), cyclin D1b
Marginal zone lymphoma (MZL) leukemic phase	+	–	–	++	++	–	No consistent chromosomal abnormalities

BCL-2a on chromosome 11 is not expressed in normal B cells and detected by immunohistochemistry. FISH: Fluorescence in situ hybridization.

Table 9.122 Rai clinical staging system in chronic lymphocytic leukemia used in North America

Stage	Features	Risk Stratification and Median Survival	Median Survival
Stage 0	Blood and bone marrow lymphocytosis	Low risk	11.5 years
Stage I	Blood and bone marrow lymphocytosis + lymphadenopathy	Intermediate risk	11 years
Stage II	Blood and bone marrow lymphocytosis + splenomegaly or hepatomegaly with or without lymphadenopathy	Intermediate risk	7.8 years
Stage III	Anemia (Hb <11 g/dl)	High risk	5.3 years
Stage IV	Thrombocytopenia (platelet count <100,000/ μl)	High risk	7 years

Table 9.123 Binet clinical staging system in chronic lymphocytic leukemia used in Europe

Stage	Features	Lymph Nodes	Median Survival
Stage A	Hb and platelet count (normal range)	Lymphadenopathy <3 lymph nodes, hepatomegaly and splenomegaly	110 months
Stage B	Hb and platelet count (normal range)	Lymphadenopathy >3 lymph nodes, hepatomegaly and splenomegaly	80 months
Stage C	Hb <10 g/dl, platelet count <100,000/µl	Lymphadenopathy, hepatomegaly and splenomegaly	20 months

lymphocytic leukemia (CLL) in Europe is given in Table 9.123. Both the classifications are based on clinical and laboratory findings and prognosis.

PROGNOSIS

Biomarkers that are independent of clinical stage especially immunoglobulin heavy chain variable region gene (IgHV) mutation status and CD49a are valuable in assessing patients with early-stage CLL disease. Later, CLL progresses and requires treatment. CLL disease stage and several biomarkers correlate with prognostic information and overall median survival. Prognostic factors in chronic lymphocytic leukemia (CLL) are given in Table 9.124.

Immunoglobulin Heavy Chain Variable Region Mutation

The immunoglobulin expressed by B cells is composed of light and heavy chains encoded by different genes. CLL patients with somatic mutated IgVH genes (M-IgVH) are associated with good prognosis. These patients may not require treatment. CLL patients without somatic mutated IgVH genes are associated with poor prognosis. These patients require treatment within few years from diagnosis with median survival of 8–10 years.

Cytogenetic Alterations

Metaphase karyotyping has been used to risk-stratify chronic lymphocytic leukemia (CLL) patients into distinct subgroups. Complex chromosomal abnormalities in CLL patients are associated with poor prognosis.

- However, metaphase chromosomal analysis using G band technique has limited sensitivity due to the low mitotic rate in CLL patients.
- Therefore, fluorescence *in situ* hybridization (FISH) by using specific probe is currently used to demonstrate cytogenetic abnormalities.
  - Using a specific probe for chromosomal regions 13q, 11q, 17p and 12 alterations are detected in about 80% of CLL cases. Deletion 17q (TP53 locus) and 11q (ATM locus) are associated with poor prognosis.
  - Patient with isolated deletion 13q has longest survival in CLL patients. Deletion 17p is most often demonstrated in refractory CLL disease to treatment and associated with poor response to chemotherapy. Cytogenetic alterations restated to clinical course of chronic lymphocytic leukemia and other disorders are given in Table 9.125.

Table 9.124 Prognostic factors in chronic lymphocytic leukemia (CLL)

Parameters	Good Prognosis	Poor Prognosis
Stage of CLL	▪ Rai clinical staging system (0–1) ▪ Binet clinical staging system (A)	▪ Rai clinical staging system (III–IV) ▪ Binet clinical staging system (B, C)
Lymphocyte doubling time (LDT)	Slow doubling time <12 months	Rapid doubling time <6 months
Bone marrow trephine biopsy morphology	Nodular pattern	Diffuse pattern
Chromosomal abnormality	13q14 deletion	17p deletion (TP53 locus), 11q deletion (ATM locus), 6p deletion, 12q trisomy, t(11;14)
Molecular genetic mutations	Not applicable	NOTCH, TP53, SF3B1
IGVH genes	Hypermutated IgVH genes	Unmutated IgVH genes
ZAP expression	Low expression	High expression
CD38 expression	Negative	Positive
Lactic dehydrogenase (LDH) level	Normal level	Raised level

CD38 expression is surrogative marker of IgVH.

Table 9.125 Cytogenetic alterations restated to clinical course of chronic lymphocytic leukemia and other disorders

Cytogenetic Alterations	Associated Hematological Disorders	Features
11q deletion involving ATM	B-CLL	Marked lymphadenopathy associated with rapid progressive disease and poor prognosis
17p deletion involving TP53	B-CLL, prolymphocytic leukemia	Patients develop resistance to drugs associated with poor prognosis
12p Trisomy	Atypical CLL	Associated with aggressive clinical course and poor prognosis
13q14 deletion	B-CLL with IgVH gene mutation	Associated with favorable prognosis
6q deletion	B-CLL	CLL associated with progressive clinical course and poor prognosis

B-CLL: B cell chronic lymphocytic leukemia.

Molecular Genetic Alterations

Whole-exome sequencing has demonstrated more than 20 driver genes in CLL cases. Recurrent gene mutations have been found in noncoding genes. Somatic gene mutations of TP53, SF3B1, ATM or NOTCH1 are associated with short survival irrespective of IgVH mutation status in CLL cases.

- **ZAP-70 expression:** The receptor tyrosine kinase ZAP-70 is essential for T cell receptor signaling in response to antigen. CLL patients without mutated IgVH demonstrate higher expression of ZAP-70 by flow cytometry and have poor prognosis. ZAP-70 is a cytoplasmic protein expressed more in T cells than B cells. Such cases pose challenge in interpretation of results.
- **CD38 expression:** Increased CD38 expression on the cell surface of neoplastic lymphocytes in CLL patients is analyzed by flow cytometry, which correlates with poor prognosis. CD38 expression does not provide additional prognostic information derived from ZAP-70, CD49d expression and IgVH mutation status.
- **CD49d expression:** CD49d is an antigen subunit that regulates trafficking of B and T cells. Expression of CD49d analyzed by flow cytometry on CLL leukemic cells is associated with poor prognosis. CD49d analysis is superior to ZAP-70 and CD38. CD49d is independent prognostic marker for overall survival.
- **Lymphocyte doubling time:** Lymphocyte doubling time (LDT) of <12 months indicates progressive CLL disease. It is associated with decreased median survival independent of stage. Lymphocyte doubling time of <6 months reflects active CLL disease and requires treatment.

TREATMENT

Many different drugs and drug combinations can be used as the first-line treatment for chronic lymphocytic leukemia. Low-dose radiation therapy may be used if the patient with CLL disease has splenomegaly and lymphadenopathy in one part of the body. Leukapheresis and hematopoietic stem cell transplantation are also used for treating CLL patients. CLL active disease is defined by one of the above mentioned criteria in Table 9.126.

Assessing Response to Therapy

The international workshop on chronic lymphocytic leukemia published standardized criteria for analyzing response to therapy in CLL cases is determined clinical history, physical examination, peripheral blood and bone marrow examination. Minimal residual disease is analyzed by multicolor flow cytometry or polymerase chain reaction-based techniques.

Table 9.126 Chronic lymphocytic leukemia (CLL) active disease and associated clinical manifestations

Active CLL Disease Criteria	Clinical Manifestations of CLL
Constitutional symptoms	Fever, night sweats, weight loss
Symptomatic CLL patients with lymphadenopathy	Massive lymphadenopathy (>10 cm size in lymph node)
Symptomatic CLL patients with splenomegaly	Massive splenomegaly (>6 cm below costal margin)
Progressive bone marrow failure	Worsening anemia and/or thrombocytopenia
Rapidly progressive lymphocytosis	Lymphocyte doubling time <6 months
Autoimmune cytopenias	Autoimmune hemolytic anemia (AIHA), immune thrombocytopenia (ITP), pure red cell aplasia (PRCA), poorly responsive to corticosteroid treatment

HAIRY CELL LEUKEMIA

Hairy cell leukemia (HCL) is a rare indolent chronic lymphoproliferative neoplasm of mature B cells arising from post-germinal center mature memory B cell in marginal zone of lymph node. HCL is recognized as an entity according to revised 2024 classification of hematolymphoid neoplasms, which affects elderly persons during 40–85 years age group. It is four to five times more frequent in men than women. Disease most often involves bone marrow, peripheral blood and spleen; however, it may also involve liver, lymph nodes and skin.

- Hairy cell leukemia (HCL) has distinct clinical presentation that includes peripheral blood pancytopenias (anemia, leukemia and thrombocytopenia), bone marrow fibrosis (myelofibrosis), splenomegaly, hepatomegaly and small number of circulating monoclonal B cells with hair-like cytoplasmic projections. Leukemic infiltrates are demonstrated in the red pulp of spleen unlike other chronic lymphoproliferative disorders involving white pulp.
- Diagnosis of hairy cell leukemia (HCL) is based on morphologic evidence of monoclonal B cells with hair-like cytoplasmic projections (hairy cells), pancytopenia, monocytopenia, splenomegaly, BRAF V600 mutation and immunophenotype positive neoplastic hairy cells for CD11c, CD19, CD20 bright, CD22 bright, CD25, CD103 and CD305.
- Bone marrow trephine biopsy is essential to specify the degree of bone marrow involvement by monoclonal B cells with hair-like cytoplasmic projections (hair cells) and the presence of BRAF V600E somatic gene mutation (BRAF gene encoding threonine kinase) in hairy cell leukemia cases. Hairy cell leukemia patients are treated by BRAF inhibitors.
- Progression of hairy cell leukemia is based on a massive splenomegaly, leukocytosis, elevated numerous clonal B cells with hair-like cytoplasmic projections (hairy cells) in the peripheral blood and the immunoglobulin heavy chain variable region gene mutational status. IgG subclasses of VH4-34 encoded antibodies positive HCL cases are associated with poor prognosis. It is worth mentioning that IgG subclasses of VH4-34 encoded antibodies are demonstrated in systemic lupus erythematosus.

PATHOPHYSIOLOGY

BRAF V600E mutation is the causal genetic event in most patients with hairy cell leukemia. Gene mutation constitutively activates BRAF by autophosphorylation of the threonine kinase protein and downstream MEK-ERK signaling pathway leading to increased expression of genes involved in unrestricted proliferation and survival of monoclonal B cells leading to development of hairy cell leukemia.

CLINICAL FEATURES

Patient of hairy cell leukemia presents with moderate to massive splenomegaly due to pooling of blood and pancytopenia (anemia, leukopenia, and thrombocytopenia) as a result of bone marrow involvement.

- Patient may develop secondary hypersplenism due to increased splenic sequestration of peripheral blood cells in the spleen. Patient less often develops slight hepatomegaly, rarely lymphadenopathy.
- Constitutional symptoms are weakness, weight loss and fever. The patients are susceptible to bacterial infections especially mycobacterial organisms.
- Poor prognostic factors of hairy cell leukemia (HCL) include massive splenomegaly, leukocytosis ($>10\times10^9$/L), high number of neoplastic B cells with hair-like cytoplasmic projections (hairy cells) in the blood ($>5\times10^9$/L), high β_2-microglobulin, unmutated IgHV status, and IgHV4-34 positive immunoglobulin heavy chain variable gene rearrangement; and CD38 expression.

LABORATORY DIAGNOSIS

Laboratory investigations of HCL include peripheral blood smear, bone marrow, bone marrow trephine biopsy, histochemistry, immunohistochemistry, immunophenotyping, splenic histology and BRAF oncogene molecular analysis using DNA sequencing to detect somatic mutations.

Pathology Pearls: Hairy Cell Leukemia—Molecular Genetic Analysis of BRAF V600E Gene Mutation

- Using whole-exome sequencing, BRAF V600E somatic mutation has been demonstrated in 80–90% hairy cell leukemia cases. BRAF V600E somatic mutation in hairy cell leukemia is associated with poor prognosis.
- BRAF V600E gene is located on chromosome 7q34 and has 18 exons. BRAF V600E gene mutation occurs in exon 15 at position 1799, in which thymine and adenine are exchanged, leading to valine (V) being substituted by glutamate (E) at codon 600 (V600E) of the BRAF protein.
- The BRAF V600E mutation constitutively activates BRAF by autophosphorylation of the protein and downstream MEK-ERK signaling pathway, resulting in increased expression of genes involved in survival and proliferation.
- BRAF V600E gene mutation is also demonstrated in few cases of multiple myeloma, CLL and various solid malignant tumors such as cutaneous melanoma, cholangiocarcinoma, sarcoma, gastrointestinal stromal tumor (GIST), cancers of lung, ovary, urinary bladder, thyroid and prostate.

Laboratory Diagnosis of Hairy Cell Leukemia

Laboratory investigations of hairy cell leukemia (HCL) include peripheral blood smear examination, bone marrow examination, bone marrow trephine biopsy examination, histochemistry, immunophenotyping, splenic histology and BRAF oncogene analysis using DNA sequencing to detect somatic mutations.

Peripheral Blood Smear Examination

- Peripheral blood smear examination shows pancytopenia with a few monoclonal B cells (hairy cells) that are medium in size with moderately abundant pale blue cytoplasm, oval to reniform nuclei, open fine chromatin, absence of nucleoli and a characteristic circumferential cytoplasmic projection (hairs).
- White blood cell count is low due to both neutropenia and monocytopenia. Hairy cell leukemia (HCL), B cell derived in Giemsa-stained peripheral blood smear is shown in Figs 9.100 and 9.101.

Bone Marrow Smear Examination

Bone marrow aspirate smear is most often a dry tap in hairy cell leukemia due to extensive and diffuse bone marrow fibrosis as demonstrable by reticulin stain.

Bone Marrow Trephine Biopsy Examination

- Bone marrow trephine biopsy section shows a monotonous infiltrate of abnormal lymphocytes with abundant palestaining cytoplasm (fried egg appearance) and small nuclei.
- Monoclonal B cells (hairy cells) infiltration can vary and have a patchy and subtle and subtle interstitial or diffuse solid pattern.
- Presence of bone marrow fibrosis is common that can be markedly hypocellular and mimic of aplastic anemia.
- Hairy cell leukemia (HCL) in hematoxylin and eosin-stained bone marrow trephine biopsy section is shown in Fig. 9.102.

Histochemistry

Monoclonal B cells (hairy cells) show acid phosphatase staining after tartrate incubation (tartrate-resistant acid phosphatase).

Immunophenotyping

- Flow cytometric immunophenotyping is performed to establish a diagnosis of HCL.
- Monoclonal B cells (hairy cells) are positive for CD19 and CD20 (strong intensity), CD22, CD25, CD103 and CD11, which also demonstrate strong intensity for monoclonal surface immunoglobulins.

Markers	Expression
▪ CD19	▪ Positive (strong intensity)
▪ CD20	▪ Positive (strong intensity)
▪ CD200	▪ Positive (strong intensity)
▪ Monoclonal surface immunoglobulin-aS-HCL1	▪ Positive (strong intensity)
▪ CD22	▪ Positive
▪ CD25	▪ Positive
▪ CD103	▪ Positive
▪ CD11c	▪ Positive

Surgical Pathology: Splenectomy Specimen in Hairy Cell Leukemia (HCL)

Gross Morphology

Spleen is enlarged and weighing above 150 g. Cut surface of spleen shows diffuse and marked enlargement without nodules.

Light Microscopy

- Splenectomy specimen in hairy cell leukemia demonstrates marked expansion of red pulp because of an infiltrate of neoplastic hairy cells with fried egg appearance.
- Monoclonal B cells (hairy cells) are larger than lymphocytes with modest pale blue agranular cytoplasm, round or folded nuclei and without distinct nucleoli. There is an increase in volume, surface and length of red pulp vessels that can classically form lakes of blood lined by neoplastic hairy cells.

Immunohistochemistry Technique

Panel of immunohistochemical markers is performed in hairy cell leukemia mentioned below.

Markers	Expression
▪ CD19	▪ Positive
▪ CD20	▪ Positive
▪ Annexin A1	▪ Positive
▪ CD123	▪ Positive
▪ BRAF V600E mutation	▪ Positive

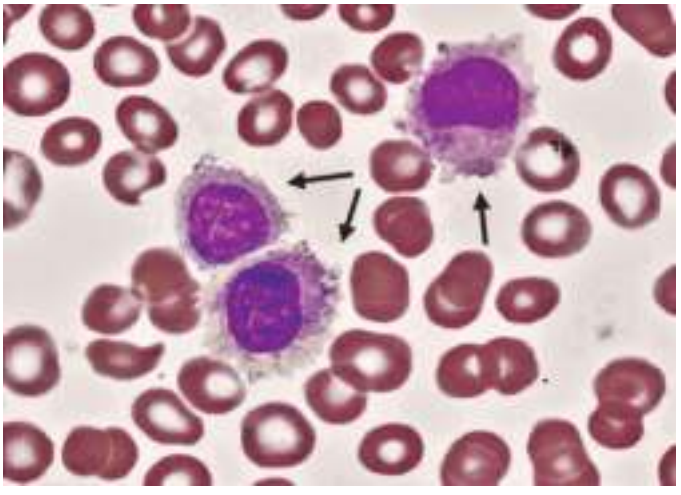


Fig. 9.100: Hairy cell leukemia (HCL), B cell derived in Giemsa-stained peripheral blood smear. It shows abnormal lymphocytes with oval or kidney cell nucleus, abundant pale-staining cytoplasm with hair-like circumferential cytoplasmic projections and relatively fine distributed chromatin (arrows). Flow cytometric analysis establishes HCL diagnosis with immunophenotype CD19, CD20 (strong intensity), CD22, CD25, CD103, and CD11C. In addition, HCL cells show acid phosphatase staining, after tartrate incubation (tartrate-resistant acid phosphatase—TRAP) (1000X).

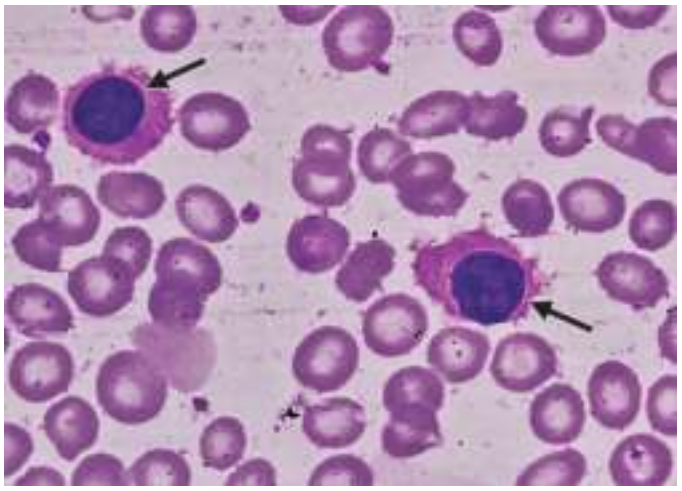


Fig. 9.101: Hairy cell leukemia (HCL), B cell derived in Giemsa-stained peripheral blood smear. It shows abnormal lymphocytes with oval or kidney cell nucleus, abundant pale-staining cytoplasm with hair-like circumferential cytoplasmic projections and relatively fine distributed chromatin (arrows) (1000X).

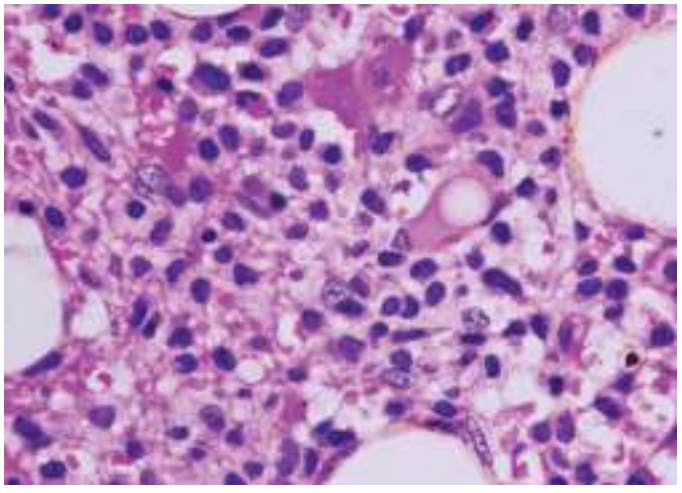


Fig. 9.102: Hairy cell leukemia (HCL) in hematoxylin and eosin-stained bone marrow trephine biopsy section. It shows a monotonous infiltrate of abnormal lymphocytes with abundant pale-staining cytoplasm (fried egg appearance) and small nuclei. Neoplastic hairy cells infiltration can vary and have a patchy and subtle and subtle interstitial or diffuse solid pattern. Presence of bone marrow fibrosis is common that can be markedly hypocellular and mimic of aplastic anemia (400X).

DIFFERENTIAL DIAGNOSIS

Hairy cell leukemia (HCL) should be differentiated from splenic lymphoma, with villous lymphocytes, prolymphocytic leukemia and large granular lymphocytic leukemia. Bone marrow aspirate is dry only in hairy cell leukemia. Hairy cells are positive with tartrate-resistant acid phosphatase (TRAP), but negative in other disorders.

TREATMENT

Hairy cell leukemia (HCL) often has an indolent course, and chemotherapy often produces long-lasting remission. Administration of chemotherapeutic agent 2-chlorodeoxyadenosine (2-CDA/cladribine) results in complete remissions for years.

B CELL PROLYMPHOCYTIC LEUKEMIA

B cell prolymphocytic leukemia (B-PLL) is an aggressive neoplasm defined by large prolymphocytes (55% of total circulating cells) in the peripheral blood. B-PLL is marked by absolute lymphocytosis **($>100\times10^9$/L).** Prolymphocytes contain abundant cytoplasm and nucleus with clumped chromatin and prominent vesicular nucleolus.

- B cell prolymphocytic leukemia involves peripheral blood, bone marrow and spleen. Although most cases of B-PLL originate *de novo* and can also be derived from chronic lymphocytic leukemia (CLL).
- Unlike CLL cases, patient with B-PLL presents with moderate to massive splenomegaly and minimal lymphadenopathy.
- Anemia and thrombocytopenia are demonstrated in 50% of cases. Bone marrow shows nodular or interstitial pattern of involvement.
- B cell prolymphocytic leukemia (B-PLL) demonstrates deletion of chromosome 17p resulting in inactivation of TP53 gene tumor suppressor gene.
- Poor prognostic factors of B cell prolymphocytic leukemia are aberrations in Myc and TP53 genes, lymphocytosis $>100\times10^9$/L and anemia. B-PLL does not respond to treatment and hence associated with worse prognosis.

Laboratory Diagnosis of B Cell Prolymphocytic Leukemia

Peripheral Blood Smear Examination

- Total leukocyte count is elevated. Differential leukocyte count shows marked lymphocytosis, most often $>100\times10^9$/L, anemia and thrombocytopenia.
- Size of prolymphocytes is larger than lymphocytes. The neoplastic B cells contain abundant pale basophilic cytoplasm, moderately condensed chromatin and prominent nucleolus.
- B cell prolymphocytic leukemia (B-PLL) demonstrates >55% prolymphocytes; and CLL cases show <10% prolymphocytes.
- In patients with B cell prolymphocytic leukemia (B-PLL), peripheral blood smear showing 11–55% prolymphocytes have an unpredictable clinical course.

Immunophenotyping	
Immunophenotyping of B cell prolymphocytic leukemia (B-PLL) most often differs from CLL demonstrating strong intensity expression of surface immunoglobulin and CD20, more variable expression of CD5, positivity with FMC7, and absence of CD23.	
Markers	**Expression**
▪ CD20 ▪ FMC-7 ▪ CD19 ▪ CD5 ▪ CD23	▪ Positive (strong intensity) ▪ Positive (strong intensity) ▪ Positive (strong intensity) ▪ Positive (variable intensity) ▪ Negative

ADULT T CELL LEUKEMIA

Adult T cell leukemia is an aggressive neoplasm associated with human T cell leukemia virus type 1 (HTLV-1) infection and prevalent in Japan and Central Africa affecting adults. It is classified into four different clinical variants that are acute, lymphomatous, chronic, and smoldering. In acute and chronic phase, condensed chromatin with a convoluted or polylobated nucleus, often called a "flower cell" or "cloverleaf".

- Patient presents with widespread lymphadenopathy, hepatosplenomegaly, skin rashes (50%) and elevated serum calcium levels due to increased osteoclastic activity.
- The number of circulatory leukemic cells does not correlate with the degree of bone marrow involvement, which suggests that circulating leukemic cells are recruited from other organs especially the skin involved in >50% of cases.
- The disease usually involves spleen, liver, skin, lungs, gastrointestinal tract and central nervous system.
- Skin lesions are classified as erythema, papules and nodules. In rare cases, tumor-like lesions on skin may be demonstrated.
- In adult T cell leukemia patients with hypercalcemia, imaging studies may reveal osteolytic lesions. FDG-PET/CT scan is most often positive in sites of disease activity.

Laboratory Diagnosis of Adult T Cell Leukemia
Total Leukocyte Count
Total leukocyte count is increased due to adult T cell leukemia spillover in blood.
Peripheral Blood Smear Examination
Leukemic cells medium- to large-sized lymphoid cells with prominent pleomorphic nuclei resembling clover leaf.

Bone Marrow Smear Examination	
Bone marrow smear examination shows patchy infiltration. Adult T cell leukemia has broad spectrum cytologic features such as pleomorphic small, medium and large cell types and rarely resembling angioblastic T cell lymphoma. Osteoclastic activity increases serum calcium levels.	
Immunophenotyping	
Leukemic cells are strongly positive with CD25.	
Marker	**Expression**
CD25	Positive

NON-HODGKIN'S LYMPHOMA: SPILLOVER IN BLOOD

Bone marrow is involved in stage IV of low-grade non-Hodgkin lymphoma in 60–80% cases and high-grade NHL in 30–40% cases. Neoplastic cells can spillover from bone marrow into peripheral blood, which can be identified in the peripheral blood of patients with non-Hodgkin's lymphoma (NHL) by several molecular genetic techniques and morphologic examination of peripheral blood smear in 8–20% of patients. It is essential to examine peripheral blood smear including blood counts and bone marrow trephine biopsy.

Laboratory Diagnosis of Non-Hodgkin's Lymphoma—Spillover in Blood
Peripheral Blood Smear Examination
Peripheral blood smears stained by Romanowsky stained demonstrate anemia (95%), thrombocytopenia (60%), neutropenia (35%) and atypical lymphoid cells in NHL patients.
Bone Marrow Examination
▪ Bone marrow shows deposits of NHL. Atypical lymphoma cells contain lobulated and notched nuclei. ▪ NHL deposits in bone marrow demonstrated in diffuse pattern is associated with poor prognosis. ▪ Deposits of follicular center cell lymphoma are demonstrated in paratrabecular pattern.
Immunophenotyping
Flow cytometry is a diagnostic tool to differentiate B and T cells origin in non-Hodgkin's lymphoma spillover in blood.

HODGKIN'S DISEASE

Hodgkin's disease is a malignant neoplasm of lymph node. It is important to determine whether the patient has only a single lymph node region involved, multiple node regions, or extranodal involvement.

- Diagnostic criteria of Hodgkin's disease include demonstration of neoplastic Reed-Sternberg cells

derived from B or T cells admixed with inflammatory cells in background due to cytokines produced by them. Hodgkin's disease has been discussed in detail under disorders of lymph node.

- Initially Hodgkin's disease involves adjacent lymph nodes then other nodes via lymphatic route in continuous and predictable fashion. Later it spreads via the blood and involves the spleen, liver and bone marrow. Spleen is practically always involved. Liver is involved if splenic hilum and retroperitoneal lymph nodes are involved. Bone marrow is involved in Hodgkin's disease depletion type. *Refer* to Hodgkin disease, details in Chapter 13.

MANTLE CELL LYMPHOMA: SPILLOVER IN BLOOD

Mantle cell lymphoma (MCL) occurs in the middle and elderly persons, which resembles small cell lymphoma except slightly different cellular details.

- Mantle cell lymphoma is disseminated, moderately aggressive incurable disease derived from the **naïve B cell** of lymphoid follicles in mantle zone of lymph node.
- Mantle cell lymphoma involves bone marrow in most cases. About 20% cases are associated with leukemia. MCL has tendency to involve gastrointestinal tract with submucosal polypoid nodules.
- Neoplastic cells in mantle cell lymphoma show t(11;14) with fusion of the cyclin D1 gene on chromosome 11 to the immunoglobulin heavy chain (IgH) promoter/enhancer region on chromosome 14. Fusion product leads to increased cyclins (CCND1/cyclin D1) expression with loss of cell cycle regulation.
- Median survival rate is 3–5 years in mantle cell lymphoma. Vast majority of patients do not respond to newer therapeutic modalities. A high proliferative rate (mitotic or Ki-67 indices) is associated with shorter overall survival. High Ki-67 proliferative index (>30%) is currently accepted cut off point.

Surgical Pathology: Lymph Node Biopsy in Mantle Cell Lymphoma (MCL)

Light Microscopy

- Mantle cell lymphoma comprises hyalinized blood vessels and scattered epithelioid histiocytes giving 'starry sky' appearance.
- Classic neoplastic cells are small with irregular indented nuclear contours. These neoplastic cells may have blastoid or pleomorphic, anaplastic morphology.
- Blastoid form of mantle cell lymphoma and sometimes classic cells may be accompanied by involvement of blood, bone marrow and spleen.

Immunophenotyping

- Neoplastic cells of mantle cell lymphoma express intense surface IgM /IgG/IgD with restriction of κ or λ.
- The lymphoma cells show positivity for BCL-2, CD5, FMC7 and CD43. These are sometimes positive for IRF4 and MUM1.
- Nuclear cyclin D1 is expressed in >95% of cases of mantle cell lymphoma.
- SOX11 positivity with monoclonal antibody is demonstrated in >90% of cases.
- Neoplastic cells are negative for CD10, CD25, CD23 and CD11C.

Markers	Expression
Surface IgM/IgG/IgD	Positive
BCL-2	Positive
CD5	Positive
CD43	Positive
FMC7	Positive (strong intensity)
Cyclin D1	Positive (strong intensity in nucleus)
IRF4	Positive (some cases)
MUM1	Positive (some cases)
CD10	Negative
CD25	Negative
CD23	Negative
CD11C	Negative

Translocation t(11; 14) creating cyclin D1–IgH fusion gene is demonstrated in mantle cell lymphoma.

Laboratory Diagnosis of Mantle Cell Lymphoma—Spillover in Blood

Mantle cell lymphoma, blastoid variant is diagnosed based on cytomorphology and flow cytometric immunophenotyping of the lymph node aspirate and peripheral blood smear examination.

Peripheral Blood Smear Examination

Peripheral blood smear examination shows a spectrum of cells ranging from small mature lymphocytes to medium- and large-sized lymphocytes with blast-like chromatin and prominent nucleoli.

Immunophenotyping

- The leukemic cells are monoclonal B cells with moderately intense surface IgM/IgG/IgD. These cells are CD5 positive, cyclin D1 positive and CD10 negative.
- The flow cytometric immunophenotyping and DNA ploidy analysis of the peripheral blood and material obtained by aspiration cytology support the diagnosis of mantle cell lymphoma, blastoid variant.

Markers	Expression
CD5	Positive
Cyclin D1	Positive
CD10	Negative

SPLENIC LYMPHOMA WITH VILLOUS LYMPHOCYTES

Splenic lymphoma with villous lymphocytes (SLVL) is a chronic B cell lymphoproliferative disorder characterized by a clonal expansion of atypical B cells with villous projections in the peripheral blood. The disease involves bone marrow and spleen resulting in anemia and splenomegaly. SLVL involves bone marrow in patchy manner and then spillover in blood. Neoplastic cells show a few short villi in one direction, which are negative for CD11 and CD103.

MULTIPLE MYELOMA AND OTHER MONOCLONAL GAMMOPATHIES

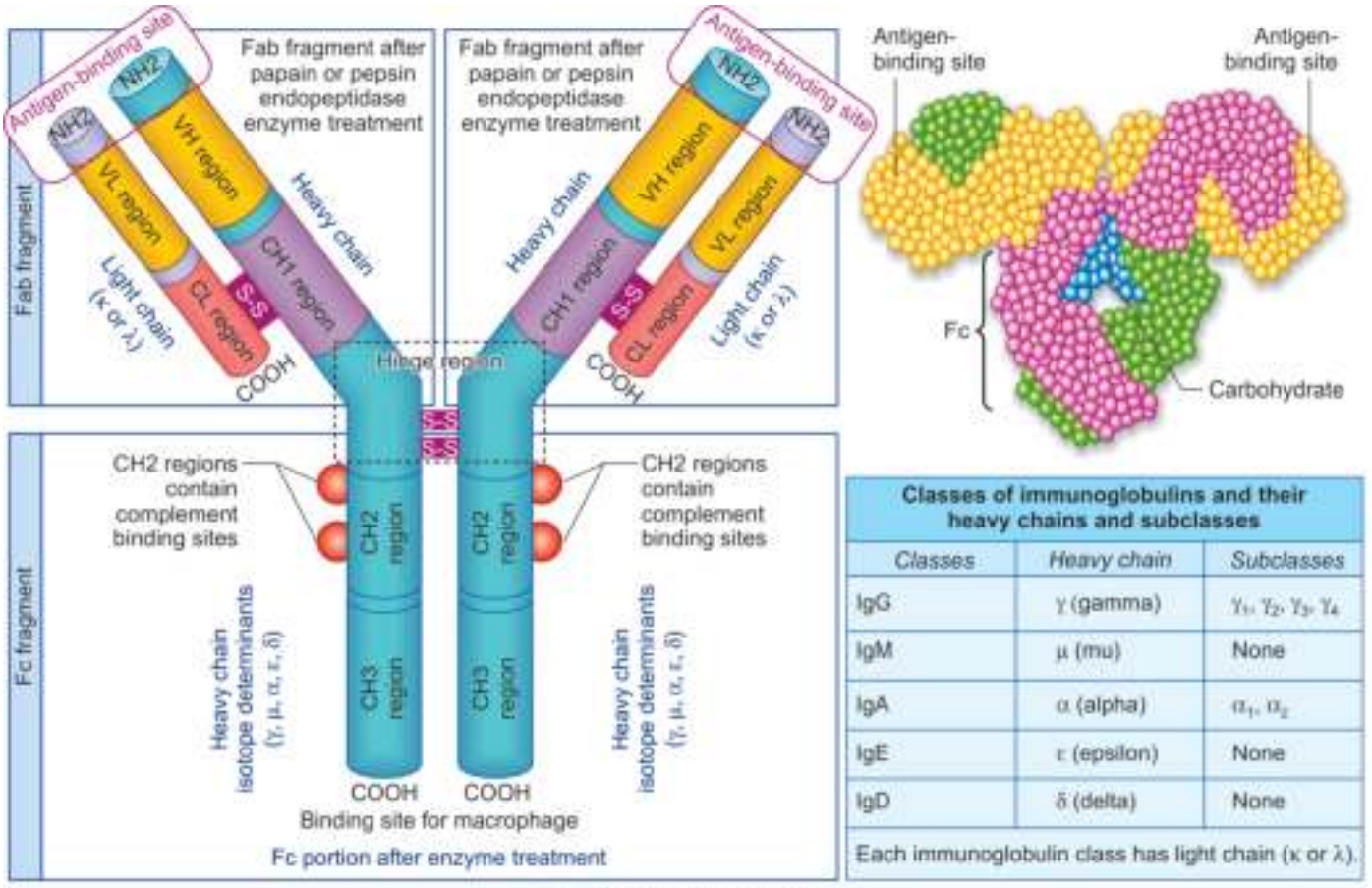
Healthy plasma cells synthesize immunoglobulins that fight infection. The immunoglobulins are made up of heavy and light chains. There are five types of immunoglobulins: IgG, IgA, IgM, IgD and IgE. Schematic representation of immunoglobulin structure is shown in Fig. 9.103.

- Clonal plasma cell proliferative disease usually produces monoclonal immunoglobulin (M protein) that may be used as a 'serologic tumor marker'. The exception to this rule is nonsecretory multiple myeloma, in which monoclonal proteins are not produced hence not detected in serum or urine. Because clonal plasma cells synthesize monoclonal immunoglobulin, clonal plasma cell disorders are also called monoclonal gammopathies or plasma cell dyscrasias.
- The secreted monoclonal immunoglobulins can be used as a diagnostic tool for the identification of the clone of plasma cells as well as quantitative serologic marker to follow-up the course of clonal plasma cell disorder and response to therapy.
- Unlike most serologic tumor markers, monoclonal immunoglobulins (M proteins) are extremely diverse. Each of the M proteins has unique variable region sequences and the molecules may range from pentameric IgM (~9,00,000 daltons) to monomeric free light chains (~24,000 daltons).
- In addition, although some plasma cell dyscrasias such as multiple myeloma, Waldenström macroglobulinemia, and benign monoclonal gammopathies of undetermined significance (MGUS) present with monoclonal immunoglobulin (M protein) concentrations expressed in grams per liter of serum, others have a little or virtually no circulating M-protein. It is the diversity of structure and concentration of M protein that make this clonal marker so interesting and challenging.
- The low-tumor burden in clonal plasma cell disorders represent a group of monoclonal gammopathies, in which there may not be increased proliferation of clonal plasma cells, but in which the cell products cause pathology. Some disorders associated with monoclonal immunoglobulins are given in Table 9.127.

MULTIPLE MYELOMA

Multiple myeloma (MM) is a clonal plasma cell proliferative disorder characterized by the excessive synthesis of monoclonal paraprotein (M protein) leading to evidence of specific end-organ damage, which constitutes 10–15% of hematopoietic stem cell neoplasms.

- Proliferation of monoclonal plasma cells in bone marrow results in extensive osteolytic skeletal lesions, hypercalcemia, anemia and/or soft tissue plasmacytomas. In addition, the excessive production of monoclonal proteins is nephrotoxic and can cause renal failure. Deficient production of functional immunoglobulin increased risk for developing life-threatening infections.
- Multiple myeloma occurs most commonly in people over 60 years of age. Median age is 65–70 years. Only 2% of cases in people under 40 years of age. Disease never affects children.
- Risk factors for development of multiple myeloma include over 60 years of age, exposure to ionizing radiation and chemical agents such as benzene, organic solvents, farmers exposed to insecticides and herbicides, and monoclonal gammopathy of undetermined significance (MGUS).
- Diagnosis of multiple myeloma is based on the combination of clinical, morphologic, radiologic and immunologic findings. Bone disease is the major cause of morbidity in patients with multiple myeloma, which can be detected on skeletal radiographs, magnetic resonance imaging (MRI), or computed tomography (CT) scans.
- Multiple myeloma is classified into four types: mature, immature, pleomorphic and plasmablastic.
- There are three patterns in which multiple myeloma infiltrates bone marrow: nodular, interstitial and diffuse. Dutcher bodies are highly specific to the clonal plasma cells. Immunophenotyping, CD138 marker is highly specific for plasma cells. Abnormal expression of CD56 is demonstrated in 70–80% of cases by flow cytometry analysis. CD56 expression definitely indicates multiple myeloma, suggesting its high diagnostic values.



Classes of immunoglobulins and their heavy chains and subclasses

Classes	*Heavy chain*	*Subclasses*
IgG	γ (gamma)	γ_1, γ_2, γ_3, γ_4
IgM	μ (mu)	None
IgA	α (alpha)	α_1, α_2
IgE	ε (epsilon)	None
IgD	δ (delta)	None

Each immunoglobulin class has light chain (κ or λ).

Immunoglobulin structure

1. Immunoglobulin consists of 2 heavy chains and 2 light chains joined together by disulfide bonds.
2. Each heavy chain consists of a variable region (VH) and constant region encompassing functional domains named CH1, CH2 and CH3. Each light chain has a variable region (VL) and a constant region (VC).
3. The carboxy (–COOH) end of both the heavy chains and light chains is highly conserved, the amino acid sequence is very similar for all immunoglobulins of the same class: IgG, IgM, IgA, IgE or IgD.
4. Both the heavy chains and light chains at the amino(–NH2) end of proteins are highly variable, which allows for the development of specific antigen-binding sites.
5. Enzyme will cleave the immunoglobulin molecule at several points in the hinge region, resulting in three fragments: one Fc fraction crystallizable and two Fab fragments (antigen-binding fraction). Hinge region is a flexible amino acid stretch in the central portion of the heavy chains of the IgG or IgA immunoglobulin classes, which link these two chains by disulfide bonds.
6. Papain cleaves the heavy chain CH2 domains above the disulfide bond that connect heavy chains and yields three different fragments. Pepsin endopeptidase enzyme cleaves IgG heavy chains at a point below the disulfide bond that links two heavy chains. The resulting fragment consists of two Fab fragments linked together by heavy chain disulfide bonds.

Fig. 9.103. Schematic representation of immunoglobulin structure. Immunoglobulins are composed of two heavy and two light chains. They can be separated functionally into variable (V) domains that bind antigens and constant (C) domains that specify effector functions such as activation of complement or binding to Fc receptors.

- Plasma cell neoplasms and their clinical variants are given in Table 9.128. Plasma cell neoplasms and their key features are given in Table 9.129. Differentiating features of multiple myeloma, smoldering myeloma and monoclonal gammopathy of undetermined significance (MGUS) are given in Table 9.130. Multiple myeloma-related organ or tissue impairment (end organ damage) due to the clonal plasma cell proliferative process is given in Table 9.131.

TERMINOLOGY

Multiple myeloma is not a single disease. There are wide variations of multiple myeloma subtypes: (a) inactive MGUS—monoclonal gammopathy of undetermined significance, (b) SMM—smoldering myeloma and (c) active multiple myeloma. Multiple myeloma can be determined by using specific tests like fluorescence *in situ* hybridization (FISH) analysis, cytogenetics testing and gene profiling through bone marrow aspirates.

Table 9.127 Some disorders associated with monoclonal immunoglobulins

Related to Clonal B Cell Proliferation
Multiple myeloma
Solitary plasmacytoma
Smoldering multiple myeloma (asymptomatic multiple myeloma)
Plasma cell leukemia
Amyloid light chain (primary) amyloidosis
Heavy chain deposition disease
Monoclonal gammopathy of undetermined significance
POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, skin changes)
Light chain deposition disease
Waldenström macroglobulinemia
Type 1 cryoglobulinemia
Non-Hodgkin lymphoma
Chronic lymphocytic leukemia
Castleman disease
Post-transplant monoclonal gammopathy
Autoimmune Disorders
Rheumatoid arthritis
Scleroderma
Systemic lupus erythematosus
Sjögren syndrome
Psoriatic arthritis
Immune complex vasculitis/type 2 cryoglobulinemia
Chronic inflammatory demyelinating polyneuropathy
Chronic Viral Infections
Human immunodeficiency virus
Hepatitis C virus (HCV)
Skin Disorders
Scleroderma
Schnitzler's syndrome
Pyoderma gangrenosum

Secretory Multiple Myeloma

Clonal proliferation of plasma cells synthesizing monoclonal immunoglobulins (paraproteins) are known as secretory multiple myeloma. Approximately 60–70% of secretory myeloma patients have IgG myeloma and about 20% have IgA myeloma. IgG or IgA monoclonal proteins are almost always found in multiple myeloma.

Table 9.128 Plasma cell neoplasms and their clinical variants

Plasma Cell Myeloma
▪ Smoldering multiple myeloma (asymptomatic multiple myeloma) ▪ Nonsecretory myeloma ▪ Plasma cell leukemia
Plasmacytoma
▪ Solitary plasmacytoma of bone ▪ Extraosseous (extramedullary) plasmacytoma in upper respiratory tract (nose, paranasal sinuses, nasopharynx and tonsils)
Monoclonal Gammopathy of Undetermined Significance (MGUS)
▪ MGUS IgM ▪ MGUS non-IgM ▪ MGUS light chain
Monoclonal Immunoglobulin Deposition Diseases
▪ Amyloid light chain (primary) amyloidosis ▪ Systemic light and heavy chain deposition
Plasma Cell Neoplasms with Associated Paraneoplastic Syndromes
▪ POEMS syndrome also known as osteosclerotic myeloma is characterized by polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, and skin changes ▪ TEMPI syndrome (provisional) is multisystem disease characterized by presence of telangiectasis, erythrocytosis with elevated erythropoietin levels, monoclonal gammopathy, perinephric fluid collections, and intrapulmonary shunting

- IgM multiple myeloma is very uncommon. Immunoglobulin D (IgD) multiple myeloma affects 1–2% of persons with multiple myeloma in elderly men under 60 years of age.
- Signs and symptoms are the same as other types. Immunoglobulin E (IgE) multiple myeloma rarest type of multiple myeloma.
- Secretory multiple myeloma tends to be aggressive and progresses to plasma cell leukemia, which spreads outside the bone marrow quickly. Multiple myeloma in Giemsa-stained bone marrow aspirate smear is shown in Fig. 9.104.

Light Chain Multiple Myeloma

Light chain multiple myeloma (LCMM) constitutes 15% of patients with multiple myeloma, which has a poor prognosis when compared to IgA or IgA variant. Patients with λ light chain multiple myeloma have a three times worse prognosis than κ light chain multiple myeloma.

- Renal failure, bone disease, and systemic light chain associated amyloidosis appear to be more frequent in patients with light chain multiple myeloma.
- Light chain multiple myeloma patients secrete either low molecular weight κ or λ chains when get filtered

Table 9.129 Plasma cell neoplasms and their key features

Key Features	Clinical Notes
Monoclonal gammopathy of undetermined significance (MGUS) non-IgM	
▪ Serum M protein (IgG or IgA;) <30 g/L ▪ Clonal bone marrow plasma cells <10% ▪ Absence of myeloma-defining events (MDE) end organ/tissue damage, e.g. hypercalcemia, renal insufficiency, anemia, and bone lesions (CRAB) and amyloidosis attributable to the plasma cell proliferative disorder	Progression risk to multiple myeloma, amyloidosis or plasmacytoma in 0.5–1.0% per year
Monoclonal gammopathy of undetermined significance (MGUS) IgM	
▪ Serum IgM protein <30 g/L ▪ Bone marrow demonstrates lymphoplasmacytic cells <10% ▪ Absence of end-organ damage (anemia, constitutional symptoms, hyperviscosity, lymphadenopathy), hepatosplenomegaly or myeloma-defining events (MDE) or amyloidosis	Progression of MGUS-IgM increases risk (1.5% per year) for Waldenström's macroglobulinemia or lymphoproliferative disorder
Monoclonal gammopathy of undetermined significance (MGUS) light chain	
▪ Abnormal free light chain ratio (<0.26 or >1.65) ▪ Increased level of the corresponding free light chain in serum ▪ No immunoglobulin heavy chains expression ▪ Urinary excretion of monoclonal protein <500 mg per 24 hours ▪ Clonal plasma cells <10% in bone marrow ▪ Absence of myeloma-defining events (MDE) end organ/tissue damage, e.g. hypercalcemia, renal insufficiency, anemia, and bone lesions (CRAB) and amyloidosis attributable to the plasma cell proliferative disorder	Progression of MGUS-light chain increases risk (0.3% per year) for multiple myeloma, amyloidosis or plasmacytoma
Plasma cell myeloma (multiple myeloma)	
▪ Bone marrow or bone marrow trephine biopsy proven plasmacytoma consisting of ≥10% clonal plasma cells ▪ M-spike in serum or urine on electrophoresis ▪ Related organ or tissue damage (CRAB), i.e. hypercalcemia, renal insufficiency, anemia, bone lesions ▪ Myeloma defining events (MDE): • Hypercalcemia (serum calcium >11 mg/dl) • Anemia (hemoglobin <10 g/dl) • Bone lesions ≥1 osteolytic lesion on conventional radiograph, CT scan, PET-CT scan • Bone marrow clonal plasma cells ≥60% • Involved/uninvolved serum free light chains ratio ≥100 with involved serum free light chains ≥100 mg/L • Presence of ≥1 focal lesion on MRI of spine	Must fulfill at least one myeloma defining events (MDE) should be related to plasma cell disorder
Asymptomatic (smoldering) myeloma	
▪ IgG or IgA; M protein spike in serum or urine >30 g/L ▪ Monoclonal plasma cells ≥10% in bone marrow ▪ Absence of myeloma-defining events (MDE) or amyloidosis	Progression risk to multiple myeloma, amyloidosis is 10% per year, then 3% per year, then 3% next 5 years per year; and then 1% per year thereafter
Nonsecretory myeloma	
▪ Monoclonal plasma cells ≥10% in bone marrow without monoclonal immunoglobulin (M proteins) synthesis hence absent in serum and urine ▪ Disorder is negative for serum protein electrophoresis (SPE)/serum immunofixation electrophoresis (IFE) ▪ There is lower incidence of renal insufficiency, hypercalcemia, and depression of normal IgG	Nonsecretory myeloma could be misdiagnosed if the workup does not include an accurate study of serum free light chain test only with small amounts monoclonal proteinuria
Osteosclerotic plasma cell myeloma	
▪ Plasma cells entrapped in areas of fibrosis in bone marrow ▪ Thickened trabecular bone present ▪ Usually, lambda light chain restricted	Excessive proliferation and improper functions of plasma cells in the bone marrow

Contd...

Table 9.129 Plasma cell neoplasms and their key features (*Contd...*)

Key Features	Clinical Notes
▪ Usually does not meet criteria for classical myeloma: • Bone marrow plasmacytosis usually <5–10%, if present • Serum M protein usually <3.5 g/dl, if present • Bence-Jones protein in urine usually <1 g/24 hours, if present	
Solitary plasmacytoma of bone	
▪ Biopsy proven solitary collection of clonal plasma cells in bone marrow (<10%) ▪ Can demonstrate M spike in serum or urine (<30 g/L) ▪ No clonal plasma cells elsewhere in bone marrow ▪ No related organ or tissue damage ▪ Absence of myeloma-defining events (MDE) or amyloidosis	Progression to multiple myeloma within 3 years in 60% of cases
Extraosseous (extramedullary) plasmacytoma	
▪ Localized tumor in soft tissues (oropharynx) other than bone ▪ M protein in serum or urine absent or lower than in myeloma	Progression to multiple myeloma within 3 years in 20% of cases
POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy and skin changes) syndrome	
▪ **Mandatory diagnostic criteria:** These include polyneuropathy, monoclonal plasma cell disorder (usually λ light chain) ▪ **Major diagnostic criteria:** These include osteosclerotic lesions, Castleman's disease, elevated VEGF (vascular endothelial growth factor) ▪ **Minor diagnostic criteria:** These must fulfill at least 1 criterion such as organomegaly, extravascular volume overload, endocrinopathy, skin changes, papilledema, thrombocytosis/polycythemia	POEMS syndrome occurs in the setting of plasma cell dyscrasia
Covert myeloma	
No tumor formation, but synthesis of abnormal paraprotein resulting in amyloidosis	Risk for amyloidosis
Primary amyloidosis	
▪ Amyloidosis occurs in the setting of plasma cell dyscrasia in which abnormal proteins build up in tissues and organs. Aggregation of abnormal proteins are called amyloid deposits ▪ Most common forms of system amyloidosis include light chain (AL amyloidosis) and reactive amyloid associated (AA amyloidosis) ▪ Monoclonal plasma cell disorder is diagnosed by demonstration of either by serum or urine electrophoresis, bone marrow, or abnormal serum free light chains ▪ Positive amyloid staining by Congo red stain in any tissue such as kidney, liver, heart or peripheral nerves	A biopsy from abdominal fat and affected organ is required to confirm amyloidosis
Light and heavy chain deposition disease	
Monoclonal immunoglobulins deposit usually light chain deposit disease (LCDD) or heavy chain deposit disease (HCDD) along basement membrane	Light and heavy chain deposition disease along basement membrane

Table 9.130 Multiple myeloma-related organ or tissue impairment (end-organ damage) due to the clonal plasma proliferative process

Hypercalcemia
Renal insufficiency
Anemia (hemoglobin 2 g/dl below the normal limit)
Bone lesions: osteolytic or osteoporosis with compression fractures possibly confirmed by MRI or CT scan
Others: amyloidosis, recurrent infections (more than two episodes in 12 years), extramedullary plasmacytomas and symptomatic hyperviscosity (rare)

at glomerulus, reabsorbed and catabolized in renal tubules.

- About 85% of patients with multiple myeloma, synthesize complete monoclonal immunoglobulins. When multiple myeloma progresses, monoclonal plasma cells start to synthesize more light chains than the heavy chains.
- In patients with light chain multiple myeloma, clonal plasma cells show rearrangements in immunoglobulin heavy chains (IgH) at the DNA level thereby leading to an inability to produce IgH.
- In most instances, one IgH allele has a germline configuration for the D, J and C domains, whereas

Table 9.131 Differentiating features of multiple myeloma, smoldering myeloma and monoclonal gammopathy of undetermined significance (MGUS)

Parameters	Multiple Myeloma	Smoldering Myeloma	Monoclonal Gammopathy of Undetermined Significance (MGUS)
Osteolytic lesions	Present	Absent	Absent
Myeloma related organs damage	Present	Absent	Absent
Clonal proliferation of plasma cells in the bone marrow	>30%	10–30%	<10%
Monoclonal protein (M protein)	>35 g/dl	>30 g/dl	<30 g/dl
Excretion of κ or λ light chains in urine	>3 g/24 hours	1–3 g/24 hours	>1 g/24 hours

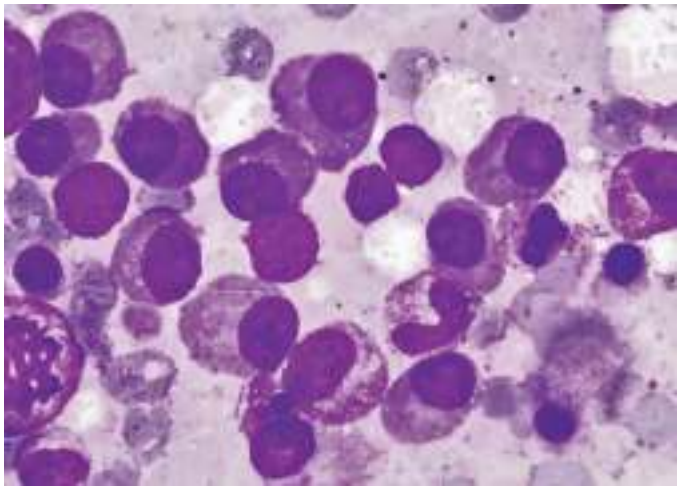


Fig. 9.104: Multiple myeloma in Giemsa-stained bone marrow aspirate smear. Replacement of bone marrow hematopoietic precursor cells by an infiltrate of malignant plasma cells is shown. Neoplastic plasma cells have eccentric nucleus and perinuclear halos. A few binuclear plasma cells are also present (1000X).

the second allele is involved in translocation. This molecular finding, in contrast with conventional multiple myeloma in which one allele has a functional rearrangement, whereas the second allele is usually involved in a translocation.

- Since light chain multiple myeloma is more aggressive disease, therefore serum-free light chain test is better predictor of clinical outcome than the amount of M protein in serum.

Nonsecretory Multiple Myeloma

Nonsecretory multiple myeloma is diagnosed by tissue infiltration by clonal plasma cells >10% without monoclonal immunoglobulin in serum or urine.

- Nonsecretory multiple myeloma is negative for serum protein electrophoresis (SPE)/serum immunofixation electrophoresis (IFE).
- About 85% cases of nonsecretory multiple myeloma demonstrate impaired secretion of immunoglobulin and have cytoplasmic immunoglobulin demonstrated by immunophenotyping and immunohistochemistry technique. There is lower incidence of renal insufficiency, hypercalcemia, and depression of normal IgG.

Hyperdiploid versus Hypodiploid Multiple Myeloma

Multiple myeloma can be categorized into hyperdiploid or hypodiploid myeloma based on the number of chromosomes found in the clonal plasma cells.

- Hyperdiploid multiple myeloma clonal plasma cells have more chromosomes than normal, which occurs in about 45% cases of multiple myeloma patients associated with less aggressive clinical course.
- Hypodiploid multiple myeloma clonal plasma cells have fewer chromosomes than normal, which occur in about 40% cases of multiple myeloma patients associated with more aggressive clinical course.

Osteosclerotic Multiple Myeloma

Osteosclerotic multiple myeloma occurs in young persons, which does not meet criteria of classical myeloma and shows (a) bone marrow plasmacytosis usually <5–10%, if present, (b) serum M protein usually <3.5 g/dl, if present, and (c) Bence-Jones protein in urine usually <1 g/24 hours, if present.

- There is excessive proliferation and improper functions of clonal plasma cells in the bone marrow. Clonal plasma cells are entrapped in areas of fibrosis in bone marrow.
- Trabecular bone shows thickening. λ light chain is usually restricted. **POEMS** (**P**: Polyneuropathies, **O**: Organomegaly, **E**: Endocrinopathy, **M**: Myeloma protein, **S**: Skin changes) is seen in rare cases of osteosclerotic multiple myeloma.

Smoldering Multiple Myeloma (Asymptomatic Multiple Myeloma)

Smoldering multiple myeloma is more likely to produce asymptomatic multiple myeloma than monoclonal gammopathy of undetermined significance (MGUS) without symptom. When compared to MGUS, smoldering multiple myeloma has a larger monoclonal protein (M protein) in the blood or presence of more monoclonal plasma cells in the bone marrow. The

levels of these markers are lower than those in active multiple myeloma.

- Smoldering multiple myeloma also progresses slowly, but in contrast to MGUS, after five years, about 50% of these patients progress to active multiple myeloma. PET-CT scan and MRI are essential to exclude bone disease.
- Diagnostic criteria of smoldering multiple myeloma are monoclonal immunoglobulins (IgG or IgA); M protein spike in serum or urine >30 g/L, and or bone marrow shows monoclonal plasma cells 10–30% and absence of myeloma-defining events (MDE) such as organ or tissue impairment including bone disease or amyloidosis.
- Risk of progression of smoldering multiple myeloma to active multiple myeloma is 10% per year in the first five years after diagnosis. Smoldering multiple myeloma in hematoxylin and eosin-stained bone marrow trephine biopsy section is shown in Fig. 9.105.

Plasmablastic Multiple Myeloma

Plasmablastic multiple myeloma is an aggressive neoplasm presenting with overlapping features of multiple myeloma and non-Hodgkin's lymphoma.

- The cytomorphologic features of plasma cell neoplasms range from mature, immature, pleomorphic and plasmablastic.
- Plasmablastic multiple myeloma is a morphologic subset of multiple myeloma, in which Giemsa-stained bone marrow aspirate smear shows 2% of plasmablasts.
  - Plasmablasts are characterized by fine reticular nuclear chromatin pattern, large nucleus (>10 µm in diameter), prominent large nucleolus and variable amount of cytoplasm.
  - Morphology of plasmablastic multiple myeloma is an independent predictor of poor survival after autologous hematopoietic stem cell transplantation for multiple myeloma.
  - There is no consensus on the management of plasmablastic multiple myeloma.
  - When combined with chemotherapy, anti-CD38 monoclonal antibody daratumumab may effectively target plasmablastic multiple myeloma clonal plasma cells.
- Plasmablastic multiple myeloma in Giemsa-stained bone marrow aspirate smear is shown in Fig. 9.106. Plasmablastic multiple myeloma in hematoxylin and eosin-stained bone marrow trephine biopsy is shown in Fig. 9.107.

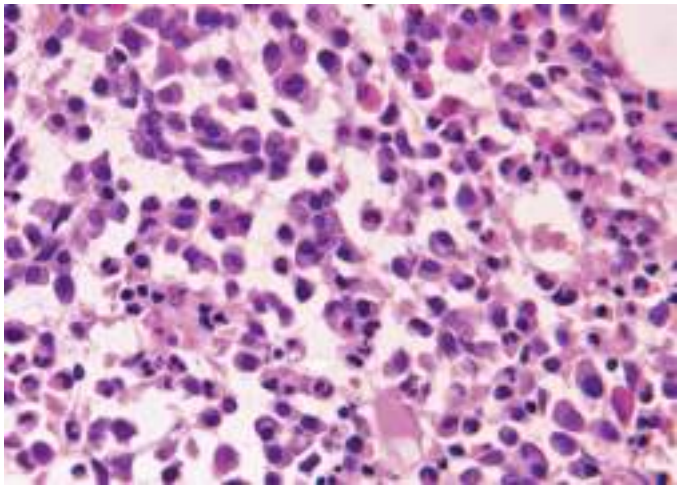


Fig. 9.105: Smoldering multiple myeloma in hematoxylin and eosin-stained bone marrow trephine biopsy section. It shows clusters of clonal plasma cells (400X).

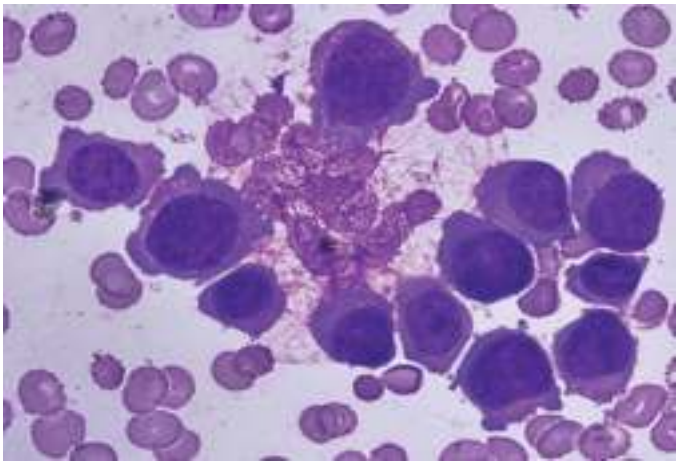


Fig. 9.106: Plasmablastic multiple myeloma in Giemsa-stained bone marrow aspirate smear. Plasmablastic clonal plasma cell contains centrally placed immature large nucleus with reticular chromatin and prominent nucleolus with high nucleocytoplasmic ratio and variable amount of cytoplasm (1000X).

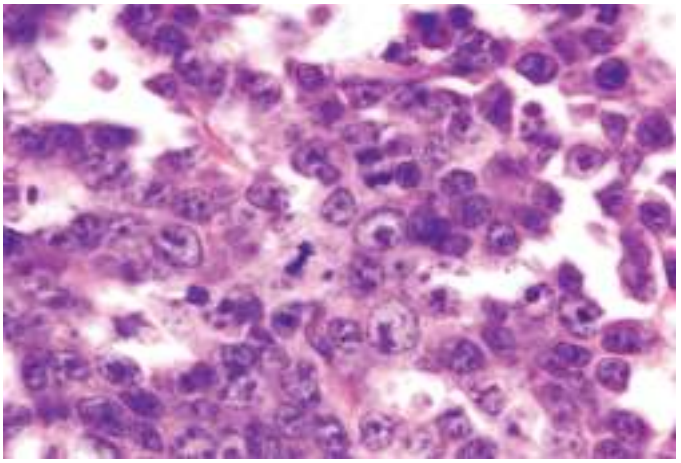


Fig. 9.107: Plasmablastic multiple myeloma in hematoxylin and eosin-stained bone marrow trephine biopsy section. It shows sheets of plasmablastic clonal plasma cells, which contain centrally placed immature large nucleus with reticular chromatin and prominent nucleolus with high nucleocytoplasmic ratio variable amount of cytoplasm (400X).

Plasma Cell Leukemia

Plasma cell leukemia (PCL) is diagnosed by absolute number of clonal plasma cells in peripheral blood $\geq 2 \times 10^9$/L or 20% of monoclonal plasma cells on differential white blood cell count. Disorder may be primary plasma cell leukemia or represent a late-stage transformation of multiple myeloma (secondary).

- Monoclonal plasma cells in plasma cell leukemia typically lack expression of CD56 and has more frequent abnormal karyotype. Genomic abnormalities analyzed by cytogenetic studies by fluorescence *in situ* hybridization (FISH) show predominance of monosomy and deletions of chromosome 13, t(11;14), del(17q), gain/amp(1q) and del(1p). Bone pain and osteolytic lesions are less common.
- Clonal plasma cells have ability to survive and grow outside of the bone marrow. Plasma cell leukemia is typically associated with extramedullary lesions (e.g. pleural effusion, lymphadenopathy, organomegaly and skin involvement).
- Plasma cell leukemia (PCL) in Giemsa-stained peripheral blood smear is shown in Fig. 9.108. Pleomorphic plasma cell leukemia in Giemsa-stained bone marrow aspirate smear is shown in Fig. 9.109.

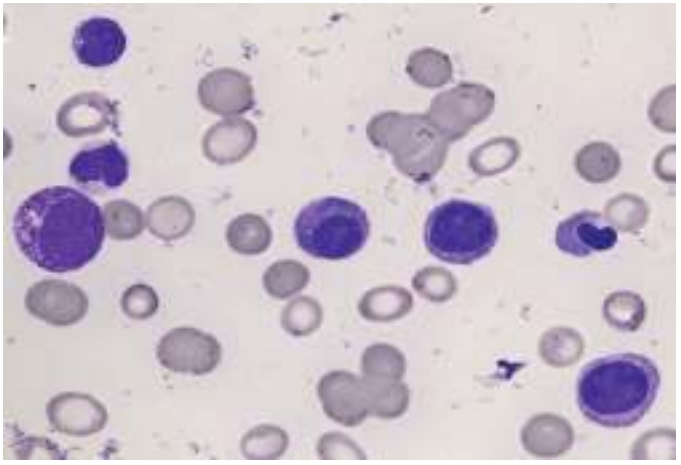


Fig. 9.108: Plasma cell leukemia (PCL) in Giemsa-stained peripheral blood smear. It shows spillover of myeloma cells in peripheral blood. Plasma cells constitute >20% of blood leukocytes in the peripheral blood (1000X).

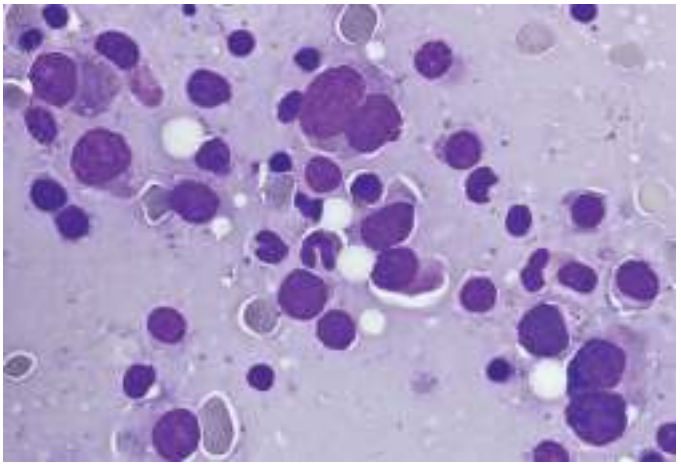


Fig. 9.109: Pleomorphic plasma cell leukemia in Giemsa-stained peripheral blood smear. It shows spillover of pleomorphic myeloma cells in peripheral blood. Plasma cells constitute >20% of blood leukocytes in the peripheral blood (1000X).

Solitary Osseous/Extraosseous Plasmacytoma

Unlike multiple myeloma, solitary osseous/extraosseous plasmacytoma occurs either in bone or soft tissues (oropharynx). Single area of bone destruction occurs due to clonal plasma cells.

- Bone marrow examination shows <10% clonal plasma cells, but findings are not consistent with multiple myeloma. There is no end-organ damage or amyloidosis other than solitary bone lesion. Most cases of solitary plasmacytoma involving bone evolve into multiple myeloma. Extraosseous plasmacytoma is commonly seen in IgD myeloma.
- Solitary plasmacytoma responds well to ionizing radiation, and/or surgery. Because patients with solitary plasmacytoma have a higher risk for multiple myeloma, these cases must be monitored closely with regular follow ups. It is essential to perform a baseline PET-CT scan and/or MRI to confirm solitary plasmacytoma. Skeletal surveys by regular X-rays are not sufficiently sensitive.

Monoclonal Gammopathy of Undetermined Significance

Monoclonal gammopathy of undetermined significance (MGUS) is the most common spectrum of diseases called plasma cell dyscrasias in elderly persons.

- The term MGUS refers to presence of a monoclonal immunoglobulin (Ig) also called M protein in the serum or urine in persons without evidence of multiple myeloma, Waldenström's macroglobulinemia, amyloidosis (AL), or lymphoproliferative disorders.
- Persons with MGUS generally remain asymptomatic, however, some persons may present with skin rash or numbness or tingling.
- Monoclonal gammopathy of undetermined significance (MGUS) patients may develop multiple myeloma, light chain amyloidosis, Waldenström's macroglobulinemia and lymphoma.

MOLECULAR CLASSIFICATION

Gene expression analysis of multiple myeloma has established the numerous genetic diversities of multiple myeloma neoplasm.

- Recently, multiple myeloma has been classified into seven different groups.

- **Favorable group** of multiple myeloma comprises low bone disease group, hyperploid (HY) group, CD1 group, and CD2 group.
- **Unfavorable group** of multiple myeloma comprises proliferation group, multiple myeloma SET domain group and MF group (MAF/MAFB).

- Each group demonstrates specific genetic structure and some of them have been associated with particular IgH translocation or ploidy status and characteristic clinical behavior. Molecular classification of multiple myeloma is given in Table 9.132.

MOLECULAR GENETIC ALTERATIONS

Multiple myeloma is B cell malignant disorder characterized by accumulation of terminally differentiated clonal plasma cells in the bone marrow, increased synthesis of monoclonal immunoglobulin detected in serum and/or urine and the presence of osteolytic lesions.

- In order to understand the pathogenesis of multiple myeloma, it is essential to review not only molecular changes involved in the development of clonal plasma cell, but also the interaction between clonal plasma cells and their microenvironment, since clonal plasma cells play relevant role in bone destruction, tumor growth, survival, migration and therapeutic drug response.

Cellular Origin of Multiple Myeloma

Normal differentiation of plasma cells from early B cells occurs by three B cell-specific DNA remodeling mechanisms that modify immunoglobulin genes: (a) VDJ rearrangement to form B cell receptor in the bone marrow; (b) somatic mutation; and (c) class switch recombination take place in the germinal center of lymph node.

- Sequencing analysis of IgVH genes supports that origin of clonal plasma cells occurs in the

Table 9.132 Molecular classification of multiple myeloma

Group	Chromosomal Translocation	Frequency	Cyclin D Expression	Genetic Signature	Prognosis
Low bone disease group	Nil	11%	CCND2	↑EDN1 ↑IL6R ↓DDK1 ↓FRZB	Favorable prognosis
Hyperploid (HY group)	Nil	26%	CCND1	↑TRAIL ↑DKK1 ↑FRZB ↓CKS1B	Favorable prognosis
CD1 group	t(11;14) CCND1 or t(6;14) CCND3	8%	CCND1 or CCND3	↑CEBPB ↑NID2 ↑SET7	Favorable prognosis
CD2 group	t(11;14) CCND1 or t(6;14) CCND3	17%	CCND1 or CCND3	↑MS4A1 (CD20) ↑PAX5 ↑CD27 ↑CXCR1	Favorable prognosis
Proliferation group	Nil	12%	CCND2	↑CCNB1 ↑CCNB2 ↑MCM2 ↑BUB1 ↑MAGEA6 ↑MAGEA3 ↑GAGE1	Unfavorable prognosis
Multiple myeloma SET domain	t(4;14) FGFR3/MMSET	18%	CCND2	↑FGR3 ↑MMSET ↑PBX1 ↑PAX5	Unfavorable prognosis
MF group (MAF/MAFB)	t(4;14) MAF or t(14;20) MAFB	8%	CCND2	↑ITGB7 ↓DKK1	Unfavorable prognosis

Prognosis of multiple myeloma: favorable prognosis (1, 2, 3, 4) and unfavorable prognosis (5, 6, 7).

postgerminal centers of the lymph node before migrating to the bone marrow.

- Multiple myeloma is B cell-derived neoplasm derived from terminally differentiating immunoglobulin producing long lived clonal plasma cells, which survive within the bone marrow over months to years.

Genomic Alterations in Multiple Myeloma

Genomic instability is the most important feature of multiple myeloma, that can contribute to therapeutic resistance and frequent relapses.

- Genomic instability occurs due to chromosomal translocations, mainly involving the IgH locus on chromosome 14q32, copy number abnormalities, mutations, methylation modifications and dysregulation of genes and microRNAs.
- A primary event in B cell neoplasm is dysregulation of an oncogene due to translocation to the IgG locus on chromosome 14q32 or somewhat less often IgL locus (κ on 2p11 or δ on 22q11) is juxtaposed near immunoglobulin enhancers.
- In multiple myeloma, IgH translocation can be classified into primary or secondary. Primary IgH chromosomal translocations occur as initiating events during pathogenesis of multiple myeloma, whereas secondary IgH translocations participate in the progression of disease.
- Most primary IgH chromosomal translocations occur due to errors in the B cell specific DNA modification process, mostly due to IgH switch recombination or, less often somatic hypermutation. The chromosomal breakpoints occur within or immediately adjacent to IgH switch regions or JH regions.
- On the other hand, secondary chromosomal translocations are mediated by the other kinds of combination, mechanism that do not specifically target B cell-specific DNA modification process. In multiple myeloma, there is a marked diversity of chromosomal loci involved in IgH translocations.
- Approximately 40% of multiple myeloma neoplasms have 14q32 IgH translocations with breakpoints involving five recurrent chromosomal patterns such as 11q13 (CCND1), 4p16 (FGFR/MMSET), 16q23 (MAF), 6p (CCND3) and 20q11 (MAFB).
- Whole genomic sequencing strategies revealed that there are about 35 nonsynonymous recurrent gene mutations per multiple myeloma sample, part from mutation in MAPK/ERK signaling pathway.
- Schematic representation of genomic instability in multiple myeloma is shown in Fig. 9.110. Multiple myeloma tumors having 14q32 IgH locus translocations involving five recurrent chromosomal patterns are given in Table 9.133.

Chromosomal Translocation t(11;14)

Chromosomal translocation t(11;14) involves and results in IgH/CCND1 fusion gene in 15–20% cases of multiple myeloma, which is detected by cytogenetic analysis and fluorescence *in situ* hybridization (FISH) technique. It is a standard risk prognostic marker in multiple myeloma based on studies conducted before the current therapies. As a result of chromosomal translocation t(11;14), CCND1 is juxtaposed to the powerful IgH 3'enhancer(s) on der(14q32) and its expression is dysregulated; as indicated by gene expression profiling and reverse transcriptase polymerase chain reaction (RT-PCR) in 100% cases of multiple myeloma.

Chromosomal Translocation t(4;14)

Chromosomal translocation t(4;14) results in overexpression of FGFR3 and MMSET domain in clonal plasma cells in 15–20% cases of multiple myeloma, which is detected by interphase fluorescence *in situ* hybridization or reverse transcriptase polymerase chain reaction (RT-PCR) and not by karyotyping techniques.

- The vast majority of patients whose tumor harbor chromosomal translocation t(4;14) also demonstrate deletion of chromosome 13.
- The chromosomal translocation t(4;14) is associated with upregulation of the fibroblast growth factor receptor 3 (FGFR3) on der(14) and multiple myeloma SET (MMSET) domain on der(14) in 15–20% cases of multiple myeloma.
- FGFR3 is one of the receptor tyrosine kinases for the FGF family of ligands. FGFR3 mutation is an adverse prognostic factor in patients with multiple myeloma. Gene expression profiling is done by reverse transcriptase polymerase chain reaction (RT-PCR) in 75% cases of multiple myeloma.
- MMSET gene plays critical transforming event in multiple myeloma. Normal plasma cells do not express both FGFR3 and multiple myeloma SET (MMSET domain) protein. In multiple myeloma patients with chromosomal translocation t(4;14) overexpress both FGFR3 and multiple myeloma SET (MMSET) domain protein, and associated with overall poor prognosis.

Chromosomal Translocation t(14;16)

Chromosomal translocation t(14;16) results in overexpression of c-MAF oncogenic transcription factor in 5–10% cases of multiple myeloma. The chromosomal

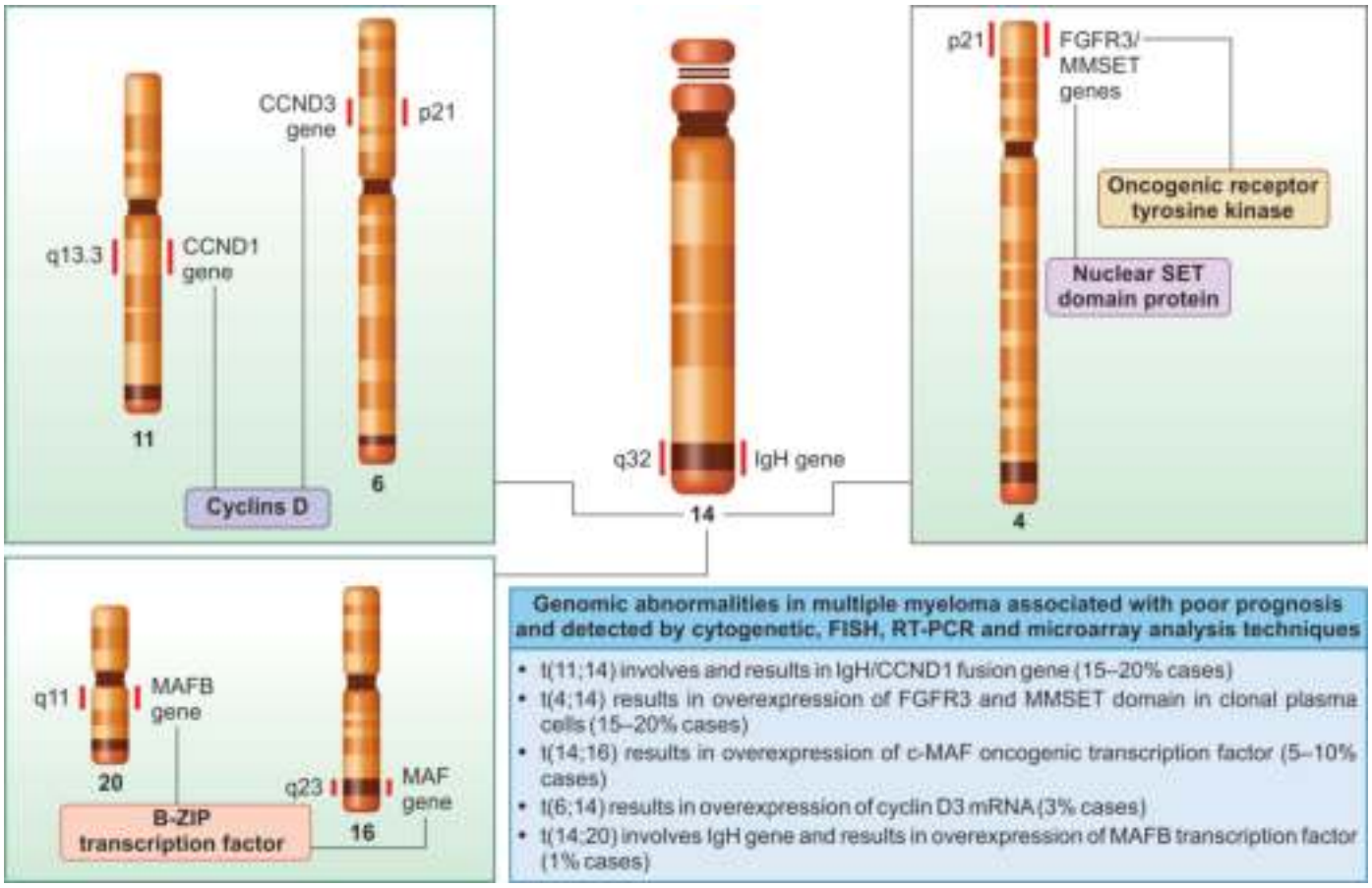


Fig. 9.110: Schematic representation of genomic instability in multiple myeloma. Genomic instability is a well-established driving force in natural history of multiple myeloma, which is difficult to treat and associated with fatal outcome. MAFB protein is a basic leucine zipper (B-ZIP) transcription factor that plays an important role in the regulation of lineage-specific hematopoiesis. MAFB gene mutation does not contain introns. The abnormal function of MAFB has been implicated in development of multiple myeloma, myeloid leukemia and multicentric carpotarsal osteolysis. Approximately 40% of multiple myeloma neoplasms have IgH translocations involving five recurrent chromosomal patterns: 11q13 (CCND1), 4p16(FGFR/MMSET), 16q23(MAF), 6p(CCND3) and 20q11(MAFB).

Table 9.133 Multiple myeloma tumors having 14q32 IgH locus translocations involving five recurrent chromosomal patterns

14q32 IGH Locus Translocations involving Recurrent Chromosomal Patterns	Frequency
Cyclins D	
▪ 11q13(CCND1) ▪ 6p21(CCND3)	20%
B-ZIP transcription factor	
▪ 20q11(MAFB) ▪ 16q23(MAF)	▪ 2% ▪ 5%
Oncogenic receptor tyrosine kinase (FGFR3) and nuclear SET domain protein	
4p16(FGFR3/MMSET)	15%

breakpoints on 16p23 occur over a region 550–1350 kb centromeric to MAF. Overexpression of c-MAF oncogenic transcription factor is demonstrated in 50% of multiple myeloma cases. Expression and stability of c-MAF transcription factor is finely tuned by several signaling pathways and the ubiquitin proteasome system. Inhibition of the signaling pathways and the ubiquitin proteasome system downregulate expression of c-MAF transcription factor.

Chromosomal Translocation t(6;14)

Chromosomal translocation t(6;14) results in overexpression of cyclin D3 mRNA in 3% cases of multiple myeloma. Using microarray analysis, elevated levels of cyclin D3 mRNA have been demonstrated in multiple myeloma cases by fluorescence *in situ* hybridization (FISH).

Chromosomal Translocation t(14;20)

Chromosomal translocation t(14;20) involves IgH gene and results in overexpression of MAFB transcription

factor in 1% cases of multiple myeloma, which like MAF encodes B-ZIP transcription factor. But in contrast to chromosomal translocation t(14;16), MAFB translocations have structural features that indicate these are secondary translocations.

Gain and Loss of Chromosomal DNA Content

Measurement of DNA content by flow cytometry and cytogenetic techniques have confirmed that all multiple myeloma cases are aneuploid.

- According to ploidy status, multiple myeloma cases have been categorized in two groups by cytogenetic analysis: (a) hyperdiploid group (>46/47 chromosomes) and (b) non-hyperdiploid group comprising hypodiploid (<44/45 chromosomes), pseudodiploid (44/45 to 46/47 chromosomes) and tetraploid (>74 chromosomes).
- Approximately 40% of multiple myeloma neoplasms have IgH translocations involving five recurrent chromosomal patterns: 11q13 (CCND1), 4p16 (FGFR/MMSET), 16q23 (MAF), 6p (CCND3) and 20q11 (MAFB).
- Monosomy/deletion 13 and gains on 1q occur predominantly in the non-hyperdiploid multiple myeloma cases. On the other hand, hyperdiploid group is associated with recurrent trisomies involving odd chromosomes (3, 5, 7, 9, 11, 15, and 19) and with a low frequency in structural chromosomal abnormalities.
- The deletion of chromosome 13 is the most common monosomy demonstrated in 40% of newly diagnosed multiple myeloma cases. This chromosomal 13 monosomy demonstrates strong association with t(4;14) and t(14;16), deletion of 17p and gains on 1q.
- Chromosomal 17p deletion includes loss of TP53 gene in 5–10% cases of multiple myeloma, which is also associated with extramedullary multiple myeloma.
- Chromosome 1 abnormality (1q gains as a result of tandem duplications and jumping duplications of the chromosome 1q band) is the most common in multiple myeloma cases demonstrated by conventional cytogenetics, fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization. Recently, frequent 1p losses especially 1p22 and 1p32 deletions have been demonstrated in multiple myeloma cases.

Epigenetic Modifications

Little is known about the epigenetic changes involved in the pathogenesis of multiple myeloma. The most important epigenetic change demonstrated includes global DNA hypomethylation and gene-specific DNA hypermethylation in multiple myeloma as compared to monoclonal gammopathy of undetermined significance (MGUS).

Late Recurrent Genetic Mutations

Based on the key landmark analysis, a number of recurrent genetic mutations have been identified in multiple myeloma such as Myc, K-RAS, N-RAS, TP53, and BRAF genes involved in the progression of multiple myeloma.

- Dysregulation of Myc is a pattern for secondary chromosomal translocations in multiple myeloma, which corresponds to complex secondary nonreciprocal chromosomal translocations and insertions of different chromosomes.
- Activated RAS (K-RAS, N-RAS) gene mutations are considered molecular markers involved in the progression of multiple myeloma in >75% cases. TP53 inactivation results in progression of the disease.
- Methylation is an epigenetic change, that inactivates tumor suppressor genes such as CDKN2B and CDKN2A, which have been demonstrated in advanced extramedullary disease of multiple myeloma.

Dysregulation of Cyclin D Genes

Dysregulation of cyclin D genes is a potential unifying event in the pathogenesis of multiple myeloma. IgH translocations induce upregulation of different oncogenes resulting in increase unrestricted proliferation of clonal plasma cells in multiple myeloma.

- Gene profiling analysis has revealed that expression of CCND1, CCND2 and CCND3 is enhanced in all cases of multiple myeloma.
- About 25% cases of multiple myeloma demonstrate overexpression of one of the cyclins, which can be triggered by IgH translocation. IgH translocation t(11;14) dysregulates CCND1 and t(6;14) dysregulates CCND3. IgH translocation indirectly involves MAF and MAFB genes, which encode transcription factor that targets cyclin D2.
- Approximately 40% cases of multiple myeloma demonstrate increased cyclin D1 through biallelic CCND1 dysregulation without apparent t(11;14). Remaining multiple myeloma cases with t(4;14) demonstrate increased expression of cyclin D2.

Dysregulation of MicroRNAs

MicroRNAs (MiRNAs) are small noncoding RNAs that regulate gene expression at the post-transcription level involved in cellular growth and differentiation; Different studies have demonstrated that dysregulation of miRNAs is present in clonal plasma cells as compared to normal plasma cells. Several miRNAs participate in the pathogenesis of multiple myeloma. Recently

abnormal expression of miRNAs has been demonstrated in multiple myeloma, where miRNAs have been found deeply dysregulated and act as oncoproteins or tumor suppressors.

MOLECULAR PATHOGENESIS

Multiple myeloma develops through a multistep transformation of normal plasma cells to monoclonal gammopathy of undetermined significance (MGUS), which results in immortalization of plasma cells and subsequently transformation to smoldering multiple myeloma and active multiple myeloma, where clonal plasma cells cause end-organ damage.

- Cytogenetic study using fluorescence *in situ* hybridization and SNP-based mapping analysis and whole genome sequencing have demonstrated genetic abnormalities at MGUS stage.
- In monoclonal gammopathy of undetermined significance (MGUS) non-IgM (IgG or IgA; M protein <30 g/L), there is increased risk of progression to multiple myeloma, amyloidosis or plasmacytoma, i.e. 0.5–1.0% per year.
- In monoclonal gammopathy of undetermined significance (MGUS) light chain, there is increased risk for progression to multiple myeloma, amyloidosis or plasmacytoma, i.e. 0.3% per year.

Interaction between Clonal Plasma Cells and their Microenvironment

Interactions between clonal plasma cells (myeloma cells) and bone marrow stromal cells (BMSCs) in the bone marrow play an important role in the pathogenesis of multiple myeloma. Clonal plasma cells adhere to extracellular matrix (ECM) and bone marrow stromal cells through series of cell adhesion molecules.

Clonal Plasma Cells Adhere Through Adhesion Molecules

Bone marrow stromal cells express vascular cell adhesion molecule 1 (**VCAM-1**) and intercellular adhesion molecule 1 (**ICAM-1**). Clonal plasma cells express adhesion molecules of β1-integrin family such as VLA-4, VLA-5 and VLA-6 also called CD49d, CD49e and CD49f, respectively.

Chemotaxis and Homing of Clonal Plasma Cells

In addition, bone marrow stromal cells synthesize stromal derived factor 1 (SDF-1), that binds to CXCR4 on the surface of clonal plasma cells and induce both chemotaxis of myeloma cells and upregulation of cell adhesion molecules such as VLA-4, VLA-5 and VLA-6. Homing of clonal plasma cells in the bone marrow is further facilitated through other cell adhesion molecules (CD138, CD38, CD44 and CD106) expressed by clonal plasma cells.

Induction of Transcription Factors and Cytokines Synthesis

Binding of clonal plasma cells to the bone marrow microenvironment results in induction of transcription factors and synthesis of various cytokines such as IL-6, IL-21, TNF-α, insulin-like growth factor 1 (IGF-1), stromal derived factor 1 (SDF-1) and vascular endothelial growth factor 1 (VEGF-1) by clonal plasma cells and/or bone marrow stromal cells.

- The cytokines induce signaling pathways (e.g. RAF/MEK/MAPK, P13K/AKT, nuclear factor kappa B (NF-κB) and JAK/STAT), that induce unrestricted proliferation of clonal plasma cells and prevent apoptosis.
- RAF/MEK/MAPK signaling pathway activated by RAS protein results in unrestricted proliferation of clonal plasma cells.
- Simultaneously, RAS induced JAK/STAT signaling pathway and cytokines induced P13K/AKT signaling pathway prevent apoptosis of the clonal plasma cells. Signaling pathways involved in pathogenesis of multiple myeloma are shown in Fig. 9.111.

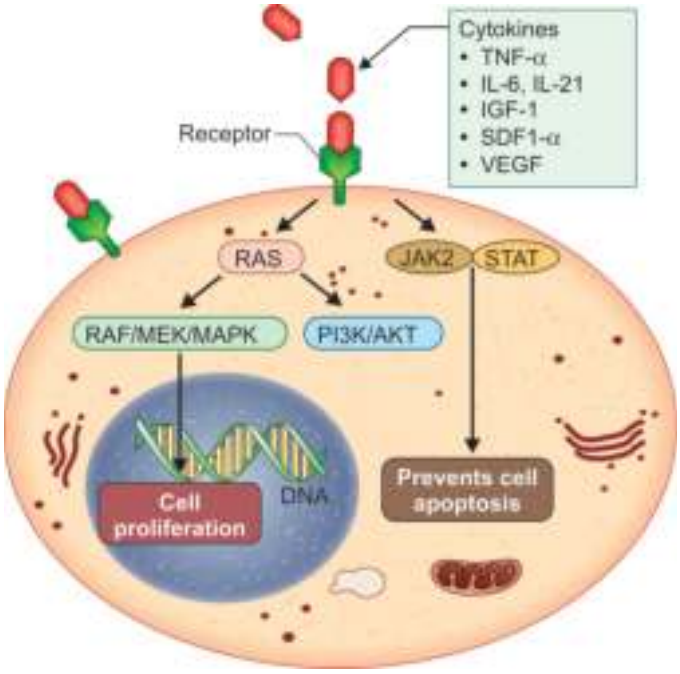


Fig. 9.111: Schematic representation of signaling pathways in the pathogenesis of multiple myeloma. Signaling pathways involved in pathogenesis of multiple myeloma. Binding of cytokines to the bone marrow microenvironment induces the transcription and synthesis of various cytokines by plasma cells and stromal cells; this trigger signaling pathways (REF/MEK/MAPK, PI13K/AKT, NF-κB and JAK2/STAT) that promote cell proliferation and prevent apoptosis. These signaling pathways are also potential targets for therapeutic intervention. In addition, cytokines modulate the synthesis of additional adhesion molecules, which, in a vicious circle further enhance cell adhesion.

Development of Drug Resistance Phenotype

Adhesion of clonal plasma cells to the bone marrow microenvironment induces a cell-mediated drug resistance phenomenon by three mechanisms: (a) cell cycle arrest at G1 by upregulation of p27, an inhibitor of cyclin-dependent kinases, (b) inhibition of apoptosis via overexpression of FLIP-L, an endogenous inhibitor of FAS (CD95) and (c) protection of clonal plasma cells from initial drug-induced DNA damage (double-stranded breaks) by reducing topoisomerase activity.

CLINICAL FEATURES

Patient with multiple myeloma presents with bone pain, pathological fractures, anemia, weakness, recurrent infections, hypercalcemia, spinal cord compression and renal failure.

- In 30% of cases, multiple myeloma is detected through routine blood screening when patients are evaluated for unrelated problems.
- In 30% of patients, multiple myeloma is diagnosed after a pathological fracture in the axial skeleton. Bone marrow involvement is multifocal and generalized in these cases. Osteolytic lesions and focal masses of clonal plasma cells are present in the sites of active hematopoiesis. Progression to advanced multiple myeloma disease involves extramedullary organs.
- Many patients with multiple myeloma present with complain of bone pain especially in the back region, long bones, skull and/or pelvis, nonspecific constitutional symptoms related to hyperviscosity and hypercalcemia.
- Diagnosis of multiple myeloma is based on the combination of clinical, morphologic features, radiological, and immunological findings by demonstration of clonal plasma cells in the bone marrow, osteolytic lesions by imaging techniques and increased M protein in serum and/or urine.
- Serum electrophoresis demonstrates M spike. Bence-Jones proteins are either κ or δ light chains excreted in the urine. These assays are used for monitoring therapeutic response and prognostic outcome.
- The interaction of clonal plasma cells (myeloma cells) and the bone marrow microenvironment favors multiple myeloma tumor growth and clinical manifestations. Clonal plasma cells synthesize excessive monoclonal protein, that is responsible for hyperviscosity, amyloidosis and renal failure.
- Clonal plasma cells fail to synthesize normal immunoglobulins responsible for life-threatening recurrent infections.
- Bone marrow infiltration by clonal plasma cells (myeloma cells) causes anemia and osteolytic lesions, i.e. bone destruction associated with hypercalcemia and bone pain. In addition to nephrotoxic effect of monoclonal protein (light chains), hypercalcemia and recurrent infections also contribute in the pathogenesis of renal failure.
- Clinical manifestations of multiple myeloma are given in Table 9.134. Schematic representation of clinical features of multiple myeloma and serum protein electrophoresis showing 'M-spike' in the gamma region are shown in Fig. 9.112. Schematic representation of clinical manifestations in a case of multiple myeloma is shown in Fig. 9.113.

Table 9.134 Clinical manifestations of multiple myeloma

Multiple Myeloma Characteristics	Clinical Manifestations	
Osteolytic bone lesions	▪ Bone pain ▪ Hypercalcemia	▪ Pathologic fractures
Bone marrow infiltration by myeloma cells	▪ Thrombocytopenia (bleeding tendencies) ▪ Paraprotein coating platelets (platelet function impaired)	▪ Anemia (fatigue and pallor) ▪ Neutropenia (decreased resistance to infection)
Failure to normal immunoglobulin synthesis	▪ Recurrent infections due to *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli* and *Haemophilus influenzae* ▪ Cellular immunity is relatively unaffected	
Plasma cell synthesis of paraprotein	▪ Hyperviscosity of body fluids	▪ Amyloid deposits in kidney and heart (AL)
Renal failure	▪ Deposition of light chains in the tubules known as myeloma kidney (most common cause of death) ▪ Hypercalcemia leads to nephrocalcinosis	▪ Hyperuricemia is directly toxic to renal tubules ▪ Bence-Jones protein clogs up glomeruli and directly toxic to renal tubules ▪ Amyloid deposit occurs in kidney
Proteinuria	Bence-Jones protein in urine	
Diagnostic testing	▪ Serum-free light chains ▪ Cytogenetic study	▪ Immunophenotype ▪ Immunohistochemistry

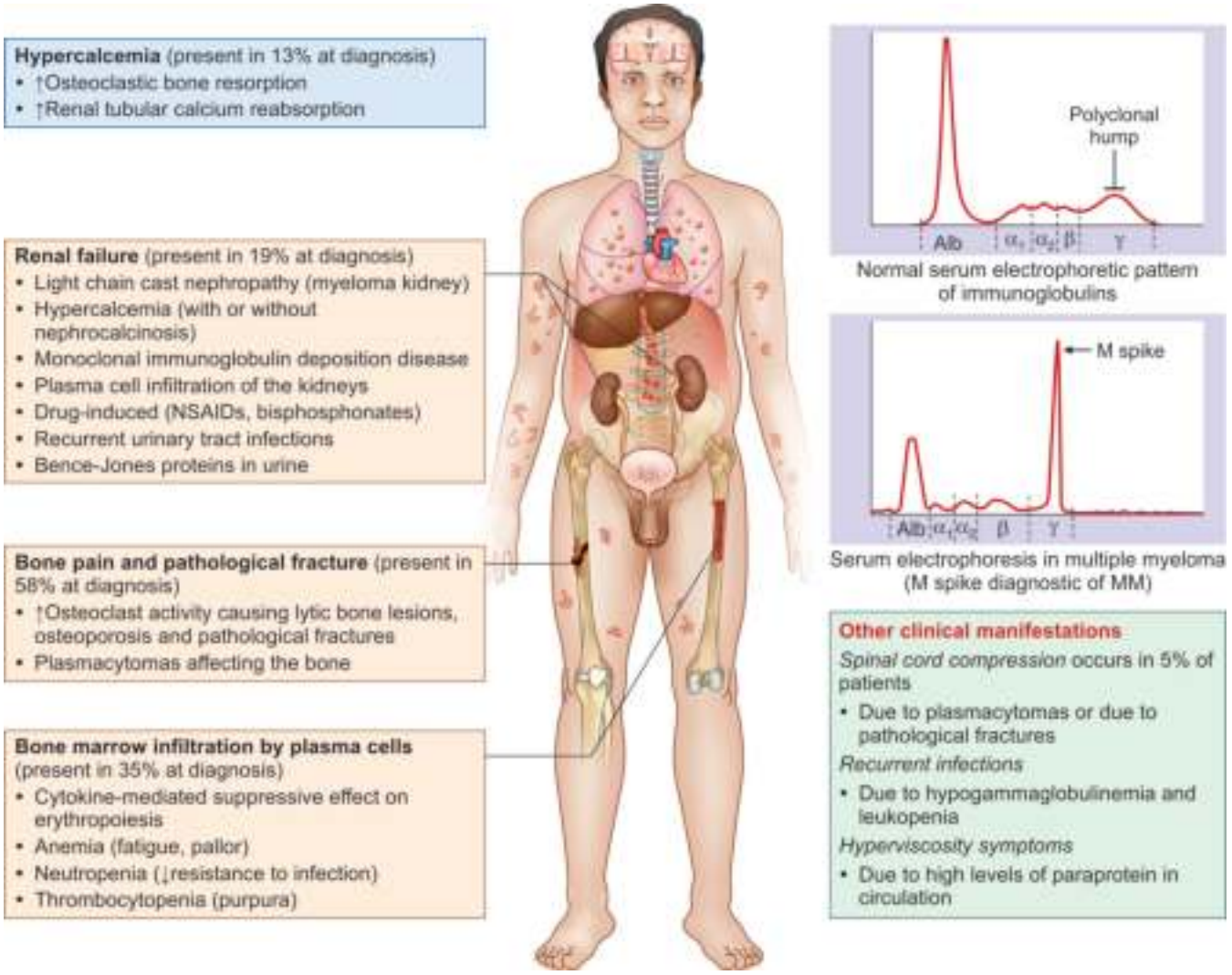


Fig. 9.112: Schematic representation of clinical features and serum electrophoresis in multiple myeloma. Diagnostic triad of multiple myeloma is characterized by presence of >10% clonal plasma cells in the bone marrow, osteolytic lesions, and presence of monoclonal protein (paraprotein) in the blood and urine. On serum electrophoresis, presence of monoclonal protein is characterized by a sharp, well-defined 'M spike' with a single heavy chain and a similar band with a κ- or λ-light chain. The absolute amount of monoclonal immunoglobulin, involved κ- or λ-light chain and κ-/λ-light chains ratio is followed by determine multiple myeloma response to therapy and progression of the disease. A broad diffuse band with one or more heavy chains and κ- or λ-light chains characterizes a polyclonal protein.

Skeletal Disease

Skeletal disease is one of the hallmarks of multiple myeloma. Osteolytic lesions begin in the medullary cavity, erode cancellous bone and progressively destroy the cortex. Bone lesions are associated with bone pain, pathologic fractures and hypercalcemia. Serum calcium is increased in multiple myeloma (IgG, IgA, IgD and Bence-Jones myelomas). Hypercalcemia results in confusion, lethargy, constipation, polyuria and renal failure.

Vertebral Column

Vertebral column is the most common site of osteolytic lesion especially in lumbar region of multiple myeloma cases. Osteolytic lesions affecting other bones in decreasing frequency include ribs, sternum, skull, pelvis, femur, clavicle and scapula. IgG and Bence-Jones myeloma produce osteolytic lesions. IgD produces extraosseous lesions. Bone pain is aggravated by movements in 65% of cases and sometimes associated with loss of vertebral height due to vertebral collapse.

Spinal Cord Compression

Spinal cord compression occurs in multiple myeloma patients due to compressed vertebral fracture and diffuse osteopenia. The symptoms that should alert clinicians to consider spinal cord compression are back pain, weakness, numbness, or dysesthesias in the extremities.

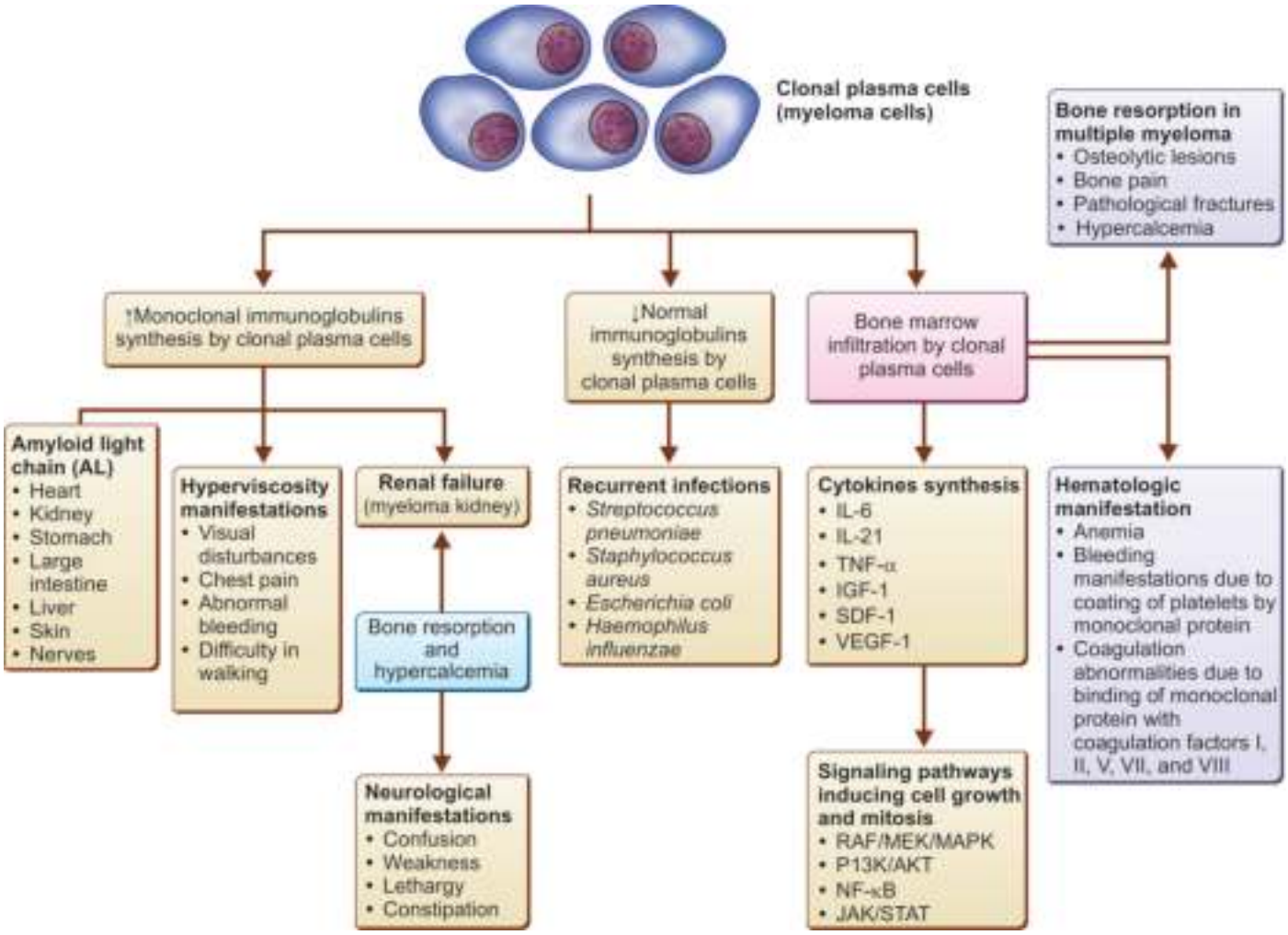


Fig. 9.113: Schematic representation of clinical manifestations in a case of multiple myeloma. Patient presents with bone pain commonly in lower back region, hypercalcemia, pathological fractures involving axial skeleton, anemia, bleeding manifestations, recurrent infection, spinal cord compression and renal failure.

Because spinal cord compression in multiple myeloma occurs at multiple levels, comprehensive evaluation of the spine is warranted. Patients who are ambulatory at the start of therapy have the best likelihood of preserving spinal cord function and avoiding paralysis.

Imaging Techniques to Detect Osteolytic Lesions in Multiple Myeloma

Osteolytic lesions are demonstrated by conventional radiography, MRI and PET-CT scan. Radiograph reveals rounded punched out osteolytic defects measuring 1–4 cm in diameter and diffuse osteopenia. Radiograph of skull demonstrates multiple punched out osteolytic lesions in a patient of multiple myeloma is shown in Fig. 9.114. Pathogenesis of bone disease in multiple myeloma because of increased synthesis of osteoclastic activating factors (OAFs) is shown in Fig. 9.115.

- Osteolytic lesions in multiple myeloma are mediated by an imbalance between osteoclastic activity of osteoblastic activity.
- Interaction of clonal plasma cells (myeloma cells) with bone marrow stromal cells and other cells in the bone marrow microenvironment induces the synthesis of numerous osteoclast-activating factors (OAFs). RANK-L, macrophage inflammatory protein-1α (MIP-1α) (also known as CCL3), vascular endothelial growth factor (VEGF), activin A, hepatocyte growth factor (HGF), interleukin 3 (IL-3), interleukin 7 (IL-7), interleukin 6 (IL-6), interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α) and macrophage inflammatory protein-3α (MIP-3α).
- Two of the most important osteoclast-activating factors (OAFs) are **RANK-L** (receptor activator of NF-κB ligand) and macrophage inflammatory protein-1α **(MIP-1α)**.

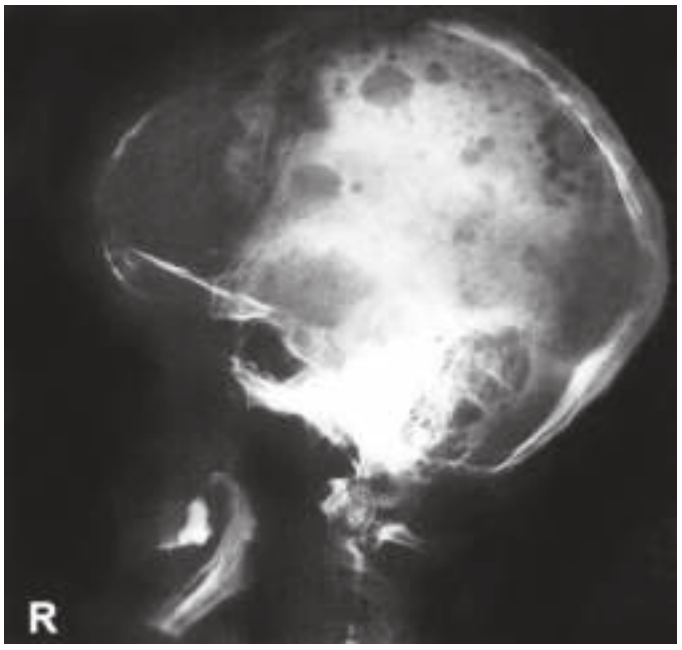


Fig. 9.114: Radiograph of skull shows osteolytic lesion in a case of multiple myeloma. It shows multiple punched-out osteolytic lesions giving 'motheaten appearance'. Skeletal disease is one of the hallmarks of multiple myeloma. Osteolytic lesions begin in the medullary cavity, erode cancellous bone and progressively destroy the cortex. Bone lesions are associated with bone pain, pathologic fractures and hypercalcemia. Vertebral column is the most common site especially in lumbar regions. Osteolytic lesions affecting other bones in decreasing frequency include ribs, sternum, skull, pelvis, femur, clavicle and scapula. IgG and Bence-Jones myeloma produce osteolytic lesions.

Pathology Pearls: Skeletal Disease in Multiple Myeloma—Pathophysiology

- Some cancers including multiple myeloma produce cytokines, which can cause resorption of bone (osteolytic activity) or cytokines that induce the proliferation of osteoblasts (osteoblastic activity), which are responsible for new bone formation.
- Cytokines are soluble proteins produced by a wide variety of hematopoietic and nonhematopoietic cells.
- Pathological fractures occur at sites of osteolytic bone lesions.
- Radionucleotide scans are very informative when osteoblastic lesions are being investigated.
- Osteolytic lesions are best detected by plain radiography or magnetic resonance imaging (MRI).

Increased Skeletal Osteoclastic Activity

In multiple myeloma, there is marked osteoclastic activity and bone resorption by following mechanisms:

Role of RANK-L (Receptor Activator of NF-κB Ligand)

- Receptor activator of NF-κB ligand (RANK-L) is a transmembrane molecule in bone marrow stromal cells/osteoprogenitors.
- RANK-L binds to functional receptor RANK on osteoclasts resulting in activation of osteoclasts and stimulation of resorption of bone. Osteoprotegerin (**OPG**) synthesized by bone marrow stromal cells blocks the RANK-L osteoclastic activity.
- Therefore, osteoclastic activity is regulated by a delicate imbalance between RANK-L present on bone marrow stromal cells and osteoprotegerin.
  - Under physiologic state, osteoprotegerin levels are higher than RANK-L.
  - RANK-L levels are higher than osteoprotegerin in multiple myeloma cases.

Role of Macrophage Inflammatory Protein-1α (MIP-1α)

- Macrophage inflammatory protein-1α (MIP-1α) is a potent stimulator of osteoclasts formation by two mechanisms: (a) MIP-1α increases the activity of RANK-L and (b) MIP-1α stimulates osteoclast precursors to differentiate into mature osteoclasts, whereas IL-3 inhibits formation of osteoclasts. Chemokines such as IL-7, TNF-α and IL-1β indirectly stimulate osteolytic activity by inducing RANK-L.
- Other osteoclast activating factors synthesized by clonal plasma cells such as IL-6, VEGF, HGF and activin A further augment osteoclastic activity and bone resorption.

Inhibition of Skeletal Osteoblastic Activity

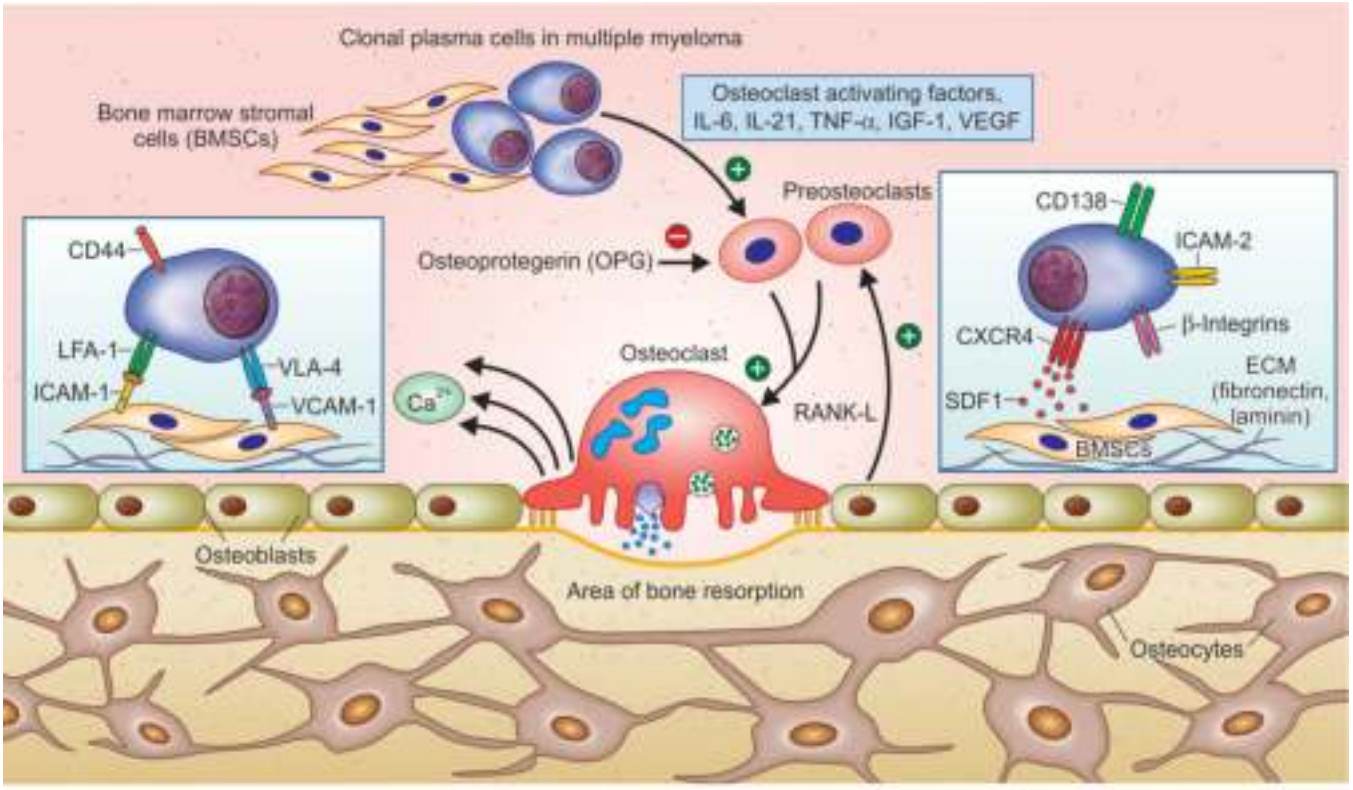
In multiple myeloma, in addition to increased osteoclast activation, there is inhibition of osteoblast formation mediated by soluble factors and direct cellular interactions between the clonal plasma cells and bone marrow stromal cells by following mechanisms.

Wnt/Bone Morphogenetic Protein Signaling Pathways

- The Wnt/bone morphogenetic protein (BMP) signaling pathways induce transcriptional factors, which participate in the osteogenic differentiation of mesenchymal stem cells to mature bone forming osteoblasts.
- RUNX2, transcriptional factor regulates osteoblastic differentiation. In multiple myeloma, osteolytic lesions demonstrate reduced RUNX2 activity.

Soluble Factors Synthesis by Clonal Plasma Cells

- Clonal plasma cells (myeloma cells) and bone marrow stromal cells synthesize numerous soluble factors that inhibit osteoblast differentiation and/or function of Wnt/bone morphogenetic protein signaling pathways.
- Various soluble factors inhibiting Wnt signaling pathways include DKK1, sclerostin and soluble frizzled related proteins 2/3 (sFRP-2/3).
  - Soluble factors inhibiting bone morphogenetic protein (BMP) include activin A, transforming growth factor-β (TGF-β) and hepatocyte growth factor (HGF).
  - Cytokines indirectly inhibiting osteoblastic differentiation include IL-7, IL-3 and TNF-α.



Skeletal lesions in multiple myeloma: pathogenesis, clinical manifestations and diagnosis

Osteoclastic activity in multiple myeloma

- RANK-L + RANK on osteoclasts → ↑Osteoclastic activity → Osteolytic lesions
- Chemokines (IL-7, TNF-α and IL-1β) stimulate RANK-L → ↑Osteoclastic activity → Osteolytic lesions
- MIP-1α stimulates RANK-L → ↑Osteoclastic activity → Osteolytic lesions
- MIP-1α stimulates RANK-L → ↑Differentiation of osteoclast precursors into mature osteoclasts → ↑Osteoclastic activity → Osteolytic lesions
- Osteoprotegerin/osteoclastogenesis inhibitory factor blocks RANK-L activity → ↓Osteoclastic activity

Skeletal lesions and manifestations

- Patients present with osteolytic bone lesions in decreasing frequency: Vertebral column in lumbar region, ribs, sternum, skull, pelvis, femur clavicle and scapula, bone pain, pathologic fracture and increased serum calcium levels (hypercalcemia)
- Presence of ≥1 osteolytic lesions on conventional radiograph, CT scan, PET-CT scan

Diagnosis of multiple myeloma

- Diagnosis of multiple myeloma is based on the combination of clinical, morphological, radiological and immunological findings by demonstration of clonal plasma cells in the bone marrow, osteolytic lesions by imaging techniques and increased M protein in serum and/or urine

Fig. 9.115: Pathogenesis of bone disease in multiple myeloma occurs as a result of synthesis of osteoclastic activating factors. Osteolytic lesions are mediated by an imbalance between osteoclastic activity of osteoblastic activity. Interaction of clonal plasma cells (myeloma cells) with bone marrow stromal cells and other cells in the bone marrow microenvironment induces the synthesis of numerous osteoclast-activating factors such as RANK-L, macrophage inflammatory protein-α (MIP-α) (also known as CCL3), vascular endothelial growth factor (VEGF), activin A, hepatocyte growth factor (HPG), interleukin 3 (IL-3), interleukin 7 (IL-7), interleukin 6 (IL-6), interleukin-1β, (IL-1β), tumor necrosis factor-α (TNF-α) and macrophage inflammatory protein-3α (MIP-3α). Two of the most important osteoclast-activating factors are RANK-L (receptor activator of NF-κB ligand) and macrophage inflammatory protein-1α (MIP-1α).

Hypercalcemia

Osteolytic skeletal lesions result in hypercalcemia in the advanced stage of multiple myeloma due to marked increase in osteoclastic bone resorption. Confusion, lethargy, bone pain, nausea, polydipsia, constipation, polyuria, and renal failure are the presenting symptoms of hypercalcemia in 30% of patients with multiple myeloma. In multiple myeloma, hypercalcemia does not adversely affect survival of multiple myeloma patients.

Recurrent Infections

Clonal plasma cells (myeloma cells) fail to synthesize normal immunoglobulins. Patients suffer from recurrent infections with *Streptococcus pneumoniae, Staphylococcus aureus, Escherichia coli* and *Haemophilus influenzae*. Cellular immunity is relatively unaffected. The absence of immunoglobulins (antibodies) directed against the bacterial capsule limits the ability of phagocytic cells to ingest and kill the bacteria.

Bleeding Diathesis, Severe Anemia and Easy Fatigue

Monoclonal protein synthesized by clonal plasma cells (myeloma cells) coats the platelets resulting in impairment of platelet functions and bleeding tendencies from gums, gastrointestinal tract and respiratory tract. Monoclonal protein binds with coagulation factors I, II, V, VII and VIII resulting in coagulation abnormalities. Patients present with easy fatigue and anemia as a result of proliferation of clonal plasma cells in the bone marrow, decreased hematopoiesis, and bleeding diathesis as a result of impaired platelet function.

Organs Damage

In multiple myeloma, excessive synthesis of light chains may lead to the amyloid light (AL) form of amyloid deposition in kidney, liver, spleen, lymph nodes and other organs. Renal amyloidosis occurs in Bence-Jones myeloma, IgG and IgD myeloma. IgD myeloma causes heavy proteinuria. Nephrotoxic effect of monoclonal protein leads to myeloma kidney. Monoclonal protein form casts in the renal tubules and induces giant cell reaction resulting in blockage of tubules and renal failure.

Cryoglobulinemia

In few multiple myeloma cases, monoclonal protein synthesized by clonal plasma cells behaves like cryoglobulins resulting in tingling sensation, numbness, and skin rashes.

Hyperviscosity Syndrome

Hyperviscosity syndrome may be associated with symptoms in multiple myeloma such as generalized malaise, infections, fever, paresthesia, sluggish motor functions and sensory losses.

- Patient presents with headache, somnolence, easy bruising and hazy vision.
- Occasionally, hyperviscosity can cause cerebral stroke or myocardial infarction. Epistaxis may be a presenting symptom of multiple myeloma with a high tumor burden.

Secondary Changes in the Bone Marrow

Due to infiltration of clonal plasma cells, secondary changes in the bone marrow are sometimes observed in cases of multiple myeloma. Interstitial acidophilic changes in bone marrow reflect hypoproteinemia, amyloid deposition and myelofibrosis. Myelofibrosis is observed in 10% of multiple myeloma cases. Genetic abnormality in FGFR3-IgH has been detected by fluorescence *in situ* hybridization (FISH).

LABORATORY DIAGNOSIS

Multiple myeloma accounts for approximately 10% of hematologic malignancies. The initial evaluation to confirm diagnosis of multiple myeloma includes clinical history, blood urine examination, bone marrow aspiration, bone marrow trephine biopsy, biochemical and immunological findings, conventional radiographs, MRI, CT scan, PET scan, and molecular genetic studies.

Clinical History

Patient presents with bone pain, backache, pathologic fractures of axial skeleton, weakness, anemia, recurrent infections (often pneumococcal), hypercalcemia, spinal cord compression and renal failure.

Physical Examination

Patient usually appear lethargic and fatigued. Physical examination reveals pallor, purpura, hepatosplenomegaly, bony tenderness, carpel tunnel syndrome, and signs of compression. Any of these physical findings may warrant further evaluation.

Skeletal Survey

- Conventional radiograph, computed tomography scan, or whole-body MRI or whole-body low-dose CT scan demonstrate osteolytic lesions in skull and axial skeleton and pathologic fracture.
- Fluorodeoxyglucose (FDG) positron emission tomography scan demonstrates areas of active multiple myeloma disease.

Molecular Genetic Alterations

Bone marrow aspirate and bone marrow trephine biopsy, cytogenetic study, or fluorescence *in situ* hybridization (FISH) and flow cytometry are performed to evaluate hyperploidy, chromosomal del 13, del 17p, t(4;14), t(14;16), t(11;14) and 1q21 amplification and aberrant plasma cell markers.

Pathology Pearls: Dutcher Bodies and Russell Bodies in Clonal Plasma Cells in Multiple Myeloma

- Dutcher bodies and Russell bodies are often found in clonal plasma cells.
- Dutcher body is an inclusion structure in the nucleus of clonal plasma cells in multiple myeloma.
- Russell body is an inclusion structure in the cytoplasm formed by immunoglobulin deposition. Cell with abundant grape-like cytoplasmic inclusions of immunoglobulin deposits is termed a Mott cell.
- Unlike Dutcher bodies, Russell bodies are sometimes found in reactive plasma cells; therefore, their presence is not definitive for neoplastic lesion.

Laboratory Diagnosis of Multiple Myeloma

- Diagnosis of multiple myeloma requires ≥10–30% clonal plasma cells in bone marrow or bone marrow trephine biopsy proven plasmacytoma plus evidence of one or more multiple defining events (MDE): CRAB (hypercalcemia, renal failure, anemia, or lytic bone lesions, features related to the plasma cell disorder, bone marrow clonal plasmacytosis ≥60%, serum involved/uninvolved free light chains (FLCs) ratio ≥100% or greater than focal lesion in bone on magnetic resonance imaging (MRI).

Blood Tests

- Hemoglobin is low in multiple myeloma patients such as <12 g/dl in women and <14 g/dl in men (i.e. <2 g/dl below normal range).
- Erythrocyte sedimentation rate (ESR) is increased often >100 mm/1st hr.
- Serum blood urea nitrogen (BUN), creatinine is elevated in renal failure in multiple myeloma.
- Serum calcium elevated due to osteolytic lesions.
- Serum β_2-microglobulin is a light chain gene of the class 1 histocompatibility antigen expressed on the surface of all nucleated cells. Increase in β_2-microglobulin in multiple myeloma reflects the tumor mass, which is excreted by the kidneys. It is most often raised in renal failure; and higher levels are associated with poor prognosis.
- Serum paraprotein levels are raised.
  - Serum paraprotein levels are increased with elevated globulins.
  - Serum paraprotein concentration is raised with suppression of normal immunoglobulin.
  - Paraprotein is IgG in 70% cases, IgA in 20%, IgM (uncommon); IgD and IgE rare.
  - Serum light chain (either κ or λ) is increased.
  - In immunofixation of serum, antibodies to IgG, IgD, IgE, κ and δ are used to show the paraprotein has a single light chain.
- Serum lactic acid dehydrogenase (LDH) is increased level not specific for diagnosis, but used as prognostic indicator.
- Serum quantitative immunoglobulins, protein electrophoresis, immunofixation, and free light chain assay are performed in multiple myeloma patients.
- Serum electrophoresis demonstrates monoclonal immunoglobulins (heavy chains and κ or δ light chains). M-spike is demonstrated in the γ-region in 80% of cases and occasionally in α or β regions). It demonstrates monoclonal IgG (50%), IgA (25%), IgD (10%) and rarely IgM or IgE.

Urine Analysis for Bence-Jones Proteins

- Urine electrophoresis is essential to demonstrate κ or λ light chains irrespective of serum electrophoresis study.
- Urine contains significant quantity of immunoglobulin κ or λ light chains, known as Bence-Jones proteins in 60–70% of cases. On heating urine specimen between 50 and 60°C, Bence-Jones proteins appear in the urine and disappear on temperature below 50°C and heating urine specimen above 60°C.
- Twenty-four hours urine analysis is done for total protein and protein electrophoresis with immunofixation.

Peripheral Blood Smear Examination

Red blood cells

- Peripheral blood smear shows '**rouleaux formation**' with a bluish background staining due to increased paraprotein concentration. Rouleaux formation of red blood cells in Giemsa-stained peripheral blood smear in multiple myeloma is shown in Fig. 9.116.
- Red blood cells are arranged in rolls and stacks (rouleaux formation), that happens with abnormal or increased plasma proteins particularly fibrinogen and globulins.
- Red blood cells can be dispersed by mixing red blood cells with saline. Rouleaux formation can be demonstrated in multiple myeloma, monoclonal gammopathy of undetermined significance (MGUS), amyloidosis, lymphomas, inflammation and infections. Leukoerythroblastic picture may be demonstrated.

White blood cells

- Clonal plasma cells (myeloma cells) fail to synthesize normal immunoglobulins resulting in recurrent infections.
- Leukocytosis is demonstrated because of recurrent infections.
- Patient with multiple myeloma may rarely develop plasma cell leukemia.
- The International Myeloma Working Group has defined the diagnostic criteria for plasma cell leukemia (PCL) by the presence of ≥20% circulating clonal plasma cells on peripheral blood and plasma cell count $>2 \times 10^9$/L may be too stringent. Organomegaly is common in plasma cell leukemia associated with unfavorable prognosis.
- **Peripheral blood plasmacytosis** is demonstrated in viral infections, bacterial endocarditis, streptococcal infections, serum sickness, sarcoidosis, Waldenström's macroglobulinemia, severe burns and kwashiorkor.

Platelets

Platelet count is decreased (thrombocytopenia) because of bone marrow infiltration by clonal plasma cells, which can lead to increased bleeding and bruising.

Bone Marrow Smear Examination

- Multiple myeloma cells are classified into four types: mature, immature, pleomorphic, and plasmablastic.
  - There are three patterns in which multiple myeloma infiltrates bone marrow: nodular, interstitial and diffuse.
  - Dutcher bodies are highly specific to the clonal plasma cells.
  - On histochemical staining, CD138 marker is highly specific for plasma cells.
  - Multiple myeloma in Giemsa-stained bone marrow aspirate smear is shown in Fig. 9.117. Grading system of clonal plasma cells in multiple myeloma is given in Table 9.135.
- **Cellularity:** Bone marrow is hypercellular because of clonal proliferation of plasma cells. Myeloid, erythroid and megakaryocytic series are relatively decreased. Bone marrow smear demonstrates ≥10% of mature clonal plasma cells, immature clonal plasma cells, plasmablastic clonal plasma cells and pleomorphic clonal plasma cells. Some clonal plasma cells are multinucleated with abnormal morphology.

- **Mature clonal plasma cells:** Mature clonal plasma cells measure 20–30 μm in diameter with abundant cytoplasm and eccentrically placed nuclei with dense clumped chromatin giving cartwheel appearance.
- **Immature clonal plasma cells:** Immature clonal plasma cells contain eccentrically placed nucleus with diffuse fine chromatin and prominent nucleus and clear cytoplasmic droplets due to aggregation of immunoglobulins known as Russell bodies. Morphology of immature clonal plasma cells is indistinguishable from normal plasma cells except that their number is increased in multiple myeloma. Presence of immature cells in the bone marrow is associated with poor prognosis.
- **Plasmablastic clonal plasma cells:** Plasmablastic clonal plasma cell contains centrally placed large immature nucleus with reticular chromatin and prominent nucleolus with high nucleocytoplasmic ratio (*refer to* plasmablastic myeloma, Figs 9.106 and 9.107).
- **Pleomorphic clonal plasma cells:** Bone marrow aspirate smear contains multilobulated pleomorphic clonal plasma cells with gray-blue basophilic cytoplasm and nuclear blebs. Some cells show monocytoid like resemblance. Pleomorphic multiple myeloma in Giemsa-stained bone marrow aspirate smear is shown in Fig. 9.118.

Methyl Green Pyronine Special Stain for Plasma Cells

- Plasma cells can be stained by methyl green pyronine stain. Plasma cells can also be demonstrated by toluidine blue and azures.
- Methyl green component stains nucleus of plasma cell as blue-green and pyronine component stains cytoplasm as red.
- Conditions associated with increase in number of plasma cells in bone marrow.
- Various disorders associated with increase in number of plasma cells (rarely >10%) in the bone marrow include metastatic bone disease, Hodgkin disease, sarcoidosis, systemic lupus erythematosus, rheumatoid arthritis, tuberculosis, syphilis, cirrhosis, and aplastic anemia.

Bone Marrow Trephine Biopsy Examination

- The single most specific test is the bone marrow trephine biopsy, which demonstrates high number of clonal plasma cells ≥10–60%. Bone marrow trephine biopsy of multiple myeloma reveals soft red tumor with gelatinous consistency typically filling bony trabeculae.
- Histologic examination of bone marrow trephine biopsy shows sheets of clonal plasma cells looking morphologically like normal plasma cells with eccentrically placed nuclei and abundant pale cytoplasm. In some cases, clonal plasma cells can be poorly differentiated.
- Immunophenotyping plays important role in quantification of clonal plasma cells in bone marrow trephine biopsy specimens. CD138 and CD38 markers are helpful for quantifying clonal plasma cells for κ and λ light chains.
- Mature multiple myeloma (MM) in hematoxylin and eosin-stained bone marrow trephine biopsy section is shown in Fig. 9.119. Dutcher body in multiple myeloma in hematoxylin and eosin-stained bone marrow trephine biopsy section is shown in Fig. 9.120. Pleomorphic myeloma with bone marrow fibrosis in hematoxylin and eosin-stained bone marrow trephine biopsy section is shown in Fig. 9.121.

Immunophenotyping

- Flow cytometry demonstrates that clonal plasma cells contain monotypic cytoplasmic immunoglobulin and lack surface immunoglobulin in multiple myeloma.
- Clonal plasma cells typically express CD38 and CD138. Normal plasma cells never demonstrate aberrant expression of antigens. Expression of CD38 positive (Syndecan 1) on plasma cell population in multiple myeloma is shown in Fig. 9.122.
- In contrast to normal plasma cells, clonal plasma cells demonstrate aberrant expression of antigens, e.g. CD56, CD200, CD28, CD117, CD20 and CD52.
- Abnormal expression of CD56 is demonstrated in 70–80% of cases by flow cytometry analysis. CD56 expression definitely indicates multiple myeloma, suggesting its high diagnostic values.

Markers	Expression
CD38	Positive (100%)
CD138	Positive (100%)
CD56	Positive (75–80%)
CD200	Positive (65–75%)
CD28	Positive (40%)
CD117	Positive (20–35%)
CD20	Positive (10–20%)
CD52	Positive (8–14%)

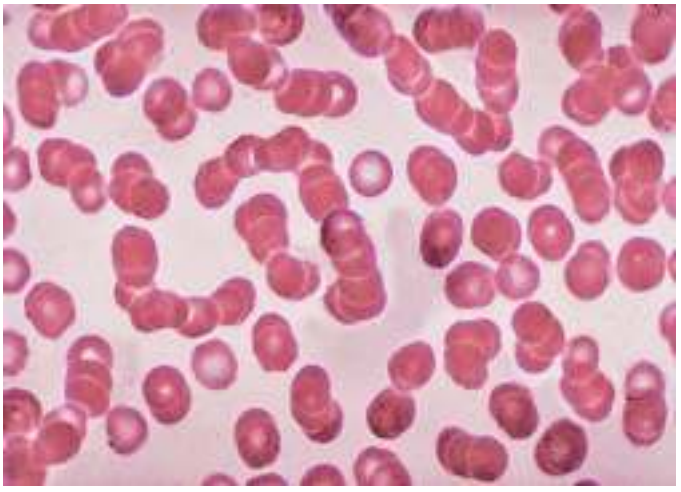


Fig. 9.116: Rouleaux formation of red blood cells in Giemsa-stained peripheral blood smear in multiple myeloma. It shows stacking of red blood cells as a result of coating of erythrocytes by increased immunoglobulin in multiple myeloma (1000X).

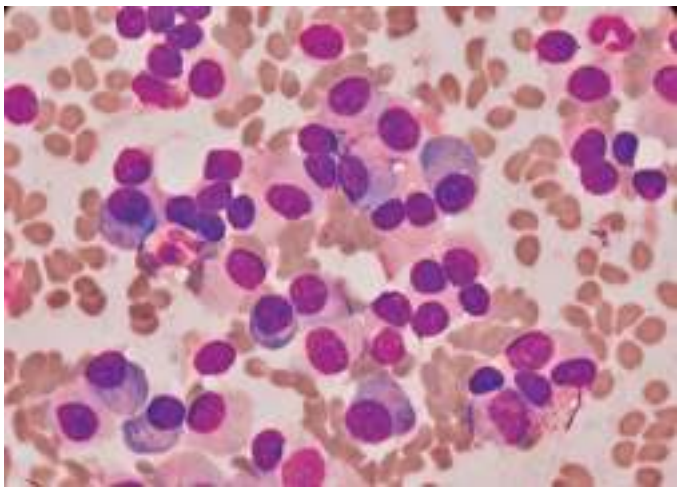


Fig. 9.117: Multiple myeloma in Giemsa-stained bone marrow aspirate smear. Replacement of bone marrow hematopoietic precursor cells by an infiltrate of malignant plasma cells is shown. Neoplastic plasma cells have eccentric nucleus and perinuclear halos. A few binuclear plasma cells are also present (1000X).

DIFFERENTIAL DIAGNOSIS

Multiple myeloma should be differentiated from reactive bone marrow changes due to infections or neoplastic process, lymphoplasmacytoid immunocytoma (Waldenström macroglobulinemia), monoclonal gammopathy of undetermined significance (MGUS), and concomitant paraproteinemia. Differential diagnosis in disorders associated with increase in number of plasma cells in the bone marrow is given in Table 9.136.

PROGNOSTIC FACTORS

On multivariable analysis of peripheral blood progenitor cell (PBPC), patient age, stage, bone marrow plasma cell leukemia 1 (PCL-1) and plasmablastic leukemia 1 (PBL1) are independent prognostic factors for survival. Circulating clonal plasma cells are also predictive for patients with smoldering multiple myeloma, who are likely to progress sooner to active multiple myeloma disease.

Table 9.135 Grading system of clonal plasma cells in multiple myeloma

Clonal Plasma Cell	Features
Mature multiple myeloma	Mature clonal plasma cell measures 20–30 µm in diameter with abundant cytoplasm and eccentrically placed nuclei with dense clumped chromatin giving cartwheel appearance
Immature multiple myeloma	▪ Immature clonal plasma cells contain eccentrically placed nucleus with diffuse fine chromatin and prominent nucleus and clear cytoplasmic droplets due to aggregation of immunoglobulins known as Russell bodies ▪ Morphology of immature plasma cells is indistinguishable from normal plasma cells except that their number is increased in multiple myeloma ▪ Presence of immature cells in the bone marrow is associated with poor prognosis
Plasmablastic multiple myeloma	Plasmablastic clonal plasma cell contains centrally placed large immature nucleus with reticular chromatin and prominent nucleolus with high nucleocytoplasmic ratio
Pleomorphic multiple myeloma	Bone marrow aspirate smear contains multilobulated pleomorphic clonal cells with gray-blue basophilic cytoplasm and nuclear blebs. Some cells show monocytoid like resemblance

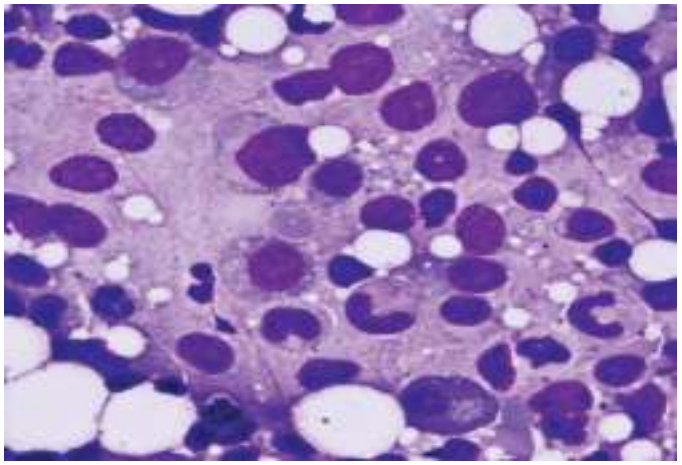


Fig. 9.118: Pleomorphic multiple myeloma in Giemsa-stained bone marrow aspirate smear. Multiple myeloma cells are classified into four types: mature, immature, plasmablastic and pleomorphic (1000X). Bone marrow aspirate smear shows pleomorphic myeloma cells, with nuclear pleomorphism, multinucleated polylobated nucleus, prominent nucleolus and abundant basophilic cytoplasm.

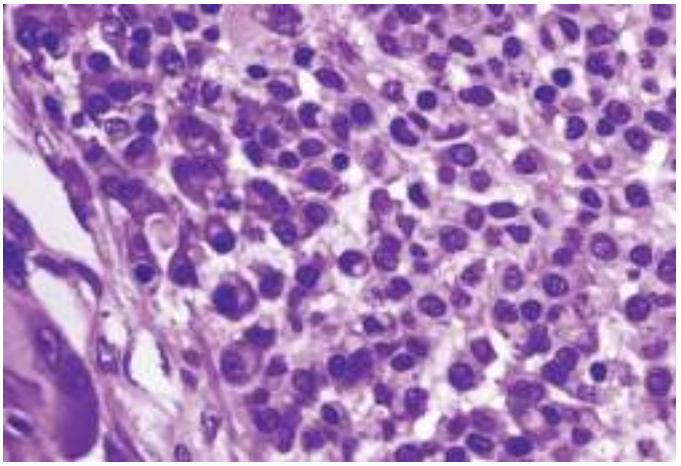


Fig. 9.119: Mature multiple myeloma in hematoxylin and eosin-stained bone marrow trephine biopsy section. Replacement of bone marrow hematopoietic precursor cells by an infiltrate of malignant mature plasma cells is shown. Neoplastic plasma cells have eccentric nucleus and perinuclear halos. A few binuclear plasma cells are also present (1000X).

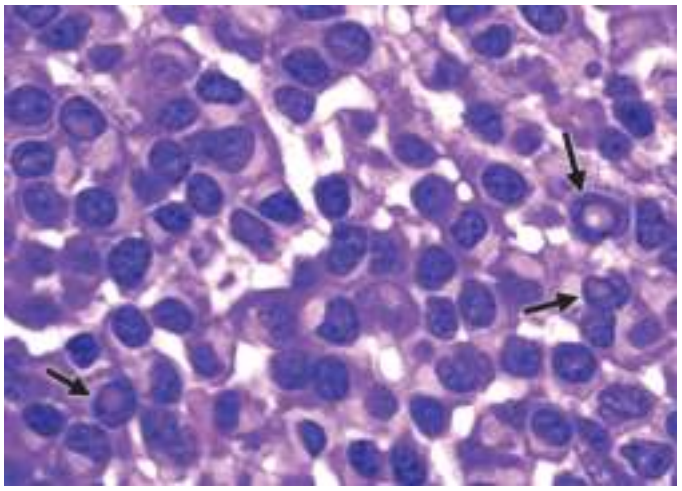


Fig. 9.120: Dutcher body in multiple myeloma in hematoxylin and eosin-stained bone marrow trephine biopsy section. A Dutcher body is an inclusion structure in the nucleus of clonal plasma cells in multiple myeloma (arrows) (1000X).

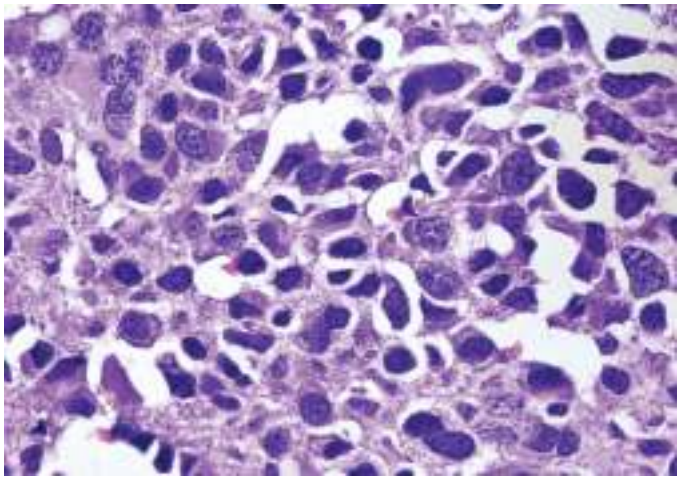


Fig. 9.121: Pleomorphic myeloma with bone marrow fibrosis in hematoxylin and eosin-stained bone marrow trephine biopsy section.

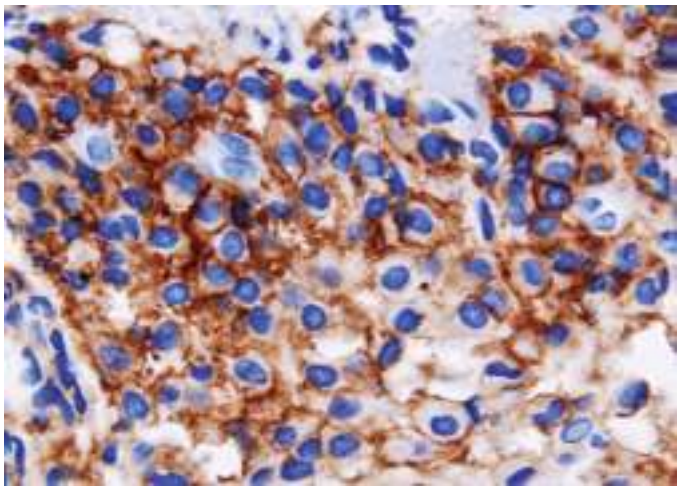


Fig. 9.122: Expression of CD38 positive (Syndecan 1) on clonal plasma cell population in multiple myeloma. CD38 expression is very high and uniform on multiple myeloma cells. CD38 is a good target for novel therapeutic strategies (400X).

Poor Prognostic Factors

Poor prognostic factors for multiple myeloma include advanced clinical stage, cytogenetic abnormalities t(4;14) (p16;q32), t(14,16), t(11;14) (q13;q32), 17p, Ip deletions, TP53 gene mutation, 1q gains, hypodiploidy tumors, diffuse involvement of bone marrow by monoclonal plasma cells, high plasma cell proliferative index, advanced osteolytic lesions, increased serum β_2-microglobulin, decreased plasma albumin, increased serum M proteins, Bence-Jones proteins in the urine, impaired renal functions, increased serum LDH, increased C-reactive protein, increased circulating clonotypic plasma cells in blood and immunophenotyping (e.g. positivity for CD28, CD19). Patients with λ light chain disease have a three-time worse prognosis than κ light chain disease.

Table 9.136 Differential diagnosis in disorders associated with increase in number of plasma cells in the bone marrow

Conditions	Number of Plasma Cells in Bone Marrow	Plasma Cell Morphology	Comments
Normal bone marrow	<5%	Small mature plasma cells	Polyclonal plasma cells
Reactive bone marrow changes due to infections or neoplastic process	5–10%	Small mature plasma cells	Plasma cells increased after HIV infection, chronic inflammatory processes
Multiple myeloma	>10–60%	Pleomorphic plasma cells (immature forms with atypical nucleoli)	Plasma cells are strongly positive for acid phosphatase
Lymphoplasmacytoid immunocytoma (Waldenström macroglobulinemia)	>10%	Plasma cells show marked pleomorphism	Marked lymphocytic infiltration and mast cells
Monoclonal gammopathy of undetermined significance (MGUS)	>10%	Plasma cells show slight pleomorphism	Transition to multiple myeloma is possible
Concomitant paraproteinemia	>10%	Plasma cells show slight pleomorphism	Occurs in systemic lymphoreticular disease and carcinoma

Favorable Prognostic Factors

Favorable prognostic factors for multiple myeloma include hyperdiploid tumors by (DNA cell content of tumor measured by flow cytometry) cytogenetic abnormalities (multiple trisomies involving chromosomes 3, 5, 7, 8, 11, 15, 19 and 21). Features of poor prognostic factors at diagnosis of multiple myeloma are given in Table 9.137.

TREATMENT

Smoldering multiple myeloma patients are asymptomatic and stable with normal blood counts and renal function, without skeletal disease and low level of paraprotein. High-risk patients with IgG paraprotein >30 g/dl may benefit from early treatment.

- Chemotherapy is indicated in multiple myeloma patients with CRAB (hypercalcemia, renal failure, anemia or bone lesion).
- In elderly multiple myeloma patients >65 years of age, induction therapy is started with melphalan, prednisolone and thalidomide. New protocol includes velcade, a proteasome inhibitor or lenalidomide instead of thalidomide.
- Multiple myeloma patients below 65 years of age benefit from intensive induction with cyclophosphamide, dexamethasone and thalidomide. Most patients will reach stable clinical stage, normal blood counts, <5% clonal plasma cells in the bone marrow, stable paraprotein levels after 4–6 cycles of treatment for a period of 1–3 years. Relapse occurs in most patients with median survival of 5–7 years.

Table 9.137 Features of poor prognostic factors at diagnosis of multiple myeloma

Features	Findings Related to Poor Prognosis
Durie and Salmon staging system	▪ Stage I (low tumor load) ▪ Stage II (intermediate tumor load) ▪ Stage III (high tumor load)
Multiple myeloma variant	Plasmablastic multiple myeloma
Cytogenetics/fluorescence *in situ* hybridization (FISH)	▪ t(4;14) (p16;q32) associated with shorter survival, and patient treated with autologous transplant ▪ t(14,16) ▪ t(11;14) (q13; q32) ▪ 17p, Ip deletions (with involvement of TP53 gene) ▪ 1q gains (independent major poor prognostic feature) ▪ Hypodiploidy of tumors ▪ TP53 gene mutation
Bone marrow	Diffuse involvement of bone marrow by monoclonal plasma cells
Clonal plasma cell proliferative index	High
Skeletal lesions	Advanced osteolytic lesions
Serum β_2-microglobulin concentration	Elevated (<6 mg/L) as a result of tumor burden and deteriorating renal functions; but not used to monitor the disease
Plasma albumin concentration	Low <30 g/L
Serum M proteins concentration	IgG >70 g/L; IgA >50 g/L
Bence-Jones protein in urine	>12 g/24 hours
Hemoglobin concentration	Low hemoglobin (<8.5 g/L)
Serum creatinine	Elevated (paraprotein blocks tubules resulting in impaired renal functions)
Serum calcium	Elevated
Serum CRP (C-reactive protein) surrogative marker for IL-6	Elevated
Serum lactic dehydrogenase (LDH) concentration	Elevated
Circulating clonotypic plasma cells in blood	High
Immunophenotyping for clonotypic plasma cells	▪ CD28 positive ▪ CD19 positive ▪ CD117 (KIT) negative ▪ CD56 negative
DNA copy number	Abnormalities detected by SNP arrays methodology

Favorable prognostic factors include hyperdiploid tumors by (DNA cell content of tumor measured by flow cytometry) cytogenetic abnormalities, i.e. with multiple trisomies involving chromosomes 3, 5, 7, 8, 11, 15, 19 and 21.

- Radiotherapy is administered to relieve pain from localized skeletal disease. Hemi body radiation may help to control systemic disease. Allogeneic hematopoietic stem cell transplantation may be curative in selected cases of <65 years. Surgery is indicated in patient developing pathologic feature and spinal cord compression.

WALDENSTRÖM MACROGLOBULINEMIA

Waldenström macroglobulinemia (WM) is a low-grade B cell lymphoproliferative disorder characterized by bone marrow infiltration with clonal post-germinal lymphoplasmacytic cells (immunophenotype: sIgM+, CD19+ and CD20+), together with immunoglobulin M (IgM) monoclonal gammopathy demonstrated on serum electrophoresis. The disorder affects elderly persons with median age 70 years and five-year survival from diagnosis.

- A point mutation in the MYD88 gene resulting in activation of NF-κB and other signaling pathways has been demonstrated in 90–100% of cases.
- Approximately 80% of patients present with monoclonal IgM spike on serum electrophoresis as a result of increased paraprotein level >3 g/dl.
- Serum IgM can be of κ or λ specificity. Bence-Jones proteinuria is demonstrated in 10% of cases. The osteolytic lesions are absent in these patients. These patients develop hyperviscosity of blood.

CLINICAL FEATURES

In Waldenström macroglobulinemia, high levels of IgM >50 g/L can cause hyperviscosity syndrome without osteolytic lesions.

- Patient presents with anemia, thrombocytopenia, fatigue, headache, blurred vision, mucosal bleeding (50% cases), retinal hemorrhages, reduced level of consciousness, hepatomegaly, splenomegaly and lymphadenopathy. Paraprotein coats the platelets and impairs their function leading to mucosal bleeding. Sometimes, plasmapheresis is performed to prevent blindness.
- Lymphoplasmacytic cells infiltrate in bone marrow, lymph nodes, liver and spleen resulting in lymphadenopathy, hepatomegaly and splenomegaly in one-third of patients.
- Other clinical manifestations include Raynaud phenomenon, hemostatic abnormalities, polyneuropathies related to anti-myelin-associated glycoprotein of antibodies, cryoglobulinemia, cold agglutinin disease and acquired von Willebrand disease and amyloidosis. Comparison of plasma cell myeloma and Waldenström disease is given in Table 9.138.

Laboratory Diagnosis of Waldenström Macroglobulinemia

- Laboratory investigations in Waldenström macroglobulinemia include assessment of serum IgM monoclonal protein, bone marrow infiltration in lymph node, liver and spleen.
- Assessment of bone marrow and serum IgM are essential taking into considerations on clinical findings.

Peripheral Blood Smear Examination

Peripheral blood smear examination demonstrates lymphocytosis and rouleaux formation of red blood cells.

Bone Marrow Smear Examination

Bone marrow aspirate smear demonstrates numerous lymphoplasmacytoid cells containing slightly eccentrically placed nucleoli and moderate amount of basophilic cytoplasm.

Immunophenotyping

Lymphoplasmacytoid cells show positivity for CD19, CD20, CD22 and light chains (κ and λ).

Markers	Expression
CD19	Positive
CD20	Positive
CD22	Positive
Kappa (κ) light chain	Positive
Lambda (λ) light chain	Positive

PREDICTIVE POOR PROGNOSTIC FACTORS

Predictive poor prognostic factors for Waldenström macroglobulinemia include hemoglobin <11 g/dl, age over 65 years, thrombocytopenia (platelet count $\leq 100 \times 10^9$/L), serum IgM monoclonal protein >70 g/L and elevated serum β_2-microglobulin (>3 g/L). Prognosis is poor in high-risk patients.

Table 9.138 Comparison of plasma cell myeloma and Waldenström disease

Feature	Multiple Myeloma	Waldenström Disease
Bone marrow lesions	Present	Absent
Serum viscosity	Present/absent	Markedly increased
Immunoglobulin	IgG (Bence-Jones proteins)	IgM (heavy chain)

MANAGEMENT

Patients with Waldenström macroglobulinemia are treated to relieve constitutional symptoms such as lymphadenopathy, hepatomegaly, splenomegaly, hyperviscosity syndrome, hematologic suppression due to bone marrow infiltration and IgM-related syndromes.

- Patients presenting with hyperviscosity syndrome should receive adjuvant plasmapheresis to prevent blindness.
- Symptomatic patients are administered alkylating agent such as chlorambucil with or without prednisolone producing remission in 50–70% of cases.
- Autologous hematopoietic stem cell (HSC) transplantation may be considered for patients with refractory or relapsing disease.

MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE

Monoclonal gammopathy of undetermined significance (MGUS) is an asymptomatic condition in which there is a presence of an abnormal M protein <3 g/dl in the blood or urine in persons, minimal or absence of Bence-Jones proteins in urine, decreased normal immunoglobulins in blood; and <10% of plasma cells in bone marrow; without evidence of multiple myeloma, Waldenström macroglobulinemia, amyloidosis or other lymphoproliferative disorders. Clinical manifestations of variants of monoclonal gammopathy of undetermined significance (MGUS) are given in Table 9.139.

- MGUS most often affect about 4% of Caucasian persons over 50 years. The prevalence of MGUS is higher in African-Americans. Other risk factors are male sex, exposure to pesticide and family history of MGUS or multiple myeloma.
- MGUS is composed of two different kinds of neoplasms: (a) lymphoid MGUS (15%) and (b) plasma cell MGUS (85%).
  - The lymphoid MGUS secretes immunoglobulin M (IgM) and progresses to Waldenström macroglobulinemia, lymphoma or other malignant lymphoproliferative disorders.
  - The plasma cell MGUS does not secrete immunoglobulin M (IgM), but rather secretes IgG, IgD, IgE, or immunoglobulin light chain, which most commonly progresses to multiple myeloma IgG.

CLINICAL FEATURES

Monoclonal gammopathy of undetermined significance (MGUS) is usually asymptomatic without clinical consequences.

- There is no evidence of **CRAB** criteria: **C**: calcium elevation (high levels of calcium in the blood; also known as 'hypercalcemia'); **R**: renal insufficiency (poor function of the kidneys that may be due to a

Table 9.139 Clinical manifestations of variants of monoclonal gammopathy of undetermined significance (MGUS)

Key Features	Clinical Notes
Monoclonal gammopathy of undetermined significance (MGUS) non-IgM	
▪ IgG or IgA; M protein <30 g/L ▪ Bone marrow plasma cells <10% ▪ Absence of myeloma-defining events (MDE) such as organ/tissue damage or amyloidosis	Progression risk to multiple myeloma, amyloidosis or plasmacytoma is 0.5–1.0% per year
Monoclonal gammopathy of undetermined significance (MGUS) IgM	
▪ IgM (M protein) <30 g/L ▪ Bone marrow demonstrates lymphoplasmacytic cells <10% ▪ Absence of end-organ damage (anemia, constitutional symptoms, hyperviscosity, lymphadenopathy, hepatosplenomegaly) or myeloma-defining events (MDE) or amyloidosis	Prognosis risk for Waldenström's or lymphoproliferative disorder is 1.5% per year
Monoclonal gammopathy of undetermined significance (MGUS) light chain	
▪ Abnormal free light chain ratio (<0.26 or >1.65) with an increased involved corresponding serum-free light chain ▪ No immunoglobulin heavy chains expression ▪ Urinary excretion of monoclonal protein <500 mg per 24 hours ▪ Bone marrow demonstrates plasma cells <10% ▪ Absence of myeloma-defining events (MDE) or amyloidosis	Progression risk for multiple myeloma, amyloidosis or plasmacytoma is 0.3% per year

reduction in blood flow to the kidneys); **A**: anemia (low RBC count); **B**: bone abnormalities (lesions).

- About 20–25% of MGUS patients can progress to multiple myeloma during their life-time. About risk of progression of MGUS to malignancy is about 20–25%.
- There are three spectrums of MGUS: (a) MGUS with an increased but **stable** number of tumor cells; (b) MGUS with **minimally progressive** multiple myeloma with end organ damage; and (c) **moderately progressive** multiple myeloma, but with end organ damage that is not yet detected.

MONONUCLEAR PHAGOCYTIC SYSTEM DISORDERS

In immunology, mononuclear phagocytic system is a part of immune system, that consists of monocytes and macrophages.

- Mononuclear phagocytes are derived from a common precursor in the bone marrow, which gives rise to monocytes. Blood monocytes migrate and differentiate into tissue macrophages known as reticuloendothelial cells diffusely distributed in the connective tissues in various organs. Reticuloendothelial cell distribution in various tissues is given in Table 9.140.
- Storage disorders of mononuclear phagocytic system are the consequence of inherited enzyme deficiencies that lead to accumulation of the substances that are incompletely catabolized and degraded in the cells of mononuclear phagocytic system in organs such as bone marrow, liver and spleen. Many of these metabolic products are derived from lipids of cell membrane.

Table 9.140 Reticuloendothelial cell distribution in various tissues

Tissue	Reticuloendothelial Cells
Blood	Monocytes
Connective tissue	Macrophages
Lung	Peritoneal macrophages
Peritoneum	Mesangial cells
Liver	Kupffer cells
Lymph node	Sinus histiocytes
Spleen	Littoral cells
Placenta	Hofbauer cells
Skin	Melanophages
Brain	Microglial cells
Synovium	Type 1 histiocytes
Bone	Osteoclasts
Adipose tissue	Lipophages
Specialized tissue macrophages (reticuloendothelial cells)	▪ Epithelioid cells ▪ Histiocytic giant cells ▪ Langerhans' giant cells ▪ Foreign body giant cells ▪ Touton giant cells

- Disorders of mononuclear phagocytic system include storage histiocytosis (e.g. Gaucher's disease, Niemann-Pick and Sea-blue histiocytosis) and Langerhans' cell histiocytosis [e.g. Letterer-Siwe disease (acute disseminated LCH), Hand-Schüller disease (multifocal LCH) and eosinophilic granuloma (unifocal LCH)].

LYSOSOMAL STORAGE DISORDERS

Lysosomal storage diseases are inherited metabolic disorders caused by gene mutations encoding lysosomal enzymes, which result in cell damage due to excessive accumulation of various toxic undegradable substrates in different organs of the body, including skeleton, skin, heart, liver, central nervous system. Lipid storage disorders include glucosylceramidase beta/GBA (Gaucher's disease), sphingomyelin phosphodiesterase 1/SMPD1(Niemann-Pick disease) and galactosidase alpha/GLA (Fabry disease).

GAUCHER'S DISEASE

Gaucher's disease is an autosomal recessive disorder most often seen in persons of European (Ashkenazi) Jewish lineage. A deficiency of β-glucocerebrosidase causes an accumulation of sphingolipid within mononuclear phagocytic cells in various organs such as bone marrow, liver, and spleen.

- Sphingolipid laden mononuclear phagocytic cells are known as Gaucher's cells. Mutations in the GBA1 gene can be demonstrated in these cases.
- Prenatal diagnosis through GBA1 gene mutation is feasible. Gaucher's disease can be confirmed by demonstration of deficiency of acid glucocerebrosidase activity in leukocytes.
- Bone marrow involvement and hypersplenism contribute to anemia and thrombocytopenia. Gaucher's cells accumulation in liver and spleen lead to hepatomegaly and splenomegaly.
- Hip fracture in a patient of any age with a palpable spleen—especially in a Jewish person of Eastern European origin—suggests the possibility of Gaucher's disease.

Diagnostic Hallmark

Diagnostic hallmark of Gaucher's disease is the presence of lipid-laden macrophages 'Gaucher's cells' with a distinctive fibrillary, wrinkled tissue paper-like cytoplasm and eccentric nuclei.

- Gaucher's cells are demonstrated in bone marrow, liver, spleen, central nervous system, tonsil, thymus, Peyer's patches, and lungs. Cytoplasm is fibrillary and crumpled (tissue paper-like).
- Definitive diagnosis requires the demonstration of deficient glucocerebrosidase activity in leukocytes.

Clinical Features

Clinical features are variable depending on three variants of type 1 (most common), type 2 (uncommon) and type 3 (uncommon) of Gaucher's disease. Comparison of three variants of Gaucher's disease types 1, 2, 3 is given in Table 9.141.

Gaucher's Disease Type 1

Gaucher's disease type 1 is an autosomal recessive lysosomal storage disorder and most common forms of Gaucher's disease affecting >90% persons in Europe and USA. It is caused by a mutation in GBA gene (1q21) that codes for the lysosomal enzyme glucocerebrosidase. The deficiency in glucocerebrosidase leads to the accumulation of glucocerebrosidase deposits in the cells of reticuloendothelial system of liver, spleen, and bone marrow (Gaucher's cells).

- **Clinical features:** Patient presents with cytopenias (anemia, leukopenia, and thrombocytopenia), splenomegaly, hepatomegaly, and bone disease. Symptoms range from mild to severe from childhood to adulthood. Gaucher disease type 1 does not involve the brain and spinal cord (central nervous system).
- **Diagnostic approach:** If Gaucher disease type 1 is suspected on the basis of clinical symptoms, diagnosis can be confirmed by molecular analysis of glucocerebrosidase (GBA) enzyme activity.
- **Treatment:** Most common complications of Gaucher's disease can be prevented by early administration of enzyme replacement therapy. The patients are treated by administration of glucocerebroside enzyme replacement therapy by intravenous route (imiglucerase, velaglucerase, or taliglucerase). Orally administered inhibitors of glucosylceramide biosynthesis can be used (miglustat of eliglustat), which reduce the total body stores of glycolipid and improve hematologic and orthopedic manifestations.
- **Prevention:** Carrier screening, especially among Ashkenazi Jews, detects those couple at high risk of having an affected infant. Prenatal diagnosis through GBA1 gene mutation analysis is feasible. Because of increased risk for developing multiple myeloma and other hematopoietic malignancies, regular screening of patients with Gaucher disease is warranted.

Gaucher's Disease Type 2

Gaucher's disease type 2 is an autosomal recessive lysosomal storage disorder caused by a mutation in GBA gene (1q21) that codes for the lysosomal enzyme glucocerebrosidase. The deficiency in glucocerebrosidase leads to the accumulation of glucocerebrosidase deposits in the cells of reticuloendothelial system of brain, liver, spleen and bone marrow (Gaucher's cells).

- **Clinical features:** Children develop symptoms by the age of three months of age, which include neurological impairment (seizures, abnormal eye movements), dysphagia, dystonia and hepatosplenomegaly.
- **Prognosis:** Many children have fatal outcome by two to four years of age. Heterozygotes for Gaucher's disease are at increased risk for developing Parkinson disease. Enzyme glucocerebrosidase replacement therapy is ineffective in these patients.

Gaucher's Disease Type 3

Gaucher's disease type 3 is an autosomal recessive lysosomal storage disorder caused by a mutation in GBA gene (1q21) that codes for the lysosomal enzyme glucocerebrosidase.

- **Pathogenesis:** The deficiency in glucocerebrosidase leads to the accumulation of glucocerebrosidase deposits in the cells of reticuloendothelial system of liver, spleen, and bone marrow (Gaucher's cells).

Table 9.141 Comparison of three variants of Gaucher's disease types 1, 2, 3

Parameters	Gaucher's Disease Type 1	Gaucher's Disease Type 2	Gaucher's Disease Type 3
Age group	Adults (>90% in Europe and USA)	Infants	Early childhood
Frequency	>90% most common	Uncommon	Uncommon
Organs involved	Liver, spleen, bone marrow, lymph nodes	Liver, spleen, bone marrow and central nervous system	Central nervous system and viscera
Clinical features	Splenomegaly, hepatomegaly, cytopenia, fracture of femoral head	Failure to thrive and neurological impairment	Myoclonic convulsions
Prognosis	Normal life span	Fatal by one year of age	Less severe disorder

It is the subacute neurological form of Gaucher's disease affecting children and adolescence age group. Neurological symptoms are variable in Gaucher's disease type 3.

- **Clinical features:** Children and adolescents can present with moderate systemic involvement and ophthalmoplegia, progressive myoclonic epilepsy, cerebellar ataxia, spasticity, and dementia.
  - Gaucher's disease type 3 can manifest as systemic disease with signs of asthenia, growth retardation, delayed puberty, and hepatosplenomegaly.
  - Skeleton anomalies may include deformities, osteopenia, pathological fractures, vertebral compression, bone infarctions or even aseptic osteonecrosis.
  - Pancytopenia is frequent leading to varying degrees of anemia, leukopenia, and thrombocytopenia.
  - Polyclonal hypergammaglobulinemia is often present and is sometimes complicated by monoclonal gammopathy.
- **Diagnostic approach:** Gaucher disease type 3 is diagnosed by ultrasonography and magnetic resonance imaging for initial; evaluation and subsequent monitoring of hepatomegaly, radiography, and bone scintigraphy to detect skeleton lesions, osteodensitometry for evaluation of osteopenia of the lumbar spine and femoral neck, and cardiac ultrasonography for the detection of pulmonary arterial hypertension.
- **Biochemical markers:** Certain biochemical markers such as chitotriosidase, an angiotensin converting enzyme, ferritin and tartrate-resistant acid phosphatases are analyzed for the initial; diagnosis and monitoring of Gaucher disease type 3.
  - Diagnosis can be confirmed by demonstrating a defect in the enzyme activity of glucocerebrosidase in circulating leukocytes.
  - Genotyping in a patient with homozygous L444P mutation in the GBA gene is at higher risk of developing neurological disease.
  - Heterozygotes for Gaucher disease are at high-risk for developing Parkinson disease.

Laboratory Diagnosis of Gaucher's Disease

Peripheral Blood Smear Examination

Peripheral blood smear shows cytopenia, i.e. anemia, leukopenia and thrombocytopenia due to involvement of bone marrow by Gaucher's disease.

Bone Marrow Examination

- Bone marrow aspiration reveals numerous lipid-laden macrophages 'Gaucher cells' with a distinctive fibrillary, wrinkled tissue paper-like cytoplasm and eccentric nuclei and PAS-positive inclusions.
- Gaucher cells are demonstrated in liver sinusoids (Kupffer cells) and bone marrow.
- Definitive diagnosis requires the demonstration of deficient glucocerebrosidase activity in leukocytes.
- Special enzymes such as chitotriosidase, an angiotensin converting enzyme, ferritin and tartrate-resistant acid phosphatases are used to demonstrate proliferation of macrophages. Gaucher's cells in bone marrow are shown in Fig. 9.123.

Histochemistry

PAS positive Gaucher cells in Gaucher disease in bone marrow trephine biopsy is shown in Fig. 9.124.

Bone Marrow Trephine Biopsy Examination

Bone marrow trephine biopsy section shows Gaucher's cells containing cigarette paper-like cytoplasmic appearance and eccentric nuclei, which are lipid-laden macrophages. Gaucher's cells in trephine bone marrow biopsy are shown in Fig. 9.125.

NIEMANN-PICK DISEASE

Niemann-Pick disease (NPD) is an inherited autosomal recessive pattern lysosomal storage disorder, which means both copies of the gene must have mutations for the manifestation of the disease.

- The three most recognized forms are Niemann-Pick disease type A and type B (NPA, NPB) also called acid sphingomyelinase enzyme deficiency, and Niemann-Pick type C (NPC).
- NPD types A and B occur due to missense mutations in the sphingomyelin phosphodiesterase 1 (SMPD1) gene. NPD type C is caused by mutations in NPC1 (located on chromosome 8) and NPC2 (located on chromosome 14) genes,

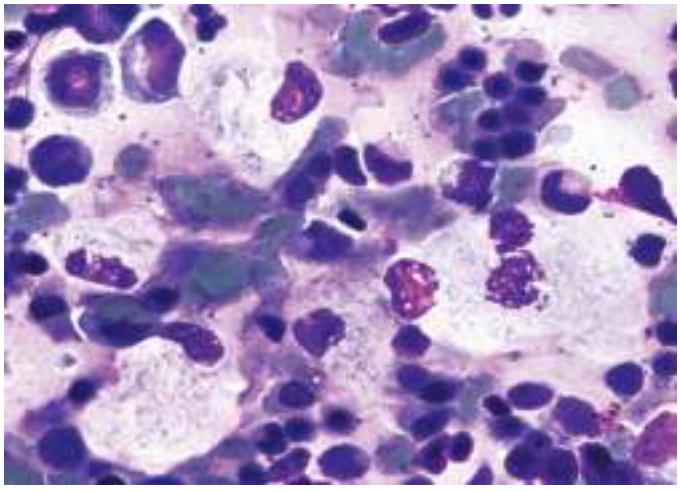


Fig. 9.123: Gaucher's disease in Giemsa-stained bone marrow aspirate smear. Bone marrow examination shows Gaucher's cells containing cigarette paper-like cytoplasmic appearance and eccentric nuclei, which are lipid-laden macrophages (400X).

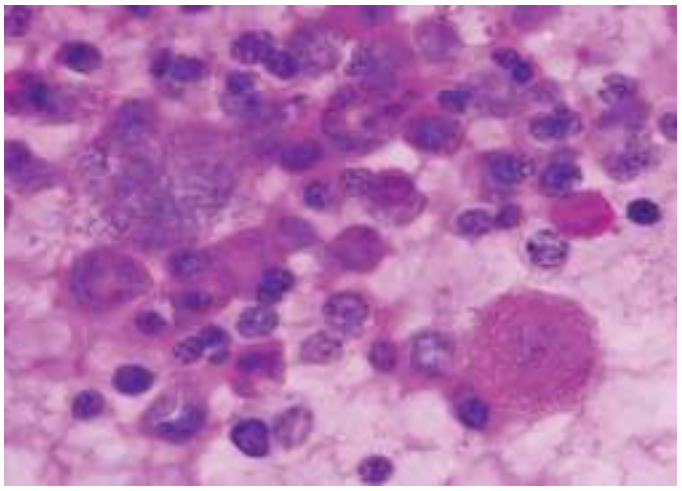


Fig. 9.124: PAS positive Gaucher cells in Gaucher disease in bone marrow trephine biopsy (400X)

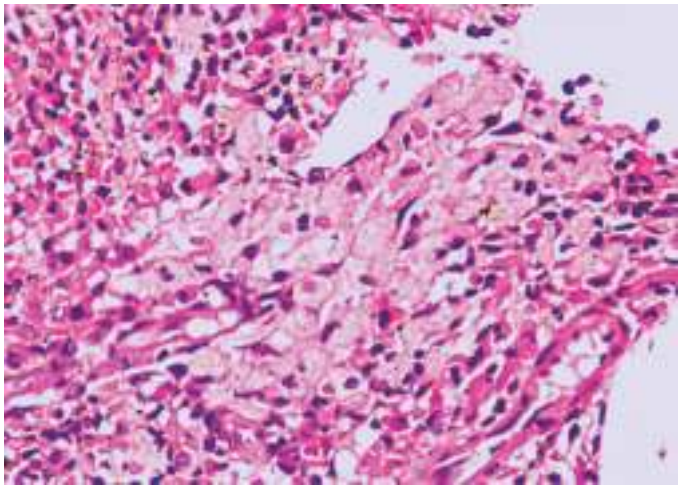


Fig. 9.125: Gaucher's disease in hematoxylin and eosin-stained bone marrow trephine biopsy section. Bone marrow trephine biopsy section shows Gaucher's cells containing cigarette paper-like cytoplasmic appearance and eccentric nuclei, which are lipid-laden macrophages (400X).

which result in synthesis of abnormal or defective proteins, which impair the movement of lipids out of the cells leading to their accumulation within macrophages.

Pathogenesis

Niemann-Pick disease type A and type B (NPA, NPB) are caused by the deficiency of lysosomal acid sphingomyelinase deficiency (ASMD). Lysosomal acid sphingomyelinase enzyme is required to metabolize lipid called sphingomyelin to ceramide and phosphocholine.

- If acid sphingomyelinase is deficient or not functioning properly, sphingomyelin cannot be metabolized properly and is accumulated within the lysosomes of macrophages. These lipid-laden macrophages deposit in the liver, spleen, bone marrow, lungs and brain, causing hepatosplenomegaly, cytopenias, lung disease and neurological symptoms.
- Diagnostic hallmark of Niemann-Pick disease is presence of foamy histiocytes containing sphingomyelin in liver, spleen, lymph nodes, skin, bone marrow, tonsil, gastrointestinal tract, and lungs.
- Niemann-Pick disease (type A and type B) is caused due to missense mutations in sphingomyelin phosphodiesterase 1 (SMPS1) gene.
  - Niemann-Pick disease type C is caused by mutations in NPC1 gene located on chromosome 18 and NPC2 gene located on chromosome 14.
  - The mutations in these genes (SMPS1, NPC1, NPC2) lead to production of abnormal of defective proteins, which impair the movement of lipids out of the mononuclear phagocytes leading to their accumulation within mononuclear phagocytes and cellular damage.
- Molecular testing is necessary to confirm the diagnosis in all persons with suspected Niemann-Pick disease.

Clinical Features

Niemann-Pick disease can affect all segments of population with cases reported in North America, South America, Europe, Africa, Australia and Asia. However, Niemann-Pick disease types A and B affect Ashkenazi Jewish population. Niemann-Pick disease type C affects French Canadian population of Nova Scotia. Comparison of three types (A, B, C) of Niemann-Pick disease is given in Table 9.142.

- **Niemann-Pick disease type A:** Niemann-Pick disease type A is also known as neurovisceral form with very low acid sphingomyelinase (ASM) activity and associated with fatal outcome before three years of age. It affects young children and results in hepatosplenomegaly, lymphadenopathy, cherry red spots inside the eyes, difficulty in feeding, neurological deficits and impaired growth.
- **Niemann-Pick disease type B:** Niemann-Pick disease type B is less severe and characterized by variable visceral symptoms and minimal neurological manifestations. The most common visceral symptoms in these phenotypes include hepatosplenomegaly, thrombocytopenia and interstitial pulmonary disease associated with relatively better prognosis.
- **Niemann-Pick dsease type C:** Niemann-Pick disease type C has heterogenous clinical manifestations as a result of systemic, neurologic and psychiatric involvement. It usually affects adults but can occur during any phase of life.

Table 9.142 Comparison of three types (A, B, C) of Niemann-Pick disease

Parameters	Type A Niemann-Pick Disease (Classic Infantile Type)	Type B Niemann-Pick Disease (Visceral Type)	Type C Niemann-Pick Disease (Visceral Type)
Age group	Infants (severe disease)	Adults (less severe)	10 and 25 years
Basic defect	SMPD1 gene mutation encoding acid sphingomyelinase (ASM) protein resulting in accumulation of sphingomyelin in the lysosomes of phagocytes	SMPD1 gene mutation encoding acid sphingomyelinase (ASM) protein resulting in accumulation of sphingomyelin in the lysosomes of phagocytes	NPC1 or NPC2 gene mutations resulting in accumulation of sphingomyelin in the lysosomes of phagocytes
Clinical features	Hepatomegaly, splenomegaly, anemia, fever, profound neurologic manifestations and cherry red spots inside eye, difficulty in feeding	Hepatomegaly, splenomegaly, pulmonary insufficiency and central nervous system manifestations	Hepatomegaly, splenomegaly, central nervous system manifestations
Prognosis	Fatal outcome by three years of age (rarely survival beyond three years of age)	Survival in adulthood	Sometimes fatal outcome

- The diagnosis of Niemann-Pick disease type C (visceral type) should be considered in persons presenting with clinical manifestations: (a) fetal ascites or neonatal liver disease accompanied by prolonged jaundice and pulmonary infiltrates, (b) infantile hypotonia without evidence of progression for months to years, (c) vertical supranuclear gaze palsy followed by progressive ataxia, dystonia, dysarthria and seizures, (d) psychiatric presentations mimicking depression or schizophrenia and (e) hepatomegaly or splenomegaly particularly in early childhood.

- Some patients can develop significant life-threatening complications including hepatocellular failure, hemorrhage, oxygen dependency, pulmonary infections, splenic rupture and coronary artery disease or valvular heart disease. There are no known treatments that effectively slow the progression of Niemann-Pick disease.

Laboratory Diagnosis of Niemann-Pick Disease (NPD)

Biochemical Analysis

- Niemann-Pick disease types A and B are diagnosed by analyzing the acid sphingomyelinase enzyme (ASM) activity in white blood cells. The test can be performed on blood sample from persons who are suspected of having Niemann-Pick disease.
- Niemann-Pick disease type C is diagnosed on skin biopsy to assess cholesterol in the mononuclear phagocytes.
- Periodic liver enzymes analysis, liver elastography and liver biopsy should be done in case of severe liver disease.
- Periodic spirometry and high-resolution computed tomography (HRCT) are performed for interstitial lung disease.
- Platelet counts and spleen volume should be analyzed. Fundoscopy is done to look for the cherry-red spot on the macula.
- Complete neurological examination should be performed at every visit. Lipid profile should be done to evaluate cardiovascular system.

Bone Marrow Smear Examination

- Bone marrow aspirate shows Niemann-Pick cells, which are large 25–100 μm in size with eccentric nuclei and foamy cytoplasm.
- Foam cells show positivity with oil red O and Sudan black B. Niemann-Pick disease in Giemsa-stained bone marrow aspirate smear is shown in Fig. 9.126.

Bone Marrow Trephine Biopsy Examination

- Niemann-Pick disease in bone marrow trephine biopsy section shows sheets of Niemann-Pick cells, which are large 25–100 μm in size with eccentric nuclei and foamy cytoplasm.
- Niemann-Pick disease in hematoxylin and eosin-stained bone marrow trephine biopsy section is shown in Fig. 9.127.

Electron Microscopy

Electron microscopy of skin, rectal neurons, liver, or brain tissues may demonstrate electron-opaque, concentrically laminated inclusions within macrophage cytoplasm.

Treatment

There is no known treatment for Niemann-Pick disease type A. Niemann-Pick disease type B can be treated by bone marrow hematopoietic stem cell transplantation, enzyme replacement therapy and gene therapy. Niemann-Pick disease type C is currently treated by miglustat, a glucosylceramide synthase inhibitor regularly for an average of two years.

SEA-BLUE HISTIOCYTOSIS

Sea-blue histiocytosis is a cutaneous condition, that may occur as familial inherited syndrome or as an acquired secondary or systemic infiltrative process.

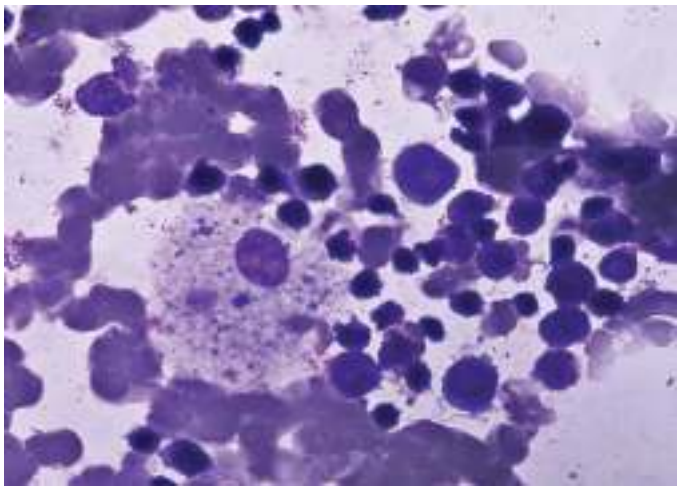


Fig. 9.126: Niemann-Pick disease in Giemsa-stained bone marrow aspirate smear (1000X).

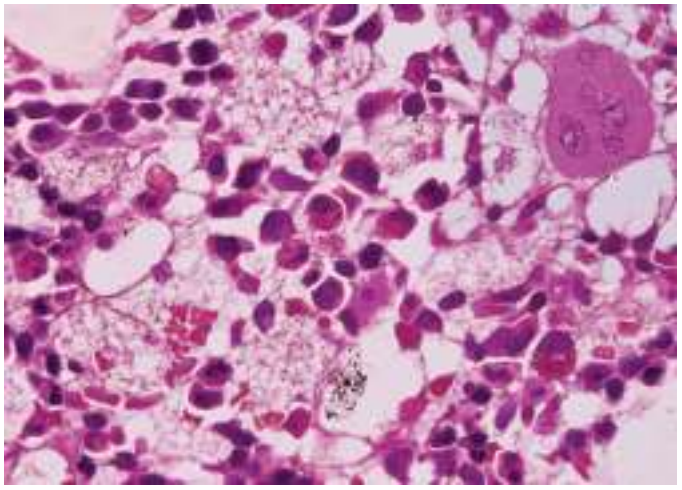


Fig. 9.127: Niemann-Pick disease in hematoxylin and eosin-stained bone marrow trephine biopsy section. Niemann-Pick cells are large 25–100 µm in size with eccentric nuclei and foamy cytoplasm. Foam cells show positivity with oil red O and Sudan black B (1000X).

- Sea-blue histiocytosis is characterized by splenomegaly, mild thrombocytopenia, bone marrow involvement, elevated blood triglyceride levels, impaired liver functions, heart disease, and numerous macrophages laden with incompletely degraded lipids and cytoplasmic granules in the bone marrow, which stain bright blue with the usual May-Grunwald Giemsa/PAS stains. Sea-blue histiocytes are ceroid-laden macrophages.
- Primary sea-blue histiocytosis is an autosomal dominant disorder caused by mutation in APOE gene.
- Secondary sea-blue histiocytosis may occur due to hematologic, lipid and ceroid metabolic and miscellaneous disorders. Causes of sea-blue histiocytosis are given in Table 9.143.

Table 9.143 Causes of sea-blue histiocytosis

Primary Sea-blue Histiocytosis
No known cause is after thorough investigation.
Secondary Sea-blue Histiocytosis
▪ **Hematological disorders** • Chronic myelogenous leukemia • Idiopathic thrombocytopenic purpura • Severe autoimmune neutropenia • Myeloproliferative dysplastic syndrome • Infectious diseases with bone marrow involvement
▪ **Lipid and ceroid metabolic disorders** • Gaucher disease • Niemann-Pick disease • Fabry disease • Hyperlipidemic disorders • Lecithin cholesterol acyltransferase deficiency
▪ **Miscellaneous disorders** • Batten's disease • Neuroaxonal dystrophy • Prolonged therapy with liposomal amphotericin B • Long-term parenteral nutrition with fat emulsion • Takayasu arteritis • Posterior column dysfunction

Laboratory Diagnosis of Sea-blue Histiocytosis

Bone Marrow Examination

Bone marrow aspirate shows numerous histiocytes with granules and sea-blue cytoplasm on May-Grunwald-Giemsa stain (Fig. 9.128).

Cytochemical Stains

Histiocytes contain granules, which are demonstrated by PAS stain and Sudan black.

Immunophenotyping

Marker	Expression
CD68	Positive

LANGERHANS' CELL HISTIOCYTOSIS

Langerhans' cells participate in immunity by presenting antigen to T cells. Langerhans' cell histiocytosis is a clonal disorder characterized by proliferation of epidermal antigen-presenting histiocytes that closely resemble the Langerhans' cells of the epidermis.

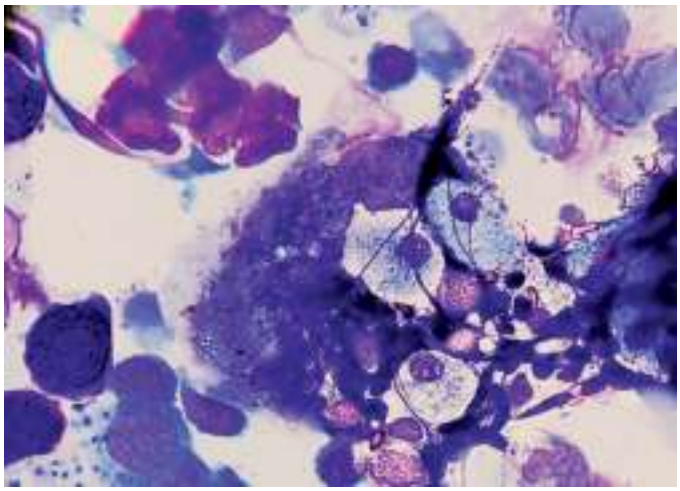


Fig. 9.128: Sea-blue histiocytosis in Giemsa-stained bone marrow aspirate smear. It shows histiocytes with sea-blue colored granules (400X).

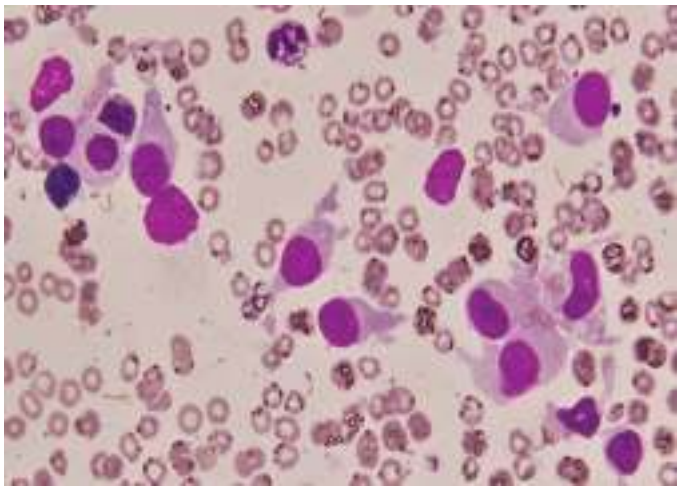


Fig. 9.129: Fine needle aspiration cytology in a case of Langerhans' cell histiocytosis. A two-year-old male child presented with fluctuant swellings measuring 2–3 cm on left frontal and occipital regions of the skull for the last one year. Fine needle aspiration from a swelling revealed hemorrhagic fluid. FNAC smear showed numerous cells and tennis racket-shaped cells consistent with Langerhans' cell histiocytosis (400X).

- Langerhans' cell histiocytosis (LCH) affects children, who can present as a solitary lesion requiring no treatment. A multiorgan life-threatening disorder requires aggressive therapy in 20% of cases.
- Langerhans' cells are tennis racket-shaped containing Birbeck granules demonstrated by electron microscopy, which contain various enzymes such as acid phosphatase, α-naphthyl acetate esterase and α-naphthyl butyrate esterase. Langerhans' cells express distinctive surface antigens, which cells show positivity with S-100, CD1a and langerin.
- BRAF gene mutation has been demonstrated in 50% cases especially in younger patients. High prevalence, recurrent BRAF mutations in LCH indicate that is a neoplastic disease, that may respond to RAF pathway inhibitors.
- Langerhans' cell histiocytosis includes Letterer-Siwe disease, Hand-Schüller-Christian disease and eosinophilic granuloma. Clinical manifestations range from involvement of single site (bone or lymph node) in 75% of cases to an aggressive systemic disorder involving multiple organs (25%).
- Fine needle aspiration cytology is helpful in achieving a rapid and accurate diagnosis of Langerhans' cell histiocytosis in an appropriate clinical and radiological setting. Fine needle aspiration cytology in a case of Langerhans cell histiocytosis is shown in Fig. 9.129.

LETTERER-SIWE DISEASE (ACUTE DISSEMINATED LCH)

Letterer-Siwe disease is **acute disseminated Langerhans' cell histiocytosis** affecting **infants** and **small children**. Disease has rapid aggressive course with fatal outcome.

Light microscopy reveals lack of lipids in histiocytes. Foci of necrosis are present.

- Langerhans' cells are normally present in the skin, which detect non-self antigens and present them to the T cells of immune system, thus allowing an appropriate immune response.
- But in Letterer-Siwe disease, Langerhans' cells disseminate to bone marrow, liver, spleen, lungs, lymph nodes, skin and brain. Granulomatous inflammatory lesions develop and may proliferate and become destructive lesions, which later become less cellular, necrotic and fibrotic.

Diagnostic Hallmarks

The hallmarks of Letterer-Siwe disease is the presence of pathologic Langerhans' cells involved tissues such as bone marrow, liver, spleen, lungs, lymph nodes, skin and brain. Specific histochemical, immunologic, immunohistochemical markers (Birbeck granules or positive S-100 protein and CD1a antigen) and multiorgan involvement.

Clinical Features

Patient presents with painless, yellow brown maculopapular skin lesions scattered over face, especially around eyes, mouth, trunk, perineum, axillae; and recurrent infection as a result of widespread histiocytic proliferation. Clinical examination reveals hepatosplenomegaly, lymphadenopathy, pulmonary involvement resulting in honeycomb appearance, spontaneous pneumothorax and pleural effusion.

Laboratory Diagnosis of Letterer-Siwe Disease	
Peripheral Blood Smear Examination	
Peripheral blood smear examination shows pancytopenia as a result of replacement of bone marrow elements due to accumulation of histiocytosis.	
Bone Marrow Smear Examination	
Bone marrow aspirate shows numerous histiocytes with abundant cytoplasm admixed with a few lymphocytes and eosinophils, which are laden with cholesterol.	
Electron Microscopy	
Birbeck granules in histiocytes may be demonstrated on electron microscopy.	
Immunophenotyping	
Panel of markers used to demonstrate Langerhans' cells includes S-100 protein, CD1a and langerin.	
Markers	**Expression**
▪ S-100 protein ▪ CD1a ▪ Langerin	▪ Positive ▪ Positive ▪ Positive

HAND-SCHÜLLER-CHRISTIAN DISEASE (MULTIFOCAL LCH)

Hand-Schüller-Christian disease is acute onset followed by chronic progressive histiocytosis affecting infants to elderly persons, which most often presents before 5 years of age and associated with better prognosis than Letterer-Siwe disease. Light microscopy shows numerous histiocytes admixed with eosinophil cells in bone, especially the skull, liver, spleen, and other tissues.

Clinical Features

Hand-Schüller-Christian disease has classic triad: (a) bone lesions, (b) diabetes insipidus and (c) exophthalmos.

- Bone lesions are demonstrated in the orbit, tooth-bearing area of mandible and mastoid bone. Diabetes insipidus occurs due to destruction of sella turcica. Exophthalmos is caused by involvement of the orbit.
- Other lesions may occur in femur, pelvis, ribs, humerus (end of shaft), spine, lung, skin and mucosa.
- Hepatosplenomegaly and lymphadenopathy may occur in 25–50% of cases.

EOSINOPHILIC GRANULOMA

Eosinophilic granuloma is the benign form of Langerhans' cell histiocytosis (LCH), which accounts for 60–80% cases and can affect children, adolescents and young adults. Disorder can cause bone lesions in solitary or multiple bones. When multiple bone lesions occur, new osseous lesions appear within a span of 1–2 years.

- Sites of bone lesion include thoracic spine, cervical spine, ramus of mandible, orbit, spine (especially vertebral bodies), ribs, clavicle, and long bones (diaphysis of femur and humerus). There may be involvement of lung and gastrointestinal tract.
- Exact pathogenesis is unknown. Epstein-Barr virus, human herpesvirus 6, bacteria and genetic factors have been implicated. An immunological dysfunction is reported with an increase in the cytokines such as IL-1 and IL-10 in the affected patients. Eosinophilic granuloma is extremely radiosensitive.

Clinical Features

Patient presents with localized bone pain, tenderness, swelling, restricted range of movements and fever. Lesions usually begin to regress within three months resulting in complete regression within two years.

- Patient with pulmonary eosinophilic granuloma presents with sudden onset, and has cough, weight loss, dyspnea and pneumothorax.
- Eosinophilic granuloma in gastrointestinal tract is solitary, which may simulate polyps, gastric carcinoma or duodenal ulcer.
- Laboratory findings in eosinophilic granuloma are usually nonspecific except for a moderate and inconsistent leukocytosis and rise in erythrocyte sedimentation rate.

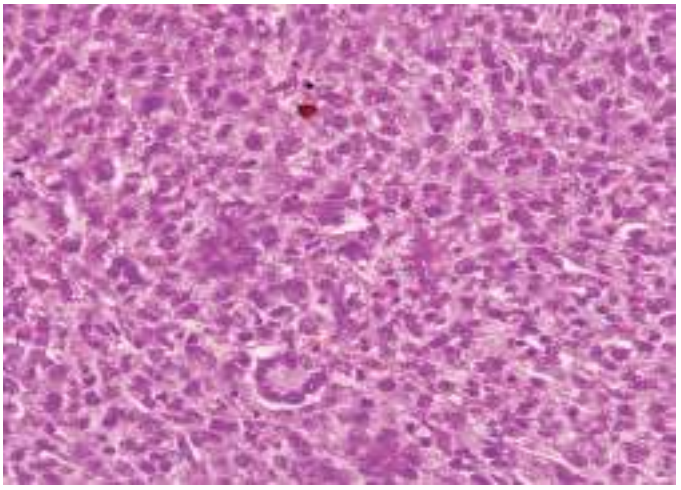


Fig. 9.130: Eosinophilic granuloma in hematoxylin and eosin-stained biopsy section. The lesion is composed of proliferation of histiocytes admixed with inflammatory cells, including ordinary macrophages, lymphocytes, and many eosinophils is characteristic (400X).

Radiologic Findings

The typical radiographic appearance of eosinophilic granuloma of the extremities is a punched-out osteolytic lesion without reactive sclerosis. In most cases, a hypervascularized soft tissue mass surrounds the affected bone.

- While reporting eosinophilic granuloma, differential diagnosis must be considered such as multiple myeloma, plasmacytoma, osteochondritis, tuberculosis, and chronic osteomyelitis.
- In the spine, imaging studies may demonstrate variable vertebral involvement ranging from isolated osteolytic lesion to a more significant vertebral collapse that involve the pedicles and posterior vertebral elements.

Laboratory Diagnosis of Eosinophilic Granuloma

Light Microscopy

Eosinophilic granuloma is composed of proliferation of histiocytes admixed with inflammatory cells, including ordinary macrophages, lymphocytes, and many eosinophils is characteristic (Fig. 9.130).

HEMATOPOIETIC STEM CELL TRANSPLANTATION

HEMATOPOIETIC STEM CELL TRANSPLANTATION: OVERVIEW

Hematopoietic stem cells (**HSCs**) develop during embryogenesis in a complex developmental process that involves multiple anatomical sites such as yolk sac, aorta-gonad-mesonephros region, placenta and fetal liver, after which hematopoietic stem cells colonize in the bone marrow at birth.

- Hematopoietic stem cells are defined as cells that are capable of self-renewal and their differentiation into myeloid and lymphoid lineages, which form the progenitor cells, which are committed to form and differentiate into mature functional cells (RBCs, WBCs and platelets) in the peripheral blood. Hematopoietic stem cells show positivity for CD34+ (most widely used, CD90, and CD123). Hematopoietic stem cells are negative for HLA-DR during postnatal life.
- Hematopoietic stem cell transplantation is the term now used in preference for bone marrow transplantation (BMT), in which a patient receives healthy hematopoietic stem cells to replace their own HSCs are destroyed by treatment with radiation or high doses of chemotherapy.
- The healthy HSCs may be derived from the blood or bone marrow of the patient or from a related or unrelated donor. HSC transplant may be autologous (using a patient's own hematopoietic stem cells that are obtained and preserved before treatment), allogeneic (using hematopoietic stem cells from a related or unrelated donor with different MHC/HLA alleles), syngeneic (using hematopoietic stem cells donated by identical twin) or umbilical cord blood (using umbilical cord blood donated after a baby is born).
- Generally, most common indications of autologous hematopoietic stem cell transplantation are multiple myeloma, non-Hodgkin lymphoma and acute myelogenous leukemia. Main indications for allogeneic hematopoietic stem cell transplantation is acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS), CML refractory to receptor tyrosine kinase inhibitors, lymphoid malignancies and nonmalignant disorders such as bone marrow dysplasia, paroxysmal nocturnal hemoglobinuria and severe immunodeficiency disorders. Indications for hematopoietic stem cell transplantation are given in Table 9.144.
- In hematopoietic stem cell transplantation procedure, blood is obtained from a vein in the arm of the donor. The blood flows through a machine that removes HSCs. Blood is now returned to the donor through a vein in the other arm. The patient receives chemotherapy to eradicate hematopoietic stem cells. The patient may receive radiation therapy. The patient receives hematopoietic stem cells through a catheter placed into blood vessel in the chest. Hematopoietic stem cell transplantation is shown in Fig. 9.131 and overview of hematopoietic stem cell transplantation is shown in Fig. 9.132.

HEMATOPOIETIC STEM CELL HARVESTING

Hematopoietic stem cells (HSCs) for allogeneic transplants can be obtained from bone marrow, peripheral blood and umbilical cord blood. These sources differ in regard to collection procedures, cellular content and transplant outcomes.

- In the related setting, the use of peripheral blood hematopoietic stem cells, when compared to bone

Table 9.144 Indications of hematopoietic stem cell (HSC) transplantation

Hematolymphoid Malignant Disorders	Hematologic Nonmalignant Disorders	Nonhematologic Disorders
Multiple myeloma	Aplastic anemia	Solid organ malignancies (breast carcinoma, ovarian carcinoma, neuroblastoma, Wilms' tumor, germ cell tumors)
Non-Hodgkin lymphoma	Thalassemia	Inborn errors of metabolism (Hurler's syndrome)
Hodgkin disease	Sickle cell disease	Severe combined immunodeficiency
Acute myelogenous leukemia	Paroxysmal nocturnal hemoglobinuria	Wiskott-Aldrich syndrome
Acute lymphoblastic leukemia	Fanconi's anemia	Congenital leukocyte dysfunction syndromes
Chronic lymphocytic leukemia	Congenital pure red cell anemia	Malignant osteopetrosis
Chronic myelogenous leukemia refractory to tyrosine kinase inhibitors	Diamond-Blackfan anemia	
Myelodysplastic syndrome	Glanzmann thrombasthenia	
Primary myelofibrosis		

Hematopoietic stem cell transplantation is performed in patients, who are chemotherapy or radiotherapy resistant leukemias and multiple myeloma.

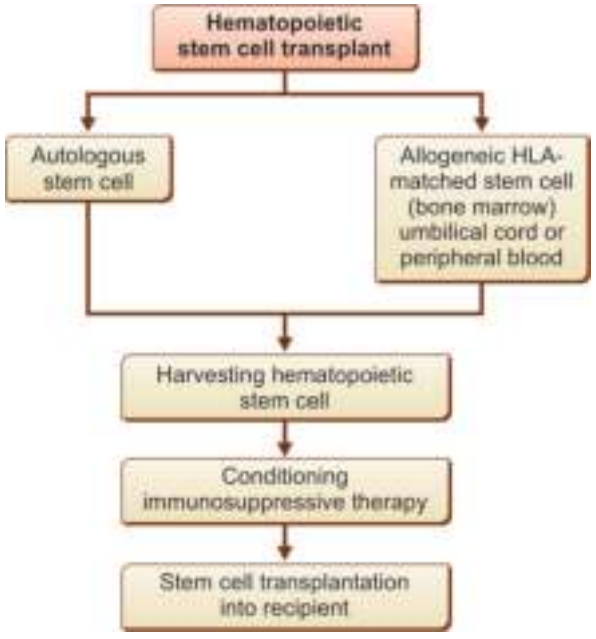


Fig. 9.131: Schematic representation of hematopoietic stem cell (HSC) transplantation.

marrow HSCs, leads to faster engraftment and may confer a survival benefit in advanced disease but also may lead to an increase chronic graft-versus-host disease. While umbilical hematopoietic stem cells are simplest to collect and permit the greater flexibility in HLA matching. Harvesting umbilical cord can save lives.

- Like bone marrow HSCs, umbilical cord blood contains HSCs capable of differentiating into mature functional blood cells (RBCs, WBCs and platelets).
  - Hematopoietic stem cells obtained from umbilical cord blood offers several advantages over those acquired from bone marrow including increased tolerance for human leukocyte antigen mismatches, decreased incidence of graft-versus-host disease, and easy availability.
  - Fetal bone marrow and liver are also rich in hematopoietic stem cells, but ethical issues limit their use. Comparison of hematopoietic stem cells obtained from umbilical cord and bone marrow is given in Table 9.145.

Bone Marrow HSCs Harvesting

Bone marrow hematopoietic stem cell is generally harvested by surgical procedure from posterior iliac crest, however bone marrow may be obtained from anterior iliac crest and sternum if required.

- The amount required to achieve hematopoiesis is 10–15 ml/kg of recipient body weight. Harvested bone marrow is filtered to remove debris. HLA typing is essential prior to bone marrow HSC transplantation.
- Bone marrow may be administered as fresh or preserved cryoprecipitate. There may be risk of infection. Patient may develop graft-versus-host disease.

Peripheral Blood HSCs Harvesting

Through a process of mobilization, hematopoietic stem cells are released from the bone marrow into the blood. Peripheral blood contains 0.1% of hematopoietic stem cells. Blood HSCs count is more than umbilical cord blood HSCs.

- Recombinant human granulocyte colony-stimulating factor (G-CSF) is used to mobilize hematopoietic

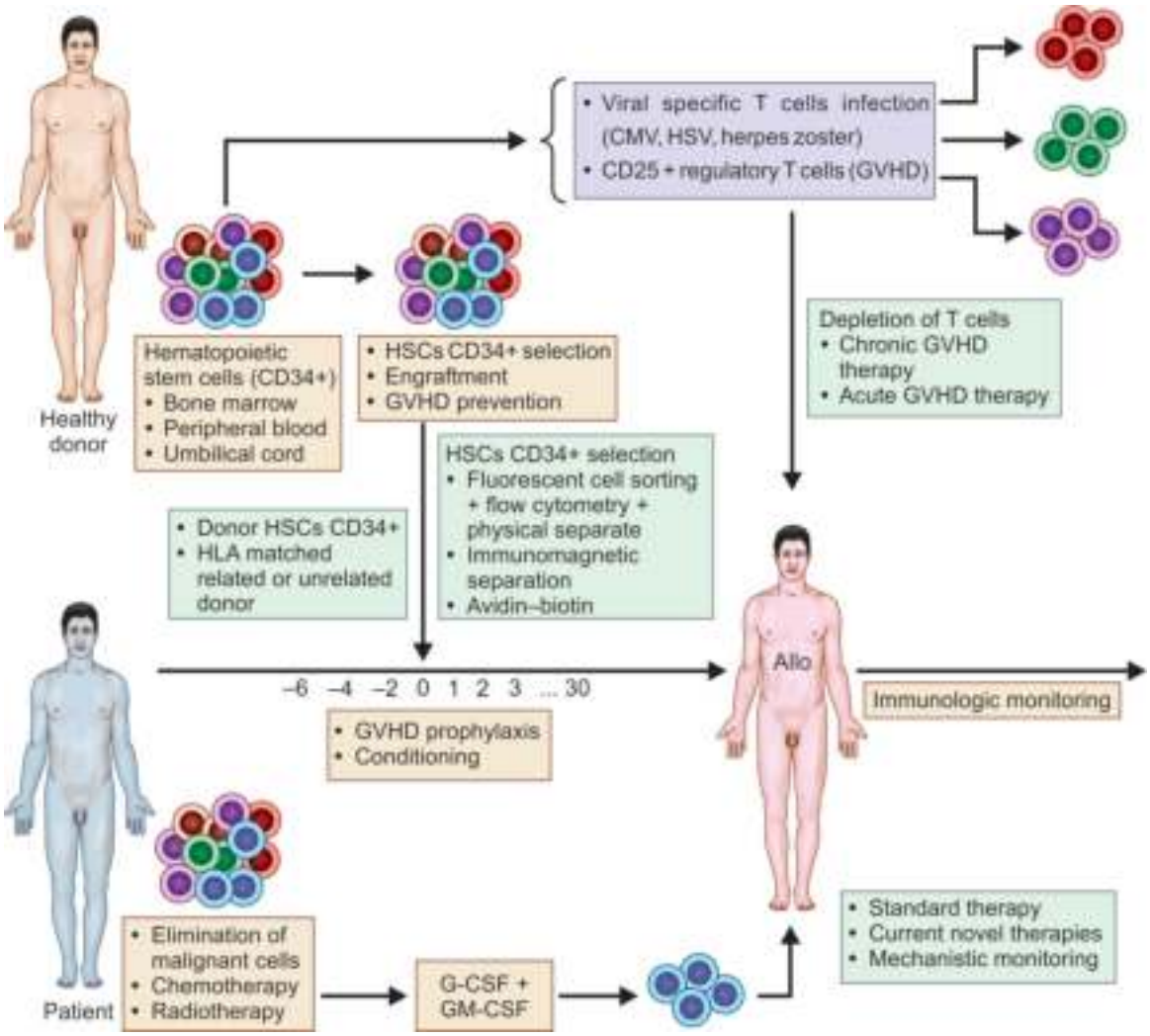


Fig. 9.132: Schematic representation of hematopoietic stem cell transplantation (HSCT) to treat malignant and nonmalignant diseases. It involves obtaining hematopoietic stem cell from the patient (autologous) or another person such as sibling or unrelated donor (allogeneic transplant) or an identical twin (synergetic transplant). Cell sources include bone marrow, umbilical cord or blood. Hematopoietic stem cells are infused by intravenous route into the recipients in order to re-establish hematopoiesis in patients whose bone marrow or immune system is damaged or defective.

Table 9.145 Comparison of hematopoietic stem cells obtained from umbilical cord blood and bone marrow

Characteristics	Hematopoietic Stem Cells (HSCs) Obtained from Umbilical Cord	Hematopoietic Stem Cells (HSCs) Obtained from Bone Marrow
Obtaining HSCs	Stored umbilical cord obtained from newborn baby	HSCs obtained from bone marrow by surgical procedure
Risk	No risk to the person as HSCs obtained from self-umbilical cord	Risk of infection to the donor; and risk to the recipient
Requirement of HLA typing	HLA typing required	HLA typing required
Time taken by engraftment of HSCs	Longer period	Shorter period
Graft-versus-host disease	Presence of immature T cells in the umbilical HSCs graft reduce risk for graft-versus-host disease	Graft-versus-host disease can occur

stem cells from the bone marrow into the blood for leukapheresis. Leukapheresis is usually performed after 4–5 days of G-CSF subcutaneous administration at as disease of 10 mg/kg of body weight.

- Through the process of apheresis, whole blood is separated into its components and hematopoietic stem cells can be collected and stored. Venous access via the antecubital vein may be used. A dual-lumen

central venous catheter may be required when venous access is inadequate. After processing, HSCs are cryopreserved in the vapor phase of liquid nitrogen for later infusion.

- Granulocytes do not survive during cryopreservation. To allow survival of granulocytes during cryopreservation, HSCs are placed in a medium containing 7.5–10% dimethyl sulfoxide. Engraftment of peripheral blood hematopoietic stem cells is more rapid than obtained from umbilical cord. There is increased in risk for developing acute/chronic graft-versus-host reactions.

Umbilical Cord Blood HSCs Harvesting

Umbilical cord blood is harvested via 16-gauze needle through the umbilical vein once the placenta has been delivered. Median volume harvested is 100 ml. Umbilical cord hematopoietic stem cells can be frozen and stored in blood bank and made readily available on demand.

- The risk of latent viral contamination due to cytomegalovirus and Epstein-Barr virus is low. Umbilical cord blood does not contain mature T cells and therefore likely to be less immunogenic and will reduce the incidence of graft-versus-host disease (GVHD).
- Umbilical cord blood HSC transplantation does not require HLA typing. Disadvantages of umbilical cord HSCs transplantation are inadequate in hematopoietic stem cells in stored umbilical cord and requirement of availability of only one unit for each transplant procedure.

Pathology Pearls: Hematopoietic Stem Cells Processing

- Prior to storage and/or administration, hematopoietic stem cells (HSCs) can be manipulated.
- Hematopoietic stem cells may be enriched with CD34+ or purged by removing T cells or malignant cells.
- Processing of HSCs include sterility testing, blood typing and a reduction of fluid volume, HSCs are cryopreserved until administration into recipients.
- Key principles of cryopreservation include: (a) reduction of the number of mature blood cells in the graft, (b) protection of HSCs from ice formation and dehydration during freezing using a cryoprotective agent such as dimethylsulfoxide or hydroxyethyl starch, (c) diluting cryoprotective agent with saline or tissue culture media, (d) reduction the risk of cell injury by adding plasma protein to the graft, and (e) cooling at controlled rate, storage at a temperature below 120°C in liquid nitrogen of mechanical freezers.
- Allogeneic hematopoietic stem cells are generally transfused into recipient within 24–72 hours of collection and do not require cryopreservation.

SELECTION OF DONOR FOR OBTAINING HSCs

Allogeneic hematopoietic stem cell transplantation (HSCT) is an established therapy for many malignancies of hematopoietic system and certain life-threatening nonmalignant disorders.

- The selection of the most suitable donor and hematopoietic stem cell (HSC) source is critical component of HSC transplantation. Donor evaluation is done for the safety of the HSCs for both donor and recipient.
- Hematopoietic stem cells (HSCs) may be collected from bone marrow, peripheral blood or umbilical cord blood. In order to minimize the risk for healthy HSC donors, thorough investigations are required before donation process.
- The donor work-up should include detailed medical history, history of previous blood transfusions, physical examination, ECG, chest radiograph, complete blood counts, coagulation screening studies, and testing for infectious disease (HIV, HBV, HCV and CMV) markers. Donors should be fully informed on the donation procedure and sign an informed consent for donation.
- Human leukocyte antigen (HLA) typing is most important contributing factor of donor selection, which is used to match patients and donors for bone marrow or umbilical cord blood transplants.
- The human leukocyte antigen system is a group of related proteins that are encoded by the major **histocompatibility complex (MHC)** gene located on the short arm of chromosome 6 in human genome. These **cell-surface proteins** are responsible for the regulation of the immune system.
- Major histocompatibility complex genes are inherited together and the most important determinants are HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1 and HLA-DPB1. Because a person inherits one HLA cluster (termed locus) from each parent. The siblings with the same parents have 25% chance to be a 'match' with the potential hemopoietic stem cell transplantation (HSCT) recipient.
- **HLA-matched identical sibling** is the **ideal donor**. Sibling in HLA-A, HLA-B, DRB1, DQB1 loci are considered identical. ABO compatibility is not essential for bone marrow transplantation.
- HLA matching promotes the growth and development of new healthy hematopoietic stem cells (called engraftment) and reduces the risk for a post-transplant complication called graft-versus-host disease (GVHD).

TECHNIQUES TO ISOLATE HSCs

Methods used to collect hematopoietic stem cells in their natural environment (bone marrow or umbilical cord blood) or the peripheral blood after stimulation by cytokines are well-defined to ensure the donor security and perform a quality hematopoietic stem cell transplantation. The hematopoietic stem cells in a graft and its characterization depend on the collection site of HSCs and on the technique used. HSCs (CD34+) selection is done by various methods.

Flow Cytometry Technique to Isolate HSCs

Hematopoietic stem cells can be identified by the use of flow cytometry technique, where combination of several different cell surface markers is used to isolate the rare HSCs from the surrounding blood cells.

- HSCs lack mature blood cell markers. Human HSCs express CD34+, EMCN+, CD59+, Thy1/CD90+, c-KIT/CD117 and lin+.
- Lack of expression of these lineage markers is used in combination with detection of several positive cell-surface markers to isolate HSCs.
- Flow cytometry is combined with fluorescent-activated cell sorting with physical separator segregates individual HSCs based on expression of molecules.
- HSCs are characterized by their small size and low staining with vital fluorescent dyes such as rhodamine.

Immunomagnetic Separation Technique of Isolate HSCs

Numerous techniques have been developed for immunomagnetic separation of human HSCs.

- A column immunoadsorption method on the high affinity between the protein avidin and the vitamin biotin has been used to obtain HSCs (CD34+) selection. Immunomagnetic separation is based on monoclonal antibodies coupled to magnetic beads.
- During incubation with cell suspension from bone marrow or peripheral blood or umbilical cord blood, the antibody/bead complex binds to cells expressing the corresponding epitope. When the cell suspension is placed into a magnetic field, magnetically labeled cells are retained, while unlabeled cells can be removed.
- To obtain the labeled cells, the sample is removed from the magnetic field. Positive selection enables the direct labeling and separation of human HSCs, whereas depletion strategies effectively remove non-HSCs to isolate untouched HSCs. Method of culture and selection of HSCs (CD34+) for transplantation is shown in Fig. 9.133.

TYPES OF HSC TRANSPLANTATION

There are two types of hematopoietic stem cell transplantation (HSCT): allogeneic and autologous. Allogeneic HSCT requires a donor to supply suitable hematopoietic stem cells. Clinicians performing allogeneic HSCT need to ensure that the donor is an appropriate match for a recipient. An autologous hematopoietic stem cell transplant uses healthy HSCs from own body to replace damaged bone marrow cells.

ALLOGENEIC HSC TRANSPLANTATION

Allogeneic HSC transplants are often indicated when the disease involves the patient's own hematopoietic stem cells in cases of acute leukemias, chronic leukemias, multiple myeloma, aplastic anemia, hemoglobinopathies, immunodeficiency disorders and metabolic inherited disorders.

- Essential criteria for successful HSC transplantation (HSCT) include: (a) infusing adequate HSCs into recipients help in engraftment in the bone marrow microenvironment, (b) recipient immune system can tolerate well the graft without rejection, and (c) patient does not develop severe graft-versus-host disease.
- Graft-versus-host disease (GVHD) occurs when immunocompetent donor T cells recognize HLA antigens on the host (recipient) cells as foreign and initiate cell injury.
  - HLA system is highly polymorphic (genetically variable). Genes present on the short arm of the chromosome 6 encode HLA antigens. Class I HLA antigens are encoded by three loci HLA-A, HLA-B, and HLA-C. On the other hand, class II HLA antigens are encoded by another three loci: HLA-DR, HLA-DP, and HLA-DQ. Serological and molecular genetic testing methods are available to analyze HLA type.
  - Graft-versus-host disease (GVHD) can occur even after compatibility testing, because serological and molecular testing do not recognize antigens that are incompatible or not encoded by major histocompatibility complex.
- **Syngeneic HSC transplantation** is performed by using HSCs derived from bone marrow or peripheral blood from identical twin to treat any disorder treated by allogeneic or autologous transplants except genetic disorders that affect both twins. Synergetic HSC transplantation has no risk of rejection or graft-versus-host disease; and not requiring special immunosuppressive drugs.

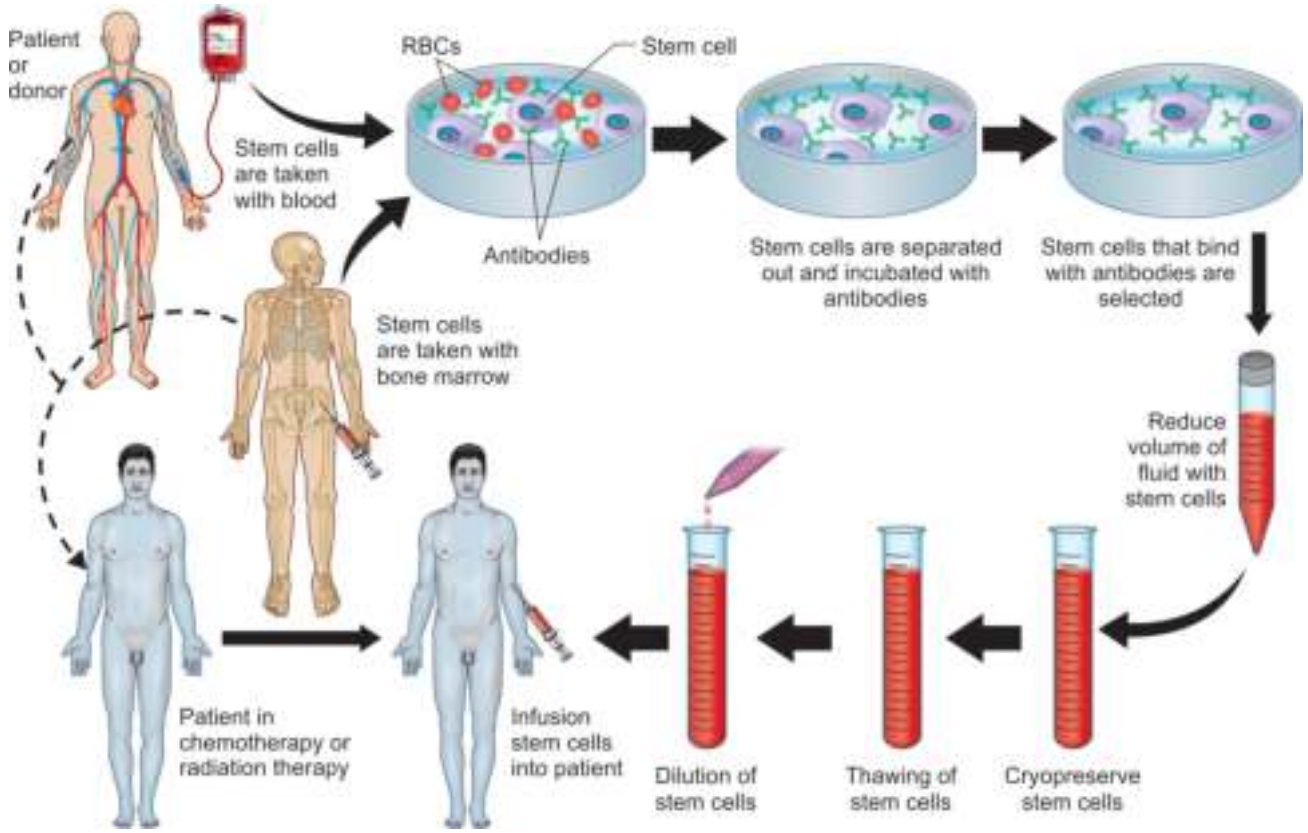


Fig. 9.133: Schematic representation of method of culture and selection of hematopoietic stem cells (HSCs) CD34+ for transplantation. HSCs and progenitor cells can be cultured under defined conditions designated to enhance self-renewal and increase the number of primitive cells or to promote lineage specific expansion and differentiation into mature blood cell types. HSCs are able to divide indefinitely, whereas progenitor cells are only able to divide defined number of times.

AUTOLOGOUS HSC TRANSPLANTATION

In autologous HSC transplantation, patient's own HSCs derived from bone marrow or peripheral blood are infused.

- Prior to collection of the hematopoietic stem cells, patient is administered intensive myeloablative chemotherapy and/or radiotherapy to eliminate the malignant cells.
- Autologous hematopoietic stem cell transplantation is usually indicated when the patient's hematopoietic stem cells are not affected by disease such as non-Hodgkin's lymphoma, Hodgkin's disease, breast carcinoma, ovarian carcinoma, neuroblastoma and Wilm's tumor.
- Autologous hematopoietic stem cell transplantation has certain advantages. Patient does not require HLA testing and secondly no graft-versus-host disease; and graft rejection does not take place.

UMBILICAL CORD HSC TRANSPLANTATION

Umbilical cord blood HSCs can be easily obtained immediately after baby birth without risk to the mother or infant, which can be frozen and stored in a blood bank and made readily available demand for HSC transplantation.

- The risk of latent viral contamination occurs due to cytomegalovirus and Epstein-Barr virus is low. Immature T cells in cord blood are less immunogenic hence reduction in graft-versus-host disease.
- Umbilical cord blood contains sufficient number of hematopoietic stem cells. Umbilical cord blood from siblings and unrelated donors can be used to reconstitute hematopoiesis in patients with malignant and nonmalignant disorders.

HEMATOPOIETIC STEM CELL TRANSPLANTATION IN THE RECIPIENTS

Hematopoietic stem cell (HSC) transplantation refers to the intravenous infusion of allogeneic or autologous HSCs, collected from bone marrow, peripheral blood, or umbilical cord blood, to replace aberrant HSCs in a patient.

CONDITIONING

In myeloablative regime, high dosage of chemotherapy and radiotherapy is given to achieve three goals: elimination of malignant cells, immunosuppression to allow engraftment and creation of physical space for adequate growth of donor HSCs. Alternative to myeloablative regimen, fludarabine based antiglobulin is administered in elderly patients to lower tumor burden in hematological malignancies.

INFUSION OF HSCs

Hematopoietic stem cells (HSCs) are obtained in a bag containing anticoagulant and infused through large-bore central catheter in recipient within a few hours of collection. Engrafting is considered as established, when peripheral blood neutrophil count reaches $>0.5 \times 10^9$/L on 3 successive days.

SUPPORTIVE MEASURES

High dosage of chemotherapy and radiotherapy induces pancytopenia in recipients may lead to infections. Therefore, antibiotics are administered to combat bacterial and fungal infections.

- Number of HSCs (CD34+) required for hematopoietic recovery is 2.5–5.0 × 10/kg.
- Blood components therapy is given in recipients. Hematopoietic growth factors such as G-CSF or GS-CSF are administered to stimulate myelopoiesis.

COMPLICATIONS OF HSC TRANSPLANTATION

Both infectious and noninfectious complications occur more frequently in allogeneic HSC transplantation.

- Complications of HSC transplantation include acute or chronic graft-versus-host disease (GVHD) and pulmonary infections.
- The goals of treatment for GVHD are to manage symptoms and to prevent further damage to the body's organs.
- Immunosuppression with corticosteroids forms the basis of therapy in both acute and chronic graft-versus-host diseases.
- Treatment decisions are determined by the severity of the patient's symptoms and concerns about complications.

Acute Graft-versus-host Disease

Acute graft-versus-host disease (GVHD) generally occurs within first 100 days after allogeneic hematopoietic stem cell transplantation, which is immunological reaction of donor's effector T cells against host tissues.

- The three main tissues that acute graft-versus-host disease (GVHD) affects the skin, gastrointestinal tract and liver.
- Recipient who develops acute GVHD presents with dermatitis (maculopapular skin rashes), cutaneous blisters, crampy pain abdomen with or without diarrhea, persistent nausea and vomiting and hepatitis with elevation of serum bilirubin and liver enzymes.

Chronic Graft-versus-host Disease

Chronic graft-versus-host disease occurs either *de novo* or progression of acute GVHD after 100 days of allogeneic HSCT.

- Chronic graft-versus-host disease is a serious, potentially life-threatening post-transplant complication. Progressive graft-versus-host disease evolving from acute graft-versus-host disease has worst prognosis.
- Pathophysiology of chronic graft-versus-host disease is different from acute GVHD and mainly characterized by impaired immune tolerance mechanisms affecting innate and adaptive immunity.
- Both alloreactive and autoreactive donor-derived T cells and B cells play a key role in pathogenesis of chronic GVHD.
- Other pathophysiologic factors for chronic GVHD include indirect presentation of alloantigens through antigen-presenting donor cells and mechanism of chronic inflammation and subsequent scar formation and fibrosis.
- There may be limited involvement of skin and liver in chronic graft-versus-host disease.
- Extensive chronic graft-versus-host disease causes oral infections, hepatic dysfunction, eye lesions and skin lesions.

Opportunistic Infections

Infections may occur 2–4 weeks prior to engraftment.

- During early post-engraftment, impaired cellular and humoral immunity leads to viral infections (cytomegalovirus, herpes simplex virus and herpes zoster) and fungal infections.
- During late post-engraftment, prolonged T cell dysfunction may cause infections with *Streptococcus pneumoniae, H. influenzae* and *N. meningitidis*.
- Patient may develop diffuse or interstitial pneumonias.

Failure of Engraftment

Failure of engraftment occurs due to inadequate HSC number, infections, GVHD and high-risk unrelated donor and HLA mismatch.

Annexure

Diagnostic workup of acute myelogenous/lymphoblastic leukemia

Comprehensive relevant data

Clinical history data

- Age, sex, and ethnicity
- History of hematologic disorder (MDS/MPN) linked to AML
- History of genetic disorders (Fanconi anemia, Bloom syndrome, ataxia-telangiectasia, Down syndrome, xeroderma pigmentosum, Li-Fraumeni linked to AML and ALL)
- History of prior malignancy and exposure to chemotherapeutic agents (alkylating agents, topoisomerases II inhibitors), immunotherapy, and radiotherapy linked to AML
- History of ionizing radiation or other cytotoxic agents
- Relevant physical examination findings: lymphadenopathy, hepatomegaly, and splenomegaly, pain and tenderness in bones and joints, skeletal muscle aches, shortness of breath, constitutional symptoms (fever, weight loss, recurrent infections, fatigue, anorexia, night sweats), anemia and skin lesions (purplish spots, easy bleeding, and bruising)
- Relevant imaging findings

Recording reporting data

- All laboratory testing performed for the initial workup and diagnosis of patient with acute leukemia and all related collection and evaluation of samples/specimens such as laboratory, morphologic, immunophenotypic and cytochemical data must be performed in the laboratory.

Collection and evaluation of samples/specimens

- Complete blood counts
- Peripheral blood smear examination
- Bone marrow aspiration and/or touch imprint
- Bone marrow trephine biopsy
- Tissue biopsy from extramedullary disease
- Cytogenetic analysis (karyotyping) to detect complex chromosomal alterations
- Flow cytometry panel to distinguish AML, B-ALL, T-ALL including early T cell precursor and AML of ambiguous lineage
- Flow cytometry to detect minimal residual disease
- Immunohistochemistry of tissue biopsy to establish diagnosis
- Immunophenotyping to establish acute leukemias
- Cerebrospinal fluid sample collection to enumerate leukemic blasts
- Cytochemical stains to diagnose and classify leukemias
- Flow cytometry of cerebrospinal fluid
- Molecular genetic analysis on cryopreserved cells, nucleic acid, formalin-fixed paraffin-embedded tissue, or unstained bone marrow aspirate or peripheral blood smear

- Acute myelogenous leukemia (AML-M0–AML-M7) based on ≥20% myeloblasts in bone marrow and peripheral blood
- Acute lymphoblastic leukemia (ALL-1, ALL-2, ALL-3) based on ≥20% lymphoblasts in bone marrow and peripheral blood

Diagnostic workup of chronic myelogenous leukemia (CML, BCR-ABL1 fusion gene) and chronic lymphocytic leukemia (CLL)

CML, BCR-ABL1 fusion gene (Philadelphia chromosome)

- CML originates in the pluripotent hematopoietic stem cells of myeloid lineage (erythroid, and granulocyte precursors and megakaryocytes) as well as in B cells but not in T cells.
- Complete blood counts and PBF show >200 × 10^9/L to 700 × 10^9/L, and unique shift to left circulating myeloblasts, myelocytes, metamyelocytes and stab forms. The hallmark is basophilia with basophil counts often >1 × 10^9/L.
- CML chronic phase: Bone marrow and peripheral blood show blasts <10% with myelocytic bulge, basophils <20%, no dysplasia in megakaryocytes and bone marrow fibrosis.
- CML accelerated phase: Bone marrow and peripheral blood show blasts 10–19% and basophils >20%, atypical megakaryocytes, bone marrow fibrosis.
- CML blast phase: Bone marrow and peripheral blood blasts >20% and basophils >20%, atypical megakaryocytes, bone marrow fibrosis.
- Philadelphia chromosome can be detected in blood and bone marrow samples by cytogenetic analysis, FISH and quantitative PCR techniques.

Chronic lymphocytic leukemia

- Complete blood counts and PBF: Absolute lymphocytosis with ≥5 × 10^9/L mature but nonfunctional clonal B cells sustained for at least 3 months. Smudge cells with bare nuclei appear squashed, damaged during the slide preparation.
- Immunophenotyping and flow cytometry: The clonality of B cells needs to be confirmed by demonstrating immunoglobulin light chain restriction using flow cytometry. A panel of CD5, CD19, CD23, and immunoglobulin light chains and an absence of FMC-7 staining are usually sufficient to establish the diagnosis of CLL.
- Bone marrow aspirate: CLL always involves bone marrow.
- Bone marrow trephine biopsy: It is only done in cases with depressed blood counts.
- Serum immunoglobulin and direct antiglobulin test (DAT) in CLL with autoimmune hemolytic anemia.
- Molecular cytogenetic alterations (FISH) for del(13q), del(11q), del(17), add(13) in peripheral blood sample.
- Molecular genetic testing reveals TP53, SF3B1, ATM, NOTCH and IGHV gene mutations.

CHAPTER 10

Platelet Disorders and Bleeding Diathesis

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LEARNING OBJECTIVES

HEMOSTASIS

- Normal endothelial hemostasis
  - Vascular component endothelial
  - Platelet component
  - Coagulation system
  - Blood clot retraction
  - Blood clot dissolution (lysis)
  - Coagulation system inhibitors
  - Tissue factor pathway inhibitor

PLATELET DISORDERS

- Thrombopoiesis
- Bleeding diathesis

QUANTITATIVE PLATELET DISORDERS

- Immune thrombocytopenia purpura
- Neonatal alloimmune thrombocytopenia

QUALITATIVE INHERITED PLATELET DISORDERS

- Platelet phospholipid plasma membrane defects
  - Glanzmann disease (thrombasthenia)
  - Bernard-Soulier syndrome
  - Scot syndrome
- Platelet storage pool defects
  - Hermansky-Pudlak syndrome
  - Chédiak-Higashi syndrome
  - Wiskott-Aldrich syndrome
  - Platelet storage pool deficiency (platelet dense granules defect)
  - Gray platelet syndrome (platelet α-granules defect)
  - Paris-Trousseau syndrome (platelet α-granule defect)
  - Jacobsen syndrome (platelet α-granule defect)
  - Quebec platelet syndrome (platelet α-granule defect)
  - X-linked macrothrombocytopenia with dyserythropoiesis
  - Thrombocytopenia with absent radii bone (tar syndrome)
- Congenital amegakaryocytic thrombocytopenia
- Platelet disorders of receptors and signal transduction
  - Cyclooxygenase enzyme deficiency disorder
  - Thromboxane A_2 receptor defect disorder
  - ADP receptors of platelets and their inhibition disorder
  - Bleeding diathesis due to glycoprotein VI deficiency disorder
  - Platelet type von Willebrand disease

QUALITATIVE ACQUIRED PLATELET DISORDERS

- Post-transfusion purpura
- Drug-induced thrombocytopenia
- Heparin-induced thrombocytopenia
- Pooling of platelets in massive splenoinegaly
- Hypoplasia of megakaryocytes
- Disseminated intravascular coagulation
- Thrombotic thrombocytopenic purpura
- Hemolytic uremic syndrome
- Dengue hemorrhagic fever
- Paraproteinemia-induced platelet dysfunction
- Uremia-induced platelet dysfunction

DIAGNOSTIC APPROACH AND TREATMENT OF PLATELET DISORDERS

- Platelet disorders treatment

SECONDARY THROMBOCYTOSIS AND ESSENTIAL THROMBOCYTHEMIA

- Secondary thrombocytosis
- Essential thrombocythemia

BLEEDING ASSOCIATED WITH VASCULAR DISORDERS

- Scurvy purpura
- Henoch-Schönlein purpura
- Senile purpura
- Simple bruising
- Hereditary hemorrhagic telangiectasia
- Marfan's syndrome
- Ehlers-Danlos syndrome

HEMOSTASIS

NORMAL ENDOTHELIAL HEMOSTASIS

Normal laminar blood flow dilutes activated clotting factors, which increases the inflow of inhibitors of clotting factors resulting in inactivation of clotting factors.

- Hemostasis is the maintenance of blood flow within vascular system by inhibiting activation of platelets, coagulation system and fibrinolytic system.
- Hemostasis involves the interaction of the blood vessels, platelets, coagulation factors leading to formation of hemostatic plug at the site of injury. Bleeding and clotting are the result of the failure of hemostatic mechanisms: (a) antithrombotic properties of resting vascular endothelium, (b) prothrombotic properties of injured or activated vascular endothelium.
- Resting vascular endothelium secretes antithrombotic molecules that inhibit hemostasis by various mechanisms. Prostacyclin (PGI_2), nitric oxide (NO) and ADPase inhibit platelet activation. Heparan-like molecules (heparan sulfate/GAG cofactor for antithrombin III (AT-III), thrombomodulin (TM), endothelial protein C receptor (EPCR), tissue factor pathway inhibitor (TFPI), tissue plasminogen

activator (tPA) inhibit activation of pathways of coagulation system cascade. Tissue-type plasminogen activator (tPA) and urinary-type plasminogen activator (uPA) activate fibrinolytic system.

- When vascular endothelium is injured, it secretes prothrombotic molecules that activate coagulation system cascade. Thromboxane A_2 (TXA_2) and platelet activating factor (PAF) activate platelets. Tissue factor (TF) initiates extrinsic pathway of coagulation system cascade. Plasminogen activator inhibitor 1 (PAI-1) inhibits fibrinolytic system. von Willebrand factor promotes platelet aggregation and acts as carrier of factor VIII in plasma and facilitates platelet activation.
- Hemostatic (antithrombotic and prothrombotic) and nonhemostatic functions of vascular endothelium are depicted in Table 10.1 and Fig. 10.1A and B.

Table 10.1 Hemostatic (antithrombotic and prothrombotic) and nonhemostatic functions of vascular endothelium

Component Characteristics	Functions
Antithrombotic functions of vascular endothelium	
Negatively charged endothelial surface	Repulsion of platelets and hemostatic proteins
Nitric oxide (NO)	Vasodilators and inhibitors of platelet recruitment and aggregation
ADPase (CD39)	▪ Antiplatelet property ▪ Degrades platelet ADP to AMP and adenosine; hence prevents platelet aggregation
Prostacyclin (PGI_2)	▪ Potent vasodilator ▪ Antiplatelet property by inhibiting platelet activation, adhesion and aggregation
Heparan-like molecules (heparan sulphate)	▪ Inhibitor of coagulation system ▪ Inhibits fibrin formation (cofactor for antithrombin III) and inactivates thrombin, factor X and other clotting factors
Thrombomodulin (TM)	▪ Inhibitor of coagulation system ▪ Binds thrombin, enhances activation of protein C, that inhibits the activity of factor V and hence inhibits conversion of fibrinogen to fibrin
Endothelial protein C receptor (EPCR)	▪ Binds protein C; facilitates activation of protein C
Tissue factor pathway inhibitor (TFPI)	▪ Binds tissue factor/factor VIIa/factor Xa complex; inhibits extrinsic pathway of coagulation
Tissue plasminogen activator (tPA)	▪ Activates fibrinolytic system
Annexin A_2 (tPA receptor A_2)	▪ Binds tissue-type plasminogen activator (tPA) and plasminogen; activates fibrinolysis
Urinary-type plasminogen activator receptor (uPAR)	Binds urinary plasminogen activator and plasminogen; activates fibrinolysis
Prothrombotic functions of vascular endothelium	
Endothelin (ET)	Vasoconstriction of blood vessel
von Willebrand factor	▪ vWF acts as a bridging molecules at the sites of vascular injury ▪ vWF promotes platelet aggregation under high-shear conditions ▪ Carrier of factor VIII in plasma; facilitates platelet adhesion
Tissue factor (TF) induced by bacterial toxins, IL-1, TNF-α	▪ Procoagulant property ▪ Initiates fibrin formation, activates factor VII
Plasminogen activator inhibitor 1 (PAI-1)	▪ Procoagulant property ▪ Inhibits activation of fibrinolytic system
Nonhemostatic functions of vascular endothelium	
Selective blood/tissue barrier	Keep blood cells and macromolecules in blood vessels; allows nutrient and gas exchange
Processing of blood-borne antigens	Contributes to cellular immunity
Basement membrane collagen synthesis	Provides back up protection for endothelial cells
Collagen of the matrix synthesis	Promotes platelet adhesion
Elastin synthesis	Vasodilates and vasoconstricts
Fibronectin synthesis	Binds one cell to another cell
Laminin	Contributes to platelet adhesion after vascular injury
Vitronectin	Binds one cell to another, possibly promotes platelet adhesion
Thrombospondin (encoded by THBS1 gene)	Adhesive glycoprotein binds one cell to another, possibly promotes platelet adhesion

Normal blood flow dilutes clotting factors, inhibits activation of vascular endothelial cells and enhances inflow of inhibitors of coagulation.

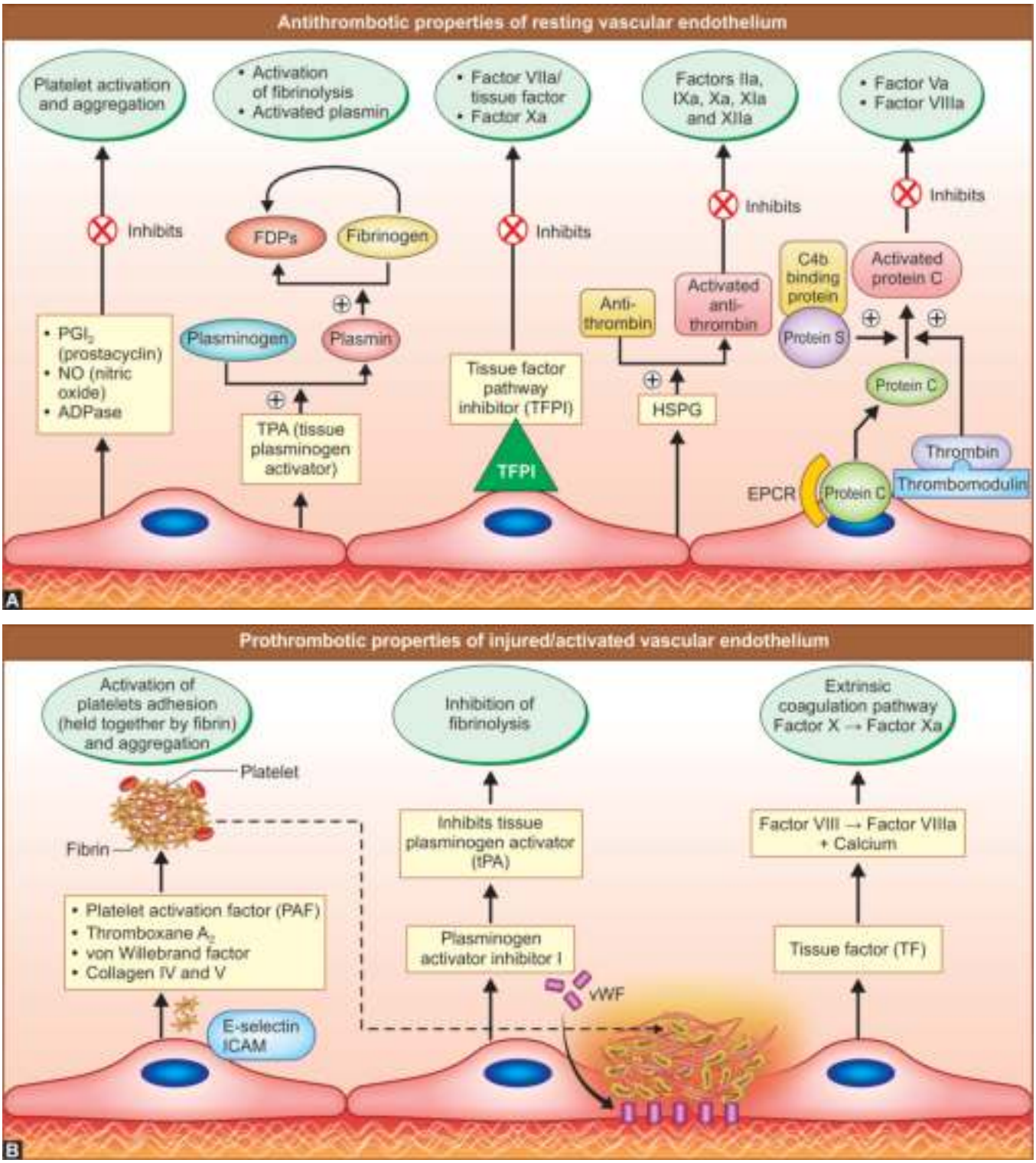


Fig. 10.1: Antithrombotic characteristics of resting vascular endothelium versus the prothrombotic effects of damaged or activated vascular endothelium. (A) Antithrombotic properties of resulting endothelium provide an environment that inhibit activation of hemostasis by secretion of substances that (1) inhibit platelet activation, e.g. PGI_2 (prostacyclin), NO (nitric oxide), ADPase; (2) inhibit coagulation (heparan sulfate/GAG as a cofactor for AT III (antithrombin III), TM (thrombomodulin) for activation of protein C, which inactivates activated FVa and FVIIIa, and TFPI 1, i.e. tissue factor pathway inhibitor 1); and (3) activate fibrinolysis (tPA, i.e. tissue-type plasminogen activator, uPA, i.e. urinary type plasminogen activator). (B) Prothrombotic properties of injured activated vascular endothelium secrete substances that (1) activate platelets (TXA_2, e.g. thromboxane, PAF, i.e. platelet activating factor) and bind them to the vessel wall (vWF, i.e. von Willebrand factor); (2) activate coagulation (TF, i.e. tissue factor which initiates formation of fibrin); and (3) inhibit fibrinolysis (PAI-1, i.e. plasminogen activator inhibitor 1). EPCR—endothelial protein C receptor. HSPG; heparin sulfate proteoglycans.

Hematology Pearls: Coagulation Factors

Coagulation factors	Synonyms	Site of synthesis
I	Fibrinogen	Liver
II	Prothrombin	Liver (vitamin K-dependent)
III	Tissue extract (thromboplastin)	Tissue
IV	Calcium	–
V	Labile factor	Liver
VI	Not assigned	NA
VII	Stable factor	Liver (vitamin K-dependent)
VIII	Antihemophilic globulin A	Liver, spleen, kidneys
IX	Christmas factor (anti-hemophilic factor B)	Liver (vitamin K-dependent)
X	Stuart-Prower factor	Liver (vitamin K-dependent)
XI	Plasma thromboplastin antecedent (antihemophilic factor C)	Unknown
XII	Hageman factor	Unknown
XIII	Fibrin stabilizing factor	Unknown
–	von Willebrand factor (factor vWF antigen assay)	Endothelial cells

Prekallikrein: Fletcher factor; Kininogen (high MW): Fitzerald factor; NA: Not applicable

Hematology Pearls: Stages of Primary Hemostasis

- Hemostasis is a stepwise process for stoppage of bleeding by sequentially transient vasoconstricting the blood vessel, forming a platelet plug and development of a blood clot.
- Blood clotting process requires the presence of platelets produced by bone marrow, von Willebrand factor generated by the vascular endothelium, and clotting factors synthesized in the liver by using vitamin K.
- Blood clot retraction and its dissolution also play significant role in hemostasis, which involves the interaction of substrates, enzymes, protein cofactors, and calcium ions in the blood or are released from platelets and cells in the blood vessel wall.

Injury to Vascular Endothelium and Vasoconstriction

- Injury ruptures the blood vessels. Injured vascular endothelium release chemical signals that cause transient vasoconstriction. It is mediated by reflex neural stimulation and endothelin release from vascular endothelial cells.
- Injury to the blood vessel causes contraction of vascular smooth muscle of the blood vessel wall and vasoconstriction, which reduces the blood flow.
- Both local nervous reflexes and local humoral factors such as thromboxane A_2 (TXA_2), which is released from platelets, contribute to the vasoconstriction.

Primary Hemostatic Plug Formation

On platelet activation, platelets synthesize their granule contents, which facilitates further activation of platelets. Platelet activation involves three overlapping machanisms.

- **Platelet adhesion:** Vascular endothelial injury causes platelets to adhere to subendothelial collagen. Shape change, secretion of granule contents, and aggregation follow. Within seconds of tissue injury, vWF (von Willebrand factor), released from the Weibel-Palade of vascular endothelium and α-granules of platelets, binds to the platelet using GpIa receptors resulting in adhesion of the platelets to the exposed collagen fibers of damaged blood vessels.
- **Platelet aggregation:** Platelets aggregate at the site of injury via GpIIb/3a using fibrinogen from the plasma as a linking molecule; results in formation of platelet primary hemostatic plug. Platelet primary hemostatic plug is weak; coagulation system cascade forms secondary hemostatic plug that stabilizes it.
- **Platelet secretion (degranulation):** The platelets become activated and release ADP and thromboxane A_2 (TXA_2), which attract additional platelets resulting in platelet aggregation and plug formation. Platelet shape change after stimulation by an agonist by ADP and thromboxane A_2 .
  - Agonist is a substance, which initiates a physiologic response when combined with a receptor. Pseudopodia develop on the surface of platelet, which contain a network of actin and myosin.
  - The microtubule circumferentially contracts resulting in activation of phospholipids and glycoprotein IIb/IIIa receptors.
  - Internal biochemical changes occur in the platelets resulting in granule secretion. Platelet-derived agonists and their receptors are given in Table 10.2.

Coagulation System

- Coagulation factors in intrinsic pathway, extrinsic pathway and common pathway are given in Table 10.3.
- Activators and inhibitors of clotting and fibrinolysis keep the coagulation system in balance.
  - Blood clotting occurs when vascular endothelium is injured and the activators of coagulation factors are exposed or released.
  - Blood clotting is controlled by fibrinolysis in response to activation of clotting factors. Inhibitors of both clotting and fibrinolysis serve to bring the coagulation system back to balance.
  - An imbalance in the activation or inhibition of either clotting and/or fibrinolysis causes thrombosis or bleeding manifestations. Activators and inhibitors of coagulation system and fibrinolytic system are given in Table 10.4.
- The terminal steps in both intrinsic and extrinsic pathways of coagulation system are same. Both coagulation system pathways lead to the activation of factor X.

- Intrinsic pathway of coagulation system is initiated by activation of circulating factor XII.
- Extrinsic pathway of coagulation system is activated by a cellular lipoprotein known as tissue factor that becomes exposed when tissues are injured.

- Calcium, factor X, factor V, platelet phospholipids combine to form prothrombin activator, which then converts prothrombin to thrombin. This interaction causes conversion of fibrinogen into fibrin strands stabilized by fibrin stabilizing factor that creates the insoluble blood clot, with platelets and red blood cells resulting in stoppage of bleeding.

Blood Clot Retraction

Within a few minutes after blood clot is formed, the actin and myosin in the platelets that are trapped in the blood clot, contract and pull fibrin strands (plasma without fibrinogen) from the blood clot resulting in shrinkage of blood clot.

Blood Clot Dissolution (Lysis)

- Blood clot dissolution begins shortly after blood clot is formed. Thrombin activates protein C. The protein C activates plasminogen activator, that gets trapped in the blood clot.
- The slow release of a very powerful plasminogen activator (tPA) from the injured tissues and vascular endothelium now converts plasminogen to plasmin, which digests the fibrin strands resulting in dissolution of the blood clot.
- Laboratory tests in defects of primary hemostasis in thrombocytopenia, platelet dysfunction and vascular purpura are given in Table 10.5.

Table 10.2 Platelet-derived agonists and their receptors

Platelet-derived Agonist	Agonist Receptors
ADP	▪ $P2Y_1$ ▪ $P2Y_2$
Serotonin	$5HT_2$
Platelet activating factor (PAF)	PAFR
Thromboxane A_2	TP

Agonist is a substance, which initiates a physiologic response when combined with a receptor.

Table 10.3 Coagulation factors in intrinsic, extrinsic and common pathways

Intrinsic Pathway of Coagulation System

- Prekallikrein
- High molecular weight kininogen
- Factor XII (Hageman factor)
- Factor XI (plasma thromboplastin antecedent)
- Factor IX (Christmas factor)
- Factor VIII (antihemophilic globulin A)

Extrinsic Pathway of Coagulation System

- Factor VII (stable factor)
- Tissue factor III (TF III)

Common Pathway of Coagulation System

- Factor X (Stuart-Prower factor)
- Factor V (labile factor)
- Factor II (prothrombin)
- Factor I (fibrinogen)

Table 10.4 Activators and inhibitors of coagulation system and fibrinolytic system

Activators	Inhibitors
Coagulation system	
▪ Coagulation factors ▪ Phospholipids ▪ Calcium ions	▪ Antithrombin ▪ Protein C ▪ Protein S, tissue factor pathway inhibitor (TFPI) ▪ α-Antitrypsin ▪ C_1-inhibitos, heparin cofactor II
Fibrinolytic system, also termed the plasminogen-plasmin system	
▪ Factor XIIa ▪ Factor XIa ▪ Kallikrein ▪ Tissue plasminogen activator (tPA) ▪ Urinary plasminogen activator (uPA)	▪ Plasminogen activator inhibitor I and II (PAI-I and PAI-II) ▪ α_2-Macroglobulin ▪ Thrombin activatable fibrinolysis inhibitor (TAFI)

Table 10.5 Laboratory tests in defects of primary hemostasis in thrombocytopenia, platelet dysfunction and vascular purpura

Screening Tests	Thrombocytopenia	Platelet Dysfunction	Vascular Purpura
Platelet count	Decreased	Usually normal	Normal
Prothrombin time (PT)	Normal	Normal	Normal
Activated partial thromboplastin time (APTT)	Normal	Normal	Normal
Template bleeding time	Abnormal	Normal or abnormal	Normal or abnormal

VASCULAR ENDOTHELIAL COMPONENT

Vascular system is composed of blood vessels, that carry blood and lymph throughout the body. Vascular endothelium is the first-line of defense for normal hemostasis, which is supported by subendothelial tissue, which maintains the integrity of structure, and lined by endothelium. Laminar blood flow dilutes clotting factors and inhibits endothelial cell activation, which increases the inflow of inhibitors of clotting factors.

- Normal (resting) vascular endothelium provides an environment that inhibits activation of hemostasis by synthesis of substances that (a) inhibit platelet activation, e.g. prostaglandin I_2 (PGI_2), nitric oxide (NO), ADPase; (b) inhibit coagulation, e.g. heparan sulfate/GAG as a cofactor for antithrombin (AT), thrombomodulin (TM) for activation of protein C, which inactivates activated factor Va, factor VIIIa; and TFPI; and (c) activates fibrinolysis, e.g. tissue-type plasminogen activator 1 (tPA-1) and urinary-type plasminogen activator (uPA). It is worth mentioning that protein C combines with endothelial protein C receptor (EPCR) and gets activated and then combine with protein C (PC) resulting in inhibition of factor V and factor VIIIa.
- Injured (activated) vascular endothelium synthesizes substances that (a) activate platelets, e.g. thromboxane A_2 (TXA_2) and platelet activating factor (PAF) and bind them to the vessel (vWF); (b) activate coagulation, e.g. tissue factor (TF), which initiates formation of fibrin; and (c) inhibit fibrinolysis.

PLATELET COMPONENT

Platelets play a central role in normal hemostasis by forming primary hemostatic plug. Platelets are membrane bound porous disc-like structures ranging from <5 to 12 femtoliter, which maintain the physical integrity of the vascular endothelium. Platelets synthesize platelet-derived growth factor (PDGF), which repairs injured vascular endothelium.

- Platelet contains α-granules and dense granules. In platelets, α-granules are the storage sites for certain proteins that are important for the hemostatic process, which contain platelet factor IV, thrombospondin, factor V, vWF, plasminogen and platelet-derived growth factor. Platelets dense granules contain ADP, ATP, calcium and serotonin, which play an important role in recruitment of additional platelets.
- β-Thromboglobulin is chemoattractant. PF4 (platelet factor 4) neutralizes chemoattractant property. Factors V and XI participate in fibrin formation. Fibrinogen is converted to fibrin.
- Fibrinogen also participates in platelet aggregation. Protein S regulates fibrin formation via protein C pathway. Tissue factor pathway inhibitor (TFPI) regulates fibrin formation by inhibiting factor VII/tissue factor complex.
- Fibronectin and von Willebrand factor (vWF) participate in adhesion of platelets to the collagen. Thrombospondin stabilizes platelets.
- Stages of thrombopoiesis are shown in Fig. 10.2. Normal megakaryocytes in bone marrow trephine biopsy are shown in Fig. 10.3. Structure of platelet is shown in Fig. 10.4. Structure and functions of platelet are given in Table 10.6. Formation of hemostatic plug by platelets adhesion, activation, aggregation and degranulation is shown in Fig. 10.5A to C.

Thrombopoiesis

Thrombopoietin, vitamin B_{12} and folic acid participate in platelets production in bone marrow. Platelets are formed by precursor cells called megakaryocytes that reside within bone marrow by a process called endomitosis where each megakaryocyte gives rise to approximately 3000 platelets. Two-thirds of platelets remain in circulation, while one-third of platelets is stored in spleen. Platelets express glycoprotein receptors of the integrin family on their surface. The survival of blood platelets is assured to be best criteria for their integrity, viability, and physiological activity. Recent studies have revealed life span of platelets is 3–7 days.

Primary Hemostatic Plug Formation

Formation of primary hemostatic plug involves platelet activation and subsequent generation of fibrin via coagulation system cascade. On platelet activation, platelets synthesize their granule contents, which facilitate further activation of platelets. Platelet activation involves three overlapping machanisms.

- **Platelet adhesion:** Exposure of subendothelial tissue activates platelets, which change their shape by producing pseudopodia. Platelets adhere to subendothelial tissue via receptor sites, which interact with von Willebrand factor (vWF) synthesized by vascular endothelial cells and megakaryocytes. The vWF binds with coagulation factor VIII in the plasma (Fig. 10.6).
- **Platelet aggregation:** Platelets interact with each other via receptor sites, which utilize fibrinogen as an intercellular bridge. Platelets contract and release α-granules and dense granules, which contain numerous substances participating in platelet aggregation. Cell membrane of platelets synthesizes

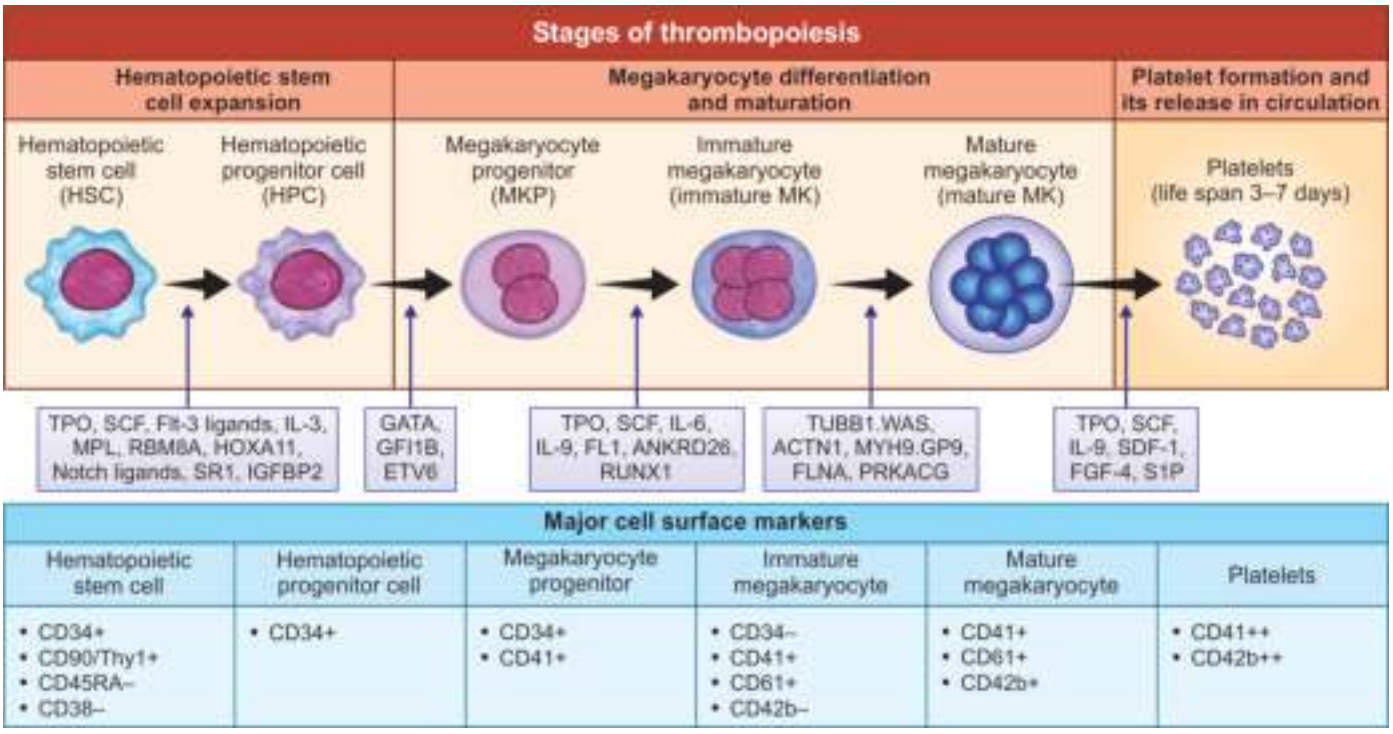


Fig. 10.2: Stages of thrombopoiesis. Thrombocytes are derived from hematopoietic stem cell (HSC) in the bone marrow. As megakaryocytes develop through stage of megakaryocyte differentiation and maturation under the influence of hematopoietic growth factors, mature megakaryocytes undergo a process of fragmentation that results in the release of approximately 3000 platelets per megakaryocyte.

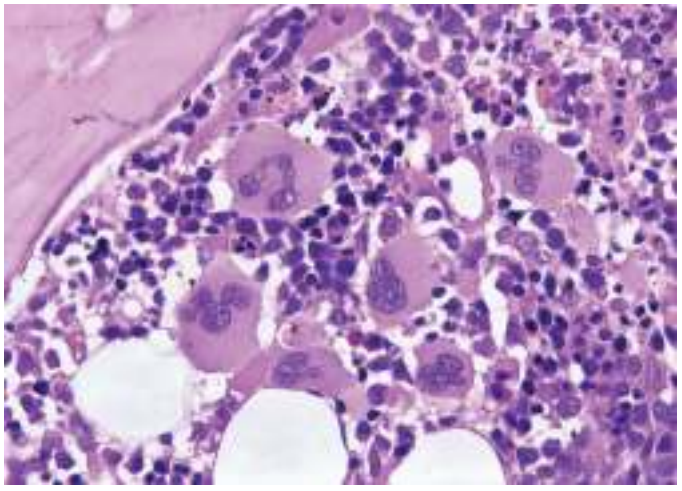


Fig. 10.3: Normal megakaryocytes in bone marrow trephine biopsy. Each megakaryocyte has a lobated nucleus responsible for the production of blood thrombocytes (platelets), which are essential for formation of primary hemostatic plug during bleeding.

metabolic arachidonic product such as thromboxane A_2, which also promotes platelet aggregation in addition to vasoconstriction (Fig. 10.7). Deficiency of GpIIb/IIIa results in Glanzmann's disease.

- **Platelet secretion (degranulation):** The platelets become activated and release ADP and thromboxane A_2 (TXA_2). The ADP and TXA_2 attract additional platelets resulting in platelet aggregation and primary hemostatic plug formation. Platelet changes its shape after stimulation by an agonist by ADP and thromboxane A_2 (TXA_2). Agonist is a substance, which initiates a physiologic response when combined with a receptor. Pseudopodia develop on the surface of platelet, which contain a network of actin and myosin. The microtubule circumferentially contracts resulting in activation of phospholipids and glycoprotein IIb/IIIa receptors. Internal biochemical changes occur in the platelets resulting in granule secretion. Formation of primary hemostatic plug occurs by platelet activation, adhesion, aggregation and degranulation.

COAGULATION SYSTEM

Exposure to tissue factor activates extrinsic pathway of coagulation system. Thrombin generation augments the platelet activation. The activated platelets provide phospholipid, which is an essential cofactor at several points in the coagulation.

- Formation of secondary hemostatic plug occurs by intrinsic and extrinsic pathways of coagulation system as a result of vascular endothelial injury, which lead to activation of factor X, conversion of prothrombin to thrombin. Thrombin converts fibrinogen to fibrin. Fibrin stabilizing factor stabilizes

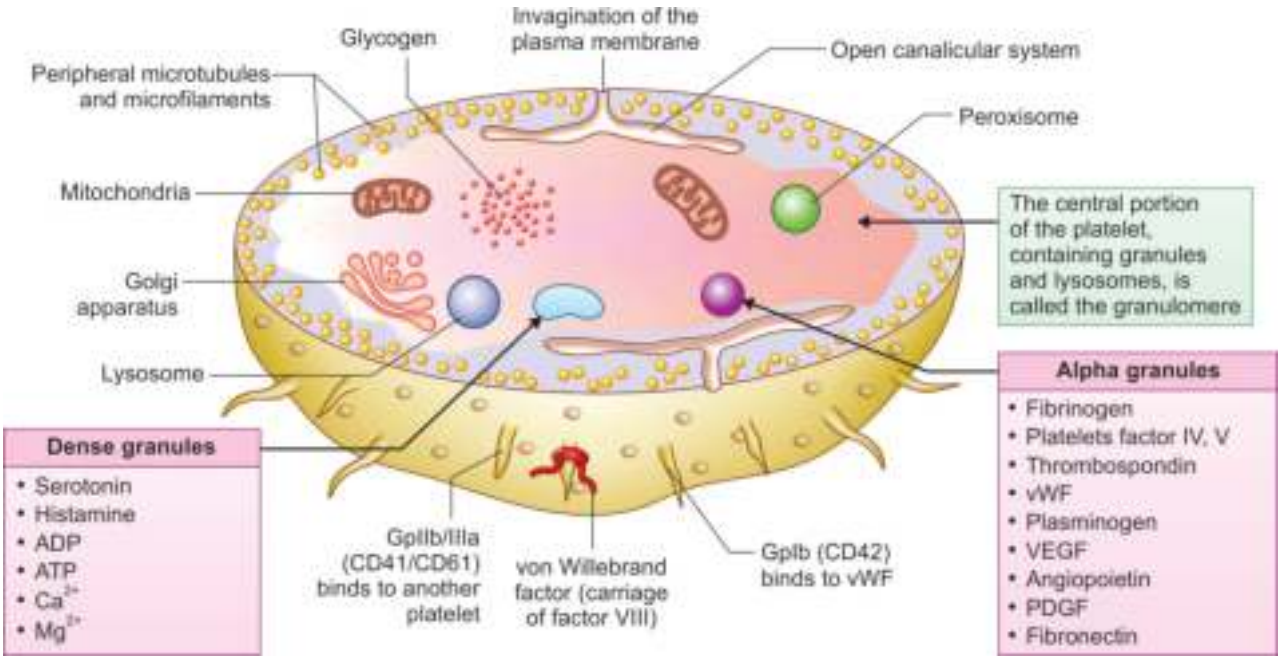


Fig. 10.4: Structure of platelets: Platelet contains α-granules and dense granules. In platelets, α-granules are the storage sites for certain proteins that are important for the hemostatic process. The α-granules contain platelet factor IV, thrombospondin, factor V, vWF, plasminogen and platelet-derived growth factor. The dense granules contain ADP, ATP, calcium and serotonin. Proteins of dense granules (ADP and serotonin) play an important role in recruitment of additional platelets. β-Thromboglobulin is chemoattractant. PF4 (platelet factor 4) neutralizes chemoattractant property. Factors V and XI participate in fibrin formation. Fibrinogen is converted to fibrin. Fibrinogen also participates in platelet aggregation. Protein S regulates fibrin formation via protein C pathway. TFPI (tissue factor pathway inhibitor) regulates fibrin formation by inhibiting factor VII/tissue factor complex. Fibronectin and vWF (von Willebrand factor) participate in adhesion of platelets to the collagen. Thrombospondin stabilizes platelets.

Table 10.6 Structure and functions of platelet

Features	Functions
Platelet membrane	
Glycocalyx	▪ Outermost coat comprising glycolipids, glycoproteins and mucopolysaccharide ▪ Negative charge due to sialic acid residue of proteins and lipids
Plasma membrane	▪ Composed of glycolipids, cholesterol and glycoproteins ▪ Lipoprotein layer containing platelet factor 3 involved in blood coagulation
Membrane glycoproteins (acting as receptors for cell-cell and ligand-cell interaction)	
Glycoprotein IIb/IIIa	▪ Cross-linking of GpIIb/3a to vWF and fibrinogen leading to platelets aggregation ▪ Deficiency of GpIIb/3a results in Glanzmann's disease
Glyoprotein Ib–IX	In Bernard-Soulier syndrome, deficiency of GpIb–IX results in bleeding diathesis
Cytoskeleton	
Short actin filament	Present under plasma membrane involved in maintaining discoid shape
Actin microfilament network	Present in cytoplasm
Microtubules	Present in peripheral part of cytoplasm involved in maintaining discoid shape
Dense granules	
ADP	Recruitment of platelets and activation of new platelets resulting in platelets aggregation
ATP	Agonist for platelets and other cells
Calcium	Extracellular source for hemostatic reactions
Serotonin	Vasoconstrictor

Contd...

Table 10.6 Structure and functions of platelet (*Contd...*)

Features	Functions
α-Granules	
Fibrinogen	▪ Aggregation of platelets ▪ Fibrinogen itself gets converted to fibrin
Platelet factor IV	Aggregation of platelets
Thrombospondin	Aggregation of platelets
Factor V	Adhesion of platelets
Hemostasis	vWF binds to factor VIII, platelets and connective tissue
Plasminogen	Plasminogen gets converted to plasmin. Plasmin participates in fibrinolysis
Platelet-derived growth factor (PDGF)	Promotes repair of injured blood vessel

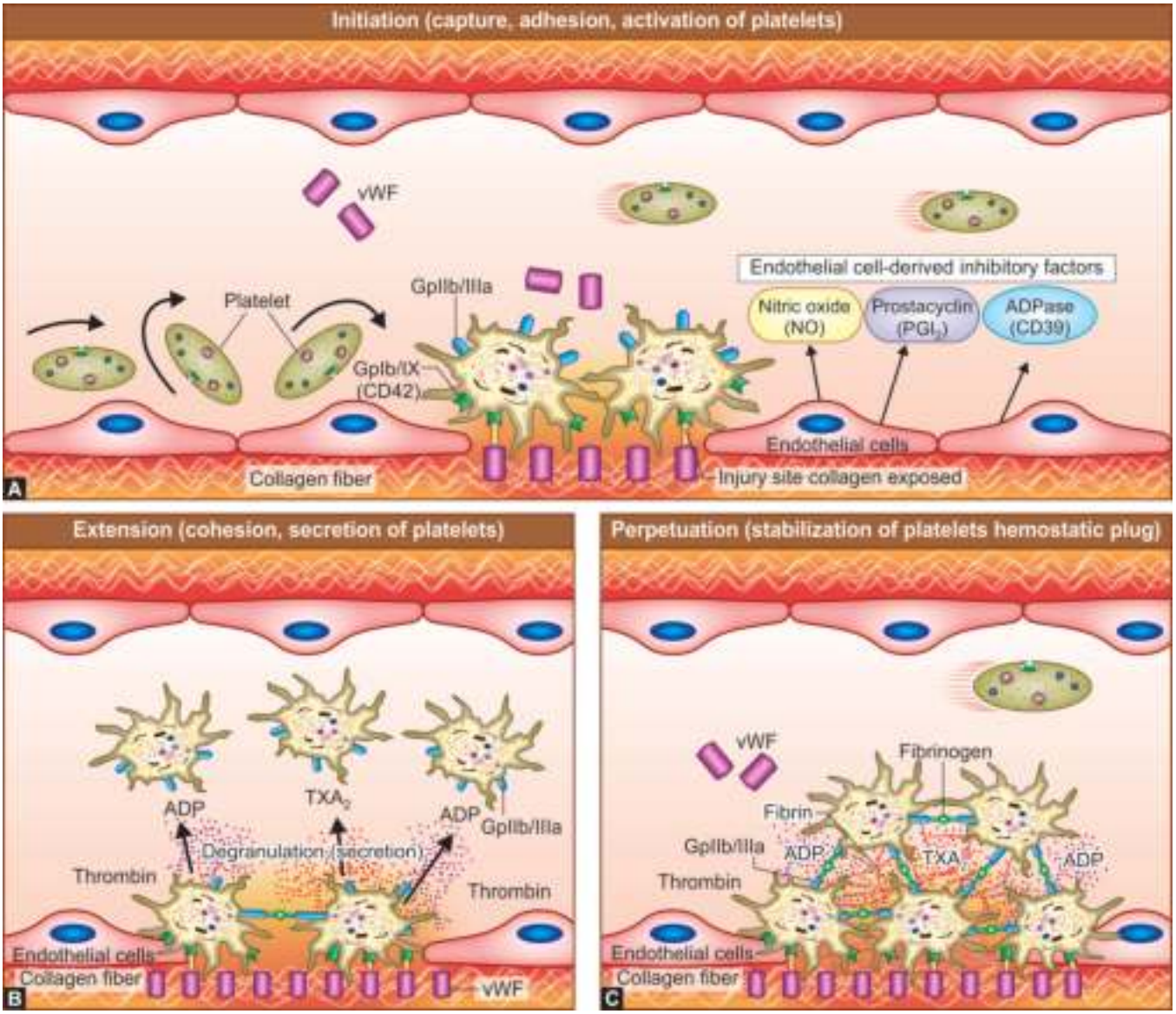


Fig. 10.5: Formation of primary hemostatic plug by platelets activation, adhesion, aggregation and degranulation. Platelet activation involves three overlapping mechanisms. (A) Adhesion of platelets to the exposed subendothelium is mediated by the binding of von Willebrand factor (vWF) to glycoprotein (Gp) Ib/IX (CD42) resulting in initiation of signal for activation of platelets. (B) Exposure of GpIIb/IIIa (CD41/61) to the fibrinogen receptor on the platelet surface permits for platelet aggregation. (C) At the same time, platelets synthesize their granule contents, which facilitate further platelets activation.

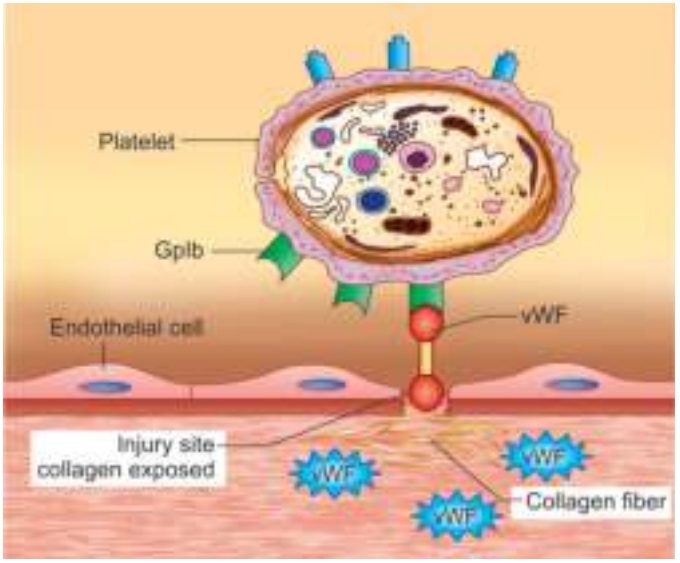


Fig. 10.6: Platelets adhesion participating in primary hemostatic plug.

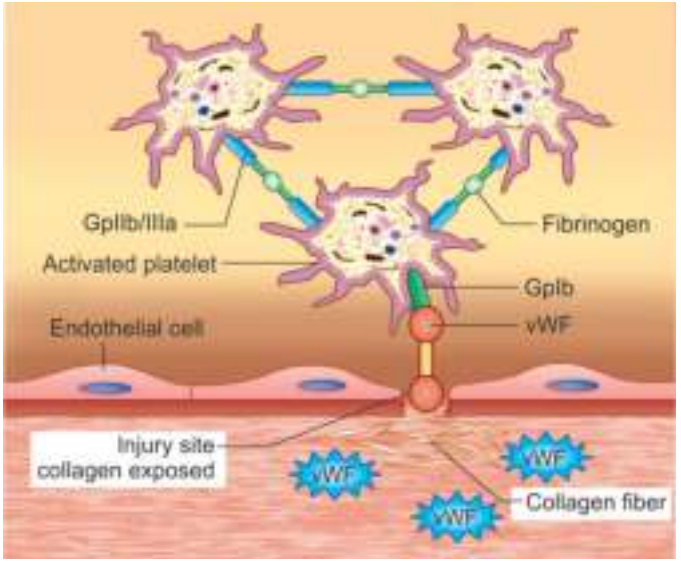


Fig. 10.7: Platelets aggregation participating in primary hemostatic plug.

fibrin resulting in holding of blood clot by insoluble fibrin strands.

- Coagulation system is composed of extrinsic and intrinsic pathways, which reflect how blood clotting occurs in the test tube during tests. Blood clotting in the body is initiated differently.
- Fibrinolysis is the controlled dissolution of the formed blood clot, that occurs when the injured blood vessel begins to heal, and initiated when formation of blood clot begins. In this way, fibrinolysis serves as regulatory mechanism to limit excess formation of blood clot. Plasminogen activator cleaves plasminogen to form plasmin. Plasmin binds to specific receptors on fibrin strands network and begins degrading the fibrin strands resulting in dissolution of blood clot. Plasmin also degrades fibrinogen, factor V, factor VIII, prothrombin, and factor XII.
- Dissolution of blood clot restores normal blood flow. Plasmin action is limited to the site of injury and formation of thrombus or clot. Systemic action of plasmin does not occur. Fibrinolytic system is shown in Figs 10.8 and 10.9.

BLOOD CLOT RETRACTION

Blood clot retraction occurs when platelets are trapped within the enlarging blood clot, which occurs within 20–60 minutes after a blood clot has formed, contributing to hemostasis. Actin and myosin of platelets pull the clot towards platelets. This phenomenon causes squeezing of serum from the blood clot resulting in shrinkage of blood clot. Blood clot retraction requires large number of platelets, and failure of clot retraction is indicative of a low platelet count. Thrombomodulin synthesized by vascular endothelium has antithrombotic activity.

BLOOD CLOT DISSOLUTION (LYSIS)

Blood clot dissolution begins shortly after a blood clot is formed.

- Thrombin activates protein C, which activates plasminogen activator, that gets trapped in the blood clot.
- The slow release of a very powerful plasminogen activator is called tissue plasminogen activator (tPA) from the injured tissues/vascular endothelium now converts plasminogen to plasmin. The plasmin digests the fibrin strands resulting in dissolution of the blood clot.

Clinical Pearls: Recombinant Tissue Plasminogen Activator (tPA)

- Tissue plasminogen activator functions by catalyzing the conversion of plasminogen to plasmin, the primary enzyme involved in dissolving blood clots.
- Recombinant biotechnology has allowed to manufacture tissue plasminogen activator (tPA) such as alteplase, reteplase and teneplase in the laboratories.
- Recombinant tissue plasminogen activators are administered in ischemic cerebral stroke within 3 hours of onset, myocardial infarction if there is delay of >1–2 hours before percutaneous transluminal coronary angioplasty, severe pulmonary thromboembolism causing severe instability due to pressure in heart, and deep vein thrombosis.

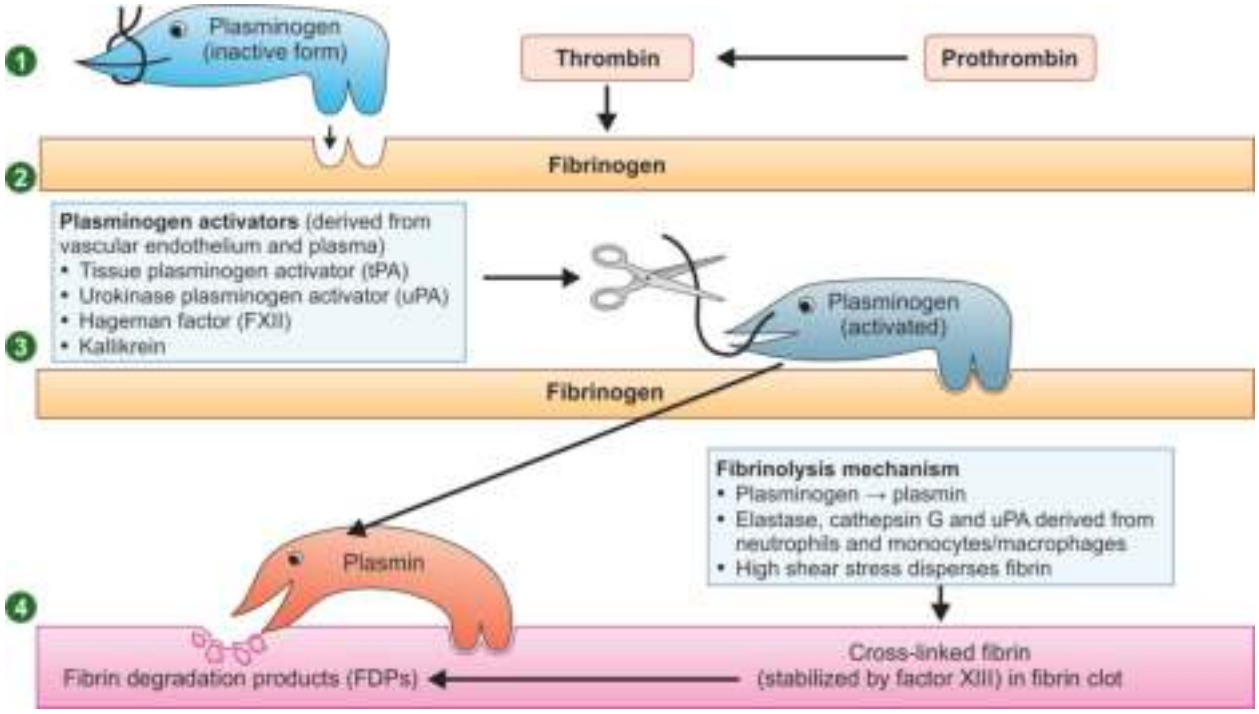


Fig. 10.8: Schematic representation of fibrinolysis. Thrombin converts plasminogen to plasmin, which breaks down the cross-linked fibrin into soluble fibrinogen degradation products (FDPs). Plasminogen activators such as tissue plasminogen activator or urokinase, activate plasminogen to form plasmin enzymatically cleaves insoluble fibrin polymers into soluble degradation products (FDPs), thereby affecting the removal of unnecessary fibrin clot. Fibrinolysis is inhibited by plasminogen activator inhibitors or plasmin inhibitors or thrombin-activable fibrinolysis inhibitors.

COAGULATION SYSTEM INHIBITORS

There are two major inhibitory pathways of coagulation system, that determine the role at which coagulation system cascade is amplified. Protein C and protein S inhibit pathways of coagulation system. An additional inhibitory mechanism is antithrombin III, that inhibits coagulation system cascade. Vitamin K-dependent clotting factors (II, VII, IX and X), protein C and protein S are synthesized by liver. Antithrombin III, protein C and protein S prevent thrombus formation. Deficiency of antithrombin III, protein C and protein S are associated with recurrent venous thrombosis and thromboembolism.

- **Antithrombin III:** Antithrombin III antagonizes thrombin activity and also inhibits factors XIIa, XIa, Xa, and IXa. Heparin-like molecules synthesized by vascular endothelium activates antithrombin III. Hence, in clinical practice, heparin is administered to minimize thrombosis.
- **Protein C:** Protein C is vitamin K-dependent natural anticoagulant presents in circulation, which inhibits factors V and VIII. Protein C is activated by modified thrombin protein. Thrombomodulin synthesized by vascular endothelium modifies thrombin molecule. The modified thrombin protein can no longer convert fibrinogen to fibrin. Protein C also enhances fibrinolysis. Protein C deficiency is the most common cause of inherited thrombophilia.
- **Protein S:** Protein S is vitamin K-dependent natural anticoagulant present in circulation. Protein C serves as a cofactor and enhances the activity of protein C resulting in inhibition of factors V and VIII. Protein S does not require activation.

TISSUE FACTOR PATHWAY INHIBITOR

Tissue factor pathway inhibitor (TFPI) is the main regulator of the tissue factor of coagulation system. TFPI encoded TFPI gene mapped on chromosome 2q32 is synthesized by vascular endothelium. Tissue factor plays a primary role in both normal hemostasis and thrombosis. With vascular injury, tissue factor binds plasma factor VIIa leading to hemostatic plug formation and vascular sealing. TFPI binds activated factor VII (VIIa) complex-activated factor X (Xa) and inactivates them rapidly in circulation, so limiting extrinsic pathway of coagulation system cascade. TFPI consists of three Kunitz domains. Kunitz domains (K1 and K2) bind with VIIa–Xa complex. The third Kunitz domain may interact with the plasma membrane.

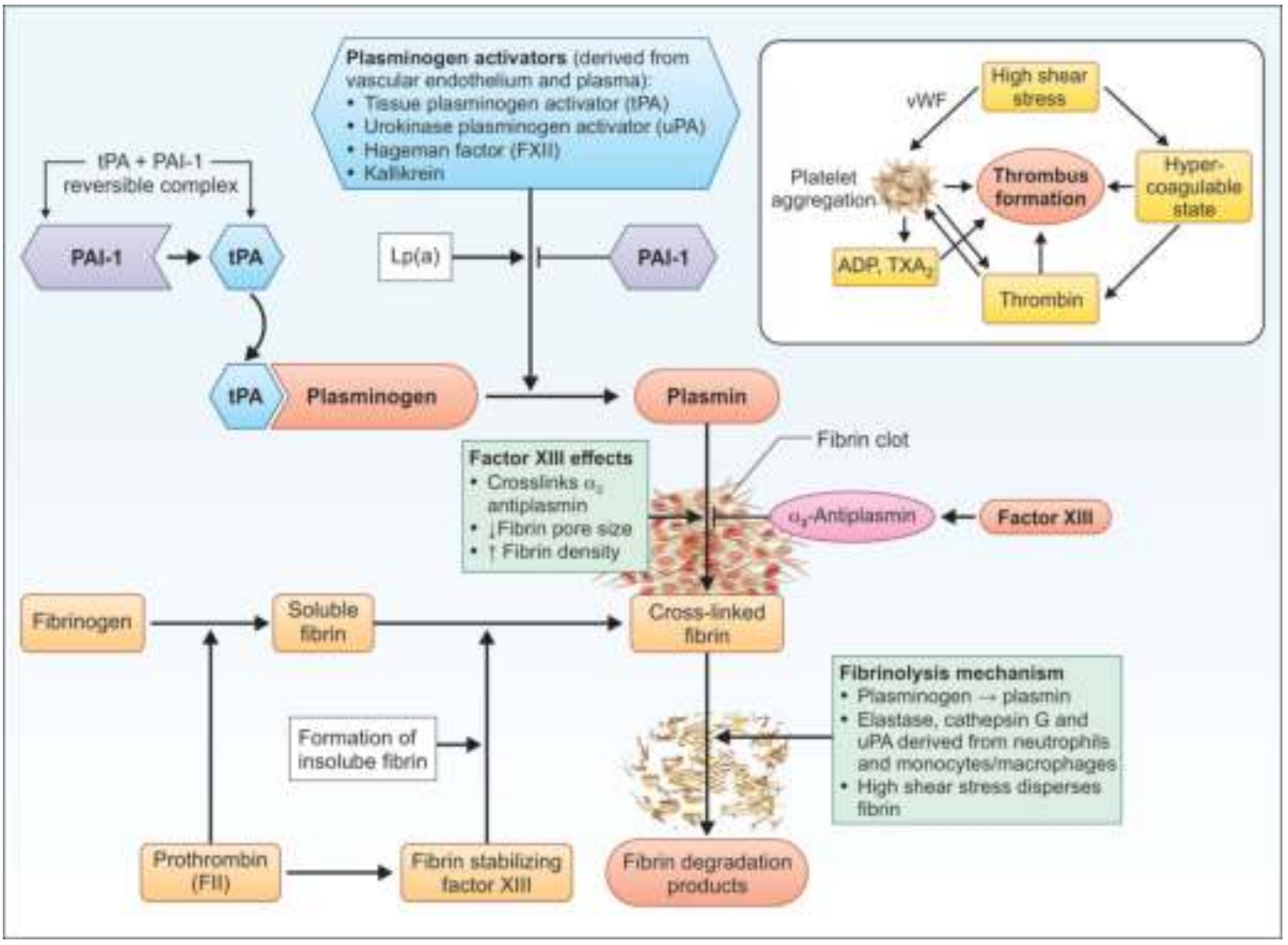


Fig. 10.9: Schematic representation of fibrinolysis. Thrombin converts plasminogen to plasmin, which breaks down the cross-linked fibrin into soluble fibrinogen degradation products (FDPs). Tissue plasminogen activator (tPA) is mainly responsible for the dissolution of fibrin formed in the circulation. Thrombolysis is inhibited by release of plasminogen activator inhibitor 1 (PAI-1) by platelets; and elastase and cathepsin G from white blood cells that become trapped in the thrombus, which directly breakdown fibrin; the plasminogen activators tPA and uPA (released from endothelial cells); and fibrin structural properties. The thickness and porosity of fibrin fibers also determines structural stability and susceptibility to thrombolysis. Lp(a), a homologue of plasminogen, can inhibit tPA-mediated plasminogen activation.

PLATELET DISORDERS

The hemostatic system consists of platelets, coagulation factors and the vascular endothelium. Platelets arise from the fragmentation of the cytoplasm of the megakaryocytes in the bone marrow and circulate in the circulation as disc-shaped anucleate particles for 3–7 days. Under physiologic state, the resistance of the vascular endothelium to interactions with platelets and coagulation factors prevent thrombosis. When vascular endothelium continuity is disrupted and the underlying subendothelial matrix is exposed, a coordinated series of events are set in motion to seal the defect. The process is known as primary hemostatic plug formation.

THROMBOPOIESIS

Developing megakaryocytes generally undergo nuclear maturation (ploidization) prior to cytoplasmic development. Megakaryocytes at any ploidy level 8N or higher can undergo cytoplasmic maturation and platelet production.

- Platelets are first released between the endothelial cells of the bone marrow sinuses as proplatelets, which break into mature platelets and released into the peripheral blood.
- Platelets are 3–4.5 μm in diameter with normal life span of 4–10 days. Normal platelet count is 150×10^9/L

to 450×10^9/L. Platelets are produced by pinching of cytoplasm of megakaryocytes situated close to sinusoids in the bone marrow. Thrombopoietin synthesized by liver regulates thrombopoiesis.

- Platelets participate in surveillance of vascular endothelium continuity, formation of primary and secondary hemostatic plugs; and healing of injured tissue. Platelets are also known to be involved in inflammation and, wound healing and cardiovascular disorders.

BLEEDING DIATHESIS

Patient presents with bleeding diathesis due to impaired blood vessel wall integrity, platelet deficiency or dysfunction; derangement in the coagulation system cascade; and combination of clotting factors. The most important cause of abnormal bleeding is a platelet disorder due to quantitative decreased production of platelets (thrombocytopenia) or defective qualitative platelet function.

- Quantitative platelet disorders (thrombocytopenias) occur when platelet count falls below $<100 \times 10^9$/L. Patient presents with bleeding into the skin and mucosa lining gastrointestinal tract and genitourinary tract.
- Qualitative platelet disorders are characterized by abnormal platelet function in the presence of normal platelet count due to various hereditary thrombocytopenias. Patient presents with spontaneous skin purpura, mucosal bleeding and prolonged bleeding after trauma; as a result of defective primary hemostatic plug.

Hematology Pearls: Type of Platelet Disorders

- Quantitative platelet disorders (thrombocytopenias) occur when platelet count falls below $<100 \times 10^9$/L. Patient presents with bleeding into the skin and mucosa lining gastrointestinal tract and genitourinary tract.
- Qualitative platelet disorders are characterized by abnormal platelet function in the presence of normal platelet count due to various hereditary thrombocytopenias.
- von Willebrand factor is a glycoprotein involved in primary and secondary hemostasis through platelet adhesion to damaged vascular subendothelium and subsequently platelet aggregation, and the intrinsic coagulation system cascade, through formation of vWF/factor VIII complex and stabilization.
- Bleeding diathesis associated with vascular disorders of blood vessel integrity result from structurally weak blood vessels or vascular damage due to inflammation and immune-mediated mechanisms.

Classification of inherited thrombocytopenias is given in Table 10.7. Classification of acquired thrombocytopenias is given in Table 10.8.

Table 10.7 Classification of inherited thrombocytopenias

Mechanism	Comments
Platelet phospholipid plasma membrane defects	
Glanzmann disease (thrombasthenia)	▪ Glycoprotein GpIIb-IIIa (GpIIB, GpIIIA) gene mutation ▪ Impaired binding of platelets to fibrinogen, hence impaired platelet aggregation ▪ Mild to severe bleeding epistaxis, petechiae **Diagnostic tests** Aggregometry (platelet aggregation impaired with platelet agonists, i.e. deficient ADP, collagen and arachidonic acid; but normal platelet aggregation with ristocetin)
Bernard-Soulier disease	▪ GpIBA; GpIBB; GpIX gene mutations ▪ Impaired binding of platelets to vWF resulting in impaired platelet aggregation ▪ Mild to severe bleeding ▪ Thrombocytopenia may be present **Diagnostic tests** ▪ Aggregometry (impaired agglutination only with ristocetin; but normal platelet aggregation normal with ADP, epinephrine, collagen and arachidonic acid) ▪ Defective clot retraction
Scot disease	▪ Autosomal recessive disorder ▪ Stem cell ANO6 gene mutation ▪ Defect in phosphatidylserine translocating to the platelet membrane resulting in impaired thrombin formation ▪ Episode of bleeding improved by platelet transfusion **Diagnostic tests** Coagulation studies

Contd...

Table 10.7 Classification of inherited thrombocytopenias (*Contd...*)

Mechanism	Comments
Storage pool defects	
Hermansky-Pudlak syndrome	▪ Autosomal recessive disorder ▪ HPSA1, AP3B1; HPS3; HPS4; HPS5; HPS6; DTNBP1 gene mutations ▪ Impaired platelet activation due to dense platelet granule defects ▪ Mucocutaneous bleeding, neutropenia, pulmonary fibrosis, and albinism (present or absent) **Diagnostic tests** ▪ Often on the basis of other abnormalities ▪ Absence of dense platelet granules on electron microscopy
Chédiak-Higashi disease	▪ Autosomal recessive disorder ▪ CHS1/LYST gene mutation ▪ Impaired activation of platelets due to dense platelet granule defects ▪ Mucocutaneous bleeding, neutropenia, recurrent pyogenic infections, peripheral neuropathy, and albinism **Diagnostic tests** ▪ Often on the basis of other abnormalities ▪ Absence of dense platelet granules on electron microscopy
Wiskott-Aldrich syndrome	▪ X-linked disorder ▪ WASp gene mutation located on chromosome Xp11, 23 ▪ Impaired activation of platelets due to dense platelet granule defects ▪ Mucocutaneous bleeding, neutropenia, immune deficiency, and bloody diarrhea **Diagnostic tests** ▪ Often on the basis of platelet count and genetic testing to defect gene mutation ▪ Absence of platelet dense granules on electron microscopy
Platelet storage pool deficiency due to defect of dense granules	▪ Autosomal dominant disorder ▪ Lack of granular nonmetabolic ADP ▪ Impaired platelet aggregation ▪ Prolonged bleeding after surgery and anemia **Diagnostic tests** ▪ Flow cytometry ▪ Bleeding time analysis ▪ Decrease in mean platelet volume
Gray platelet syndrome (platelet α-granule defect)	▪ Autosomal recessive disorder ▪ NBEAL2 gene mutation ▪ Impaired platelet activation due to α-granule defect ▪ Thrombocytopenia ▪ Large platelets ▪ Absence of α-platelet granule **Diagnostic tests** Absence of α-platelet granule on electron microscopy
Paris-Trousseau syndrome (platelet α-granule defect)	▪ Deletion of chromosome 11q23 involving FLI-1 gene ▪ Impaired platelet function due to α-granule defect ▪ Mild bleeding tendencies due to chronic thrombocytopenia **Diagnostic tests** Peripheral blood smear shows abnormal platelets with giant granules confirmed on electron microscopy
Jacobsen syndrome (platelet α-granule defect)	▪ Loss of genetic material of chromosome 11q ▪ Impaired platelet function due to α-granule defect ▪ Bleeding manifestations, distinctive facial features with delayed development of motor skills and speech; and cognitive impairment **Diagnostic tests** Genetic testing to demonstrate broken chromosome

Contd...

Table 10.7 Classification of inherited thrombocytopenias (*Contd…*)

Mechanism	Comments
Quebec platelet syndrome (platelet α-granule defect)	▪ Autosomal dominant disorder ▪ PLAU gene mutation leads to overexpression of urokinase plasminogen activator ▪ Impaired platelet function due to α-granule disorder with overexpression of urokinase plasminogen activator resulting in platelet dependence fibrinolysis ▪ Bleeding manifestations **Diagnostic tests** Genetic testing to demonstrate PLAU gene mutation
X-linked macrothrombocytopenia with dyserythropoiesis	▪ X-linked recessive disorder ▪ GATA1 gene mutation ▪ Bleeding manifestations **Diagnostic tests** Genetic testing to demonstrate GATA1 gene mutation
Thrombocytopenia with absent radius bone (TAR syndrome) (platelet dense granules defect)	▪ Deletion of genes on chromosome 1q21.1 ▪ Thrombocytopenia with absent radius bone **Diagnostic tests** ▪ Genetic testing to demonstrate chromosome breakage
Congenital amegakaryocytic thrombocytopenia	
Congenital amegakaryocytic thrombocytopenia	▪ cMPL gene mutation located on chromosome 3 ▪ Insensitivity to thrombopoietin ▪ Bleeding manifestations, severe thrombocytopenia from birth **Diagnostic tests** ▪ Bone marrow aspirate examination ▪ Elevated TPO (thrombopoietin) level ▪ Genetic testing to demonstrate cMPL gene mutation
Disorders of receptors and signal transduction	
Cyclooxygenase enzyme deficiency	▪ PTGS1 and PTGS2 gene mutations ▪ Quantitative deficiency of cyclooxygenase deficiency resulting in thromboxane A_2 synthesis ▪ Thrombotic and bleeding phenotype **Diagnostic tests** Bleeding and thrombosis work up
Thromboxane A_2 receptor defect	▪ Autosomal recessive disorder ▪ TBXA2R gene mutation ▪ TXA_2 functional activity impaired with loss of prothrombotic property ▪ Mucocutaneous gastrointestinal or surgical bleeding **Diagnostic tests** ▪ Platelet aggregometry ▪ Impaired platelet aggregation to low dose adenosine diphosphate (ADP), collagen and arachidonic acid) ▪ Normal platelet aggregation to thrombin
ADP (adenosine diphosphate) receptors of platelets and their inhibition	▪ Autosomal recessive disorder ▪ P2YC12 gene mutation (3q24–q25) ▪ Impairment of platelet aggregation ▪ Bleeding manifestations **Diagnostic tests** Selective impairment of platelet responses to ADP
Bleeding diathesis due to glycoprotein VI deficiency	▪ Autosomal recessive disorder ▪ GpVI gene mutation ▪ Bleeding manifestations **Diagnostic tests** Genetic testing to demonstrate GpVI gene mutation

Contd...

Table 10.7 Classification of inherited thrombocytopenias (*Contd...*)

Mechanism	Comments
Platelet-type von Willebrand disease	▪ Autosomal recessive disorder ▪ GpXA2R gene mutation ▪ Bleeding manifestations **Diagnostic tests** Genetic testing to demonstrate GpXA2R gene mutation

von Willebrand disease is also inherited autosomal dominant/recessive platelet function disorder; characterized by platelet aggregation with adenosine diphosphate (ADP), collagen and arachidonic acid; but impaired with ristocetin. Platelet aggregation abnormality is corrected by cryoprecipitate therapy.

Table 10.8 Classification of acquired thrombocytopenias

Mechanism	Causes
Failure of platelet production	
Generalized bone marrow failure	▪ Leukemias ▪ Myelodysplastic syndrome (MDS) ▪ Aplastic anemia ▪ Human immunodeficiency virus (HIV) infection ▪ Myelofibrosis ▪ Megaloblastic anemia ▪ Multiple myeloma ▪ Metastatic bone disease ▪ Lymphoma involving bone marrow ▪ Cytotoxic drugs
Selective megakaryocytic depression	▪ Drugs (aspirin, indomethacin, butazolidin, sulfinpyrazone, carbenicillin, phenothiazine, dipyridamole, tricyclic antidepressants) ▪ Viral agents ▪ Plasma expanders (dextran or hydroxyethyl starch coat platelets) ▪ Chemical agents ▪ Alcohol
Increased destruction of platelets	
Immune-mediated thrombocytopenia	▪ Alloantibodies (neonatal, post-transfusion) ▪ Autoantibodies causing primary or secondary thrombocytopenia (e.g. systemic lupus erythematosus, chronic lymphocytic leukemia, post-infection, HIV infection, post-stem cell transplantation)
Drug-induced thrombocytopenia either by immune mechanism or decreased platelet aggregation	▪ Aspirin inhibits prostaglandin synthesis ▪ Nonsteroidal anti-inflammatory drugs ▪ Platelet function inhibitors (e.g. carbenicillin, phenothiazines, dipyridamole)
Disseminated intravascular coagulation (DIC)	▪ Blood transfusion reactions ▪ Leukemia ▪ Pancreatitis ▪ Liver disease ▪ Retained placenta after delivery
Microangiopathic process	▪ Thrombotic thrombocytopenic purpura (TTP) ▪ Hemolytic uremic syndrome (HUS) ▪ Extracorporeal circulation ▪ Hemolysis, elevated liver enzymes and low platelet count in association with pre-eclampsia (HELLP) syndrome
Giant hemangioma (Kasabach-Merritt syndrome)	Giant hemangioma is associated with thrombocytopenia and bleeding manifestations
Pooling (abnormal distribution) of platelets	
Massive splenomegaly	Hyperfunctioning of phagocytic activity (e.g. chronic myelogenous leukemia, myelofibrosis, Gaucher disease, visceral leishmaniasis, β-thalassemia and malaria)
Malignant disorders	Pooling of platelets
Dilution loss of platelets	
Massive blood transfusions of stored blood	Dilutional thrombocytopenia
Pregnancy	Dilutional thrombocytopenia
Malignant disorders	Pooling of platelets
Platelet coated by paraproteins	
Paraproteinemias	▪ Multiple myeloma (coating of platelets by paraprotein) ▪ Waldenström's syndrome (coating of platelets by paraprotein)

QUANTITATIVE PLATELET DISORDERS

Quantitative platelet disorders (thrombocytopenias) occur when platelet count falls below $<100 \times 10^9$/L. Patient presents with bleeding into the skin and mucosa lining gastrointestinal tract and genitourinary tract. Failure to produce platelets by bone marrow and increased destruction in the peripheral blood are the causes of thrombocytopenia.

- Drug toxicity or viral infections lead to selective megakaryocyte depression. Aplastic anemia, leukemia, myelofibrosis, cytotoxic chemotherapy or bone marrow infiltration and decreased numbers of megakaryocytes can be part of a generalized bone marrow failure.
- Neonatal thrombocytopenia occurs in the newborn infants as a result of intrauterine rubella or other infections, platelet antibodies, disseminated intravascular coagulation, congenital absence of megakaryocytes, giant hemangiomas and inherited thrombocytopenias.

Hematology Pearls: Pathophysiology of Thrombocytopenia

- **Decreased platelet production by bone marrow:** Decreased platelet production by bone marrow occurs due to bone marrow hypoplasia, replacement of bone marrow by metastatic deposits, bone marrow failure syndrome, drug toxicity, viral infections and cytotoxic chemotherapeutic agents.
- **Ineffective maturation of platelets:** Ineffective maturation of platelets in the cytoplasm of megakaryocytes occurs due to megaloblastic anemia, myelodysplastic syndrome and myeloproliferative neoplasms.
- **Increased platelet destruction:** Increased platelet destruction due to immune-mediated mechanism, hypersplenism and heparin therapy.
- **Pooling of platelets:** Pooling of platelets in the spleen in cases of massive splenomegaly results in lowering of platelet counts in peripheral blood.

IMMUNE THROMBOCYTOPENIC PURPURA

Immune thrombocytopenic purpura (ITP), also known as autoimmune thrombocytopenic purpura, is an acquired autoimmune pathology characterized by isolated thrombocytopenia (peripheral blood platelet count $<100 \times 10^9$) due to pathogenic **a**ntiplatelet autoantibodies (IgG, IgA) directed against platelet membrane glycoproteins (GpIIb/ GpIIIa) or (GpIb–IX) or antibodies (IgG, IgA) directed against megakaryocyte antigen in the bone marrow leading to splenic sequestration of platelets coated by antiplatelet autoantibodies and their destruction by mononuclear macrophages in spleen and Kupffer cells in liver, and impaired bone marrow megakaryocyte function. In addition, impairment of T cells, cytokine imbalances (IL-6 and IL-11), and the contribution of the bone marrow niche have now been recognized to play role in pathogenesis of immune thrombocytopenic purpura, which manifests with hemorrhagic episodes. Normal platelet count is in the range of 150,000 to 450,000/L. With immune thrombocytopenic purpura, the platelet count is less than 100×10^9/L. By the time significant bleeding occurs, patient may have low-platelet count $<10 \times 10^9$/L. The lower the platelet count, the greater is the risk of bleeding episodes.

- **Predisposing factors:** Immune thrombocytopenic purpura (ITP) can occur following viral infection (chicken pox, HBV, HCV, HIV, EBV), bacterial infection (*Helicobacter pylori*), parasitic infection (malarial parasite) and in association with autoimmune disorders (rheumatoid arthritis, systemic lupus erythematosus), and hematological disorders (chronic lymphocytic leukemia, and low-grade non-Hodgkin lymphoma). In the infectious immune thrombocytopenic purpura (ITP) cases, viral antigen is recognized as being similar to platelet antigen, a process termed 'molecular mimicry', which then gives rise to cross-reactive anti-platelet autoantibodies and anti-megakaryocyte autoantibodies.
- **Clinical features:** Patient of immune thrombocytopenic purpura presents with petechiae (small red and purple dots), purpura, ecchymosis (larger red brown and purple dots), bleeding gums, hematuria, hematochezia, heavy menstrual bleeding and extreme tiredness. Serious and possible fatal complications occur due to very low-platelet count, which include subarachnoid hemorrhage or intracerebral hemorrhage, lower gastrointestinal bleeding or internal bleeding.

CLASSIFICATION OF IMMUNE THROMBOCYTOPENIC PURPURA

Immune thrombocytopenic purpura (ITP) can be observed in both children and adults, with both sexes being affected, however, the underlying mechanisms of pediatric immune thrombocytopenic purpura and adult immune thrombocytopenic purpura may be different. Immune thrombocytopenic purpura can be acute ITP (short-term following infection and resolves within two months) and/or chronic ITP (long-term persists longer than six months). Comparison between acute and chronic immune thrombocytopenic purpura is given in Table 10.9. Low-platelet count and clinical manifestations are given in Table 10.10.

Table 10.9 Comparison between acute and chronic autoimmune thrombocytopenic purpura

Features	Acute Immune Thrombocytopenic Purpura	Chronic Immune Thrombocytopenic Purpura
Age group	2–6 years of age	20–40 years of age
Sex predilection	Boys and girls equally affected	Males are more affected than females
Viral infection	Antecedent viral infection	Common 1–3 weeks of prior viral infection
Platelet destruction	Autoimmune mediated disorder	Autoimmune mediated disorder
Platelet count	$<20 \times 10^9$/L	$30–80 \times 10^9$/L
Onset of disorder	Abrupt onset	Gradual onset
Duration of disorder	2–8 weeks	Months to years (lifelong)
Spontaneous remission of disorder (% cases)	85–90% within six months	Uncommon more than six months
Nature of disorder	Self-limited	Not self-limited
Type of bleeding	Petechiae and superficial (e.g. epistaxis, bleeding gums, hematuria, subarachnoid hemorrhage and intracranial bleeding)	Petechiae and superficial (e.g. epistaxis, bleeding gums, hematuria, excessive menstrual flow, extensive soft tissue hemorrhages after minor surgery, melena, hematuria and excessive menstrual blood flow)
Splenomegaly	Uncommon	Common
Peripheral blood smear	▪ Platelet counts low ▪ Eosinophilia or basophilia present (common)	▪ Few giant platelets ▪ Eosinophilia or basophilia absent
Bone marrow aspirate	Compensatory hyperplasia of megakaryocytes (not indicated)	Compensatory hyperplasia of megakaryocytes (mature and immature megakaryocytes with smooth borders)
Prothrombin time	Normal	Normal
Activated partial thromboplastin time	Normal	Normal
Spontaneous remission	95% of cases	<15% of cases; course of disease fluctuates
Therapy	Corticosteroids, anti-D, immunoglobulin	Corticosteroids, splenectomy, rituximab

Table. 10.10 Low-platelet count and clinical manifestations

Platelet Count	Site of Bleeding
Platelet count $<10 \times 10^9$/L	▪ Genitourinary tract bleeding ▪ Gastrointestinal tract bleeding ▪ Intracranial bleeding
Platelet count $10–50 \times 10^9$/L	▪ Spontaneous bleeding in skin and mucosal surface ▪ Post-traumatic bleeding
Platelet count $>50 \times 10^9$/L	▪ No spontaneous bleeding

- **Acute immune thrombocytopenic purpura:** Acute immune thrombocytopenic purpura (acute ITP) is the most common disorder that usually affects 2–7 years old young children, which usually starts suddenly and the symptoms usually disappear spontaneously within 6 months, and often within few weeks.
  - The symptoms may follow a viral illness such as chicken pox, hepatitis C and human immunodeficiency virus during winter and spring season, which can prompt production of autoantibodies that can cross-react with platelets in blood and megakaryocyte in bone marrow leading to platelet destruction in macrophages in spleen and Kupffer cells in liver; and impaired function of bone marrow megakaryocytes. Patient presents with petechial hemorrhages, ecchymosis, epistaxis, bleeding gums, hematuria, subarachnoid hemorrhage, intracranial bleeding and easy bruising at joints of elbows and knees just from movement.
  - There is no significant splenomegaly. Minority of patients develop mucosal bleeding and should be treated with prednisolone or intravenous immunoglobulin.
- **Chronic immune thrombocytopenic purpura:** Onset of chronic immune thrombocytopenic purpura

(chronic ITP) can happen at any age and the symptoms usually can last for a minimum of six months, several years, or a life-time. Adults are more affected than children, but it does affect adolescents.

- Disorder is more common in females than males. Autoantibodies are demonstrated on the platelet surface and in free form in blood. Patient with chronic ITP is less likely to resolve without therapy, which can recur often and patient requires continual follow-up care with a clinical hematologist.
- Diagnosis of chronic ITP should be made only after exclusion of other causes of thrombocytopenia (e.g. chronic lymphocytic leukemia, non-Hodgkin lymphoma, HBC, HBV, HIV, Epstein-Barr virus infection, malarial parasite and systemic lupus erythematosus, rheumatoid arthritis, drug-induced thrombocytopenia). Evans syndrome is characterized by thrombocytopenia, autoimmune hemolytic anemia and positive Coombs' test.

PATHOPHYSIOLOGY

Cellular pathogenetic mechanisms in immune thrombocytopenic purpura involve dysregulation of B cells and autoreactive plasma cells, which produce autoantibodies (IgG, IgA), which bind to membrane glycoproteins (GpIIb/GpIIIa) or (GpIb–IX) on circulating platelets inducing their destruction in the spleen and liver or impaired functions of megakaryocytes.

- Immune response is also adversely affected, leading to decrease of CD4+ regulatory T cells (Treg) and B regulatory cells (Breg) which contributes to autoreactive plasma cell survival (supporting autoantibody production). Moreover CD8+ cytotoxic T cells are also activated, inducing platelets and megakaryocytes apoptosis as well as dysregulation of bone marrow niche homeostasis. Therefore, immune thrombocytopenic purpura (ITP) does not only result in platelet destruction by macrophages in spleen and Kupffer cells in liver, but also defects in the megakaryopoiesis and thrombopoiesis.
- The spleen plays a critical role in the pathogenesis of immune thrombocytopenic purpura due to destruction of platelets. Splenic macrophages expressing FcyR mediate the uptake of autoantibody-coated platelets with phagocytosis mediated through SYK signaling pathways.

LABORATORY DIAGNOSIS

Thrombocytopenia is common hematologic presentation with a variety of potential causes. Immune thrombocytopenic purpura (ITP) is diagnosed by identifying a low platelet count and based on the exclusion of other causes of low-platelet count (thrombocytopenia) such as exposure to drugs, infections (HBV, HCV, HIV, *Helicobacter pylori*) and other disorders, clinical history, physical examination (evidence of bleeding in the mucous membrane and skin, lymphadenopathy, splenomegaly or hepatomegaly) and addition investigation such as bone marrow trephinebiopsy.

- Peripheral blood smear from a patient with immune thrombocytopenic purpura shows low-platelet count ($<100 \times 10^9$/L) with typical normal morphology with varying numbers of large platelets, and normal morphology of red blood cells and leukocytes. An elevated reticulocyte count in immune thrombocytopenic anemia is an evidence of compensatory increased red blood cell production.
- Diagnostic value of bone marrow examination in immune thrombocytopenic purpura remains unresolved and data from a large perspective study would be helpful. A reasonable approach would be to limit the bone marrow aspirate examination to patients with typical clinical or hematologic feature (e.g. abnormal bleeding and clotting studies) or with a poor response to corticosteroids or those patients in whom septectomy is being considered.
- Bone marrow trephine biopsy is often performed in children with acute immune thrombocytopenic purpura (acute ITP) to rule out leukemia, particularly when treatment with corticosteroids is contemplated.
- Direct antiglobulin test (DAT, direct Coombs' test) is performed to detect autoantibodies attached to red blood cells. Quantitative platelet-bound immunoglobulin (IgG) sensitizing the circulating platelets and megakaryocytes in bone marrow are analyzed to diagnose immune thrombocytopenic purpura.

Laboratory Diagnosis of Immune Thrombocytopenic Purpura

Hemoglobin

Hemoglobin—low due to blood loss

Bleeding and Coagulation Studies

- Bleeding time—prolonged
- Clotting time—normal range
- Blood clot retraction—decreased
- Prothrombin time and international normalized ratio (PT–INR) within normal range
- Activated partial thromboplastin time (APTT) within normal range

Peripheral Blood Smear Examination

- Peripheral blood smear examination shows low-platelet count, few large platelets, normal morphology of red blood cells and leukocytes.
- Immune thrombocytopenic purpura is shown in Fig. 10.10.

Bone Marrow Trephine Biopsy Examination

- Bone marrow aspiration trephine biopsy shows normal number or increased number of mature and immature megakaryocytes. Borders of the megakaryocytes are smooth indicating nonfunctional megakaryocytes.
- Bone marrow trephine biopsy shows normoblastic or micro-normoblastic erythropoiesis and normal myelopoiesis.
- Iron stores are depleted due to bleeding. Iron is demonstrated by Perls Prussian blue stain.
- Immune thrombocytopenic purpura is shown in Fig.10.11.

Other Hematologic Tests

- Glycoprotein immobilization assays are performed to demonstrate antiplatelet antibodies.
- Plasma glycocalcin—high level
- Thrombopoietin—normal or slightly elevated

TREATMENT

Treatment strategies of immune thrombocytopenic purpura (ITP) are aimed at restoration of platelet counts compatible with adequate hemostasis rather than achieving platelet counts in physiologic range.

- First-line treatments consist of corticosteroids alone or in combination with intravenous immunoglobulin or anti-D, with an aim to inhibit anti-platelet autoantibody presentation by antigen-presenting cells (APCs) to restore normal immune response. These therapeutic agents also act on B cells and plasma cells, thus decreasing autoantibodies induced destruction of platelets in spleen and restoration of megakaryocytes function in bone marrow.
- Second-line treatments include immunosuppressive drugs (e.g. cyclophosphamide, azathioprine, ciclosporin, vincristine, rhesus anti-D, rituximab targeting B cells), and splenectomy. **Splenectomy** is indicated for non-responders with continuing symptoms, and/or very low-platelet count. Immunosuppressive drugs and splenectomy also modulate T cell compartment, notably increasing CD4+ regulatory T cell (Treg) function.
- Finally, third-line treatments aim at to stimulate production of megakaryocytes in the bone marrow by administration of thrombopoietin (TPO) receptor agonists (romiplostim and eltrombopag). Minority of patients develop mucosal bleeding and should be treated with prednisolone or intravenous immunoglobulin. Thrombopoietin (TPO) agonists present indirect immunoregulatory effects on regulatory B cells (Bregs) and CD4+ regulatory T cells (Tregs). Combining multiple therapeutic approaches are often required to ensure the restoration of a physiologic platelet count range. Treatment of immune thrombocytopenic purpura (ITP) is given in Table 10.11.

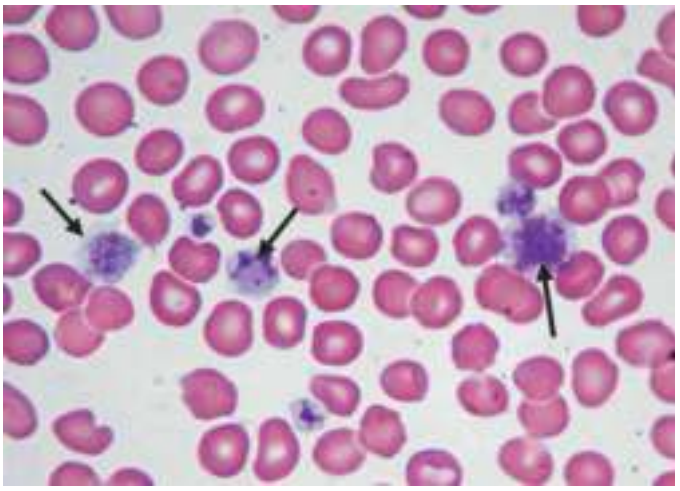


Fig. 10.10: Immune thrombocytopenic purpura. Peripheral blood smear shows lack of platelets and a few giant platelets (arrows).

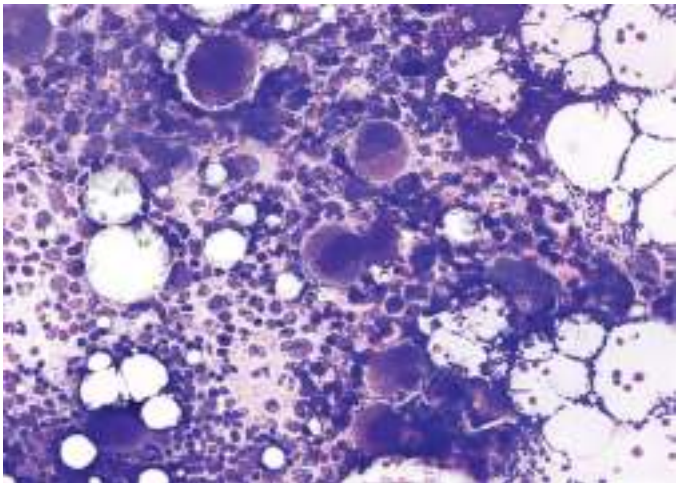


Fig. 10.11: Immune thrombocytopenic purpura. Bone marrow aspirate shows marked hyperplasia of megakaryocytes. Borders of megakaryocytes are smooth indicating nonfunctional megakaryocytes.

NEONATAL ALLOIMMUNE THROMBOCYTOPENIA

Neonatal alloimmune thrombocytopenia is similar to hemolytic disease of newborn except that antigenic stimulus comes from platelet specific antigens rather than red blood cell antigens.

- In about 80% of cases, the antigen is HPA-Ia (human platelet antigen Ia). The antibody is then directed against the HPA-Ia (human platelet antigen Ia) on platelets resulting in platelet destruction by reticuloendothelial cells.
- Transplacental passage of antibody from a mother with immune thrombocytopenia can lead to neonatal thrombocytopenia, which most often resolves spontaneously over a few weeks.

Table 10.11 Treatment of immune thrombocytopenic purpura

Findings	Treatment
Platelet count >50,000/mm^3	No treatment
Platelet count <50,000/mm^3 with minor bleeding	Prednisolone
Platelet count <10,000/mm^3 with severe bleeding	IVIg or RHo GAM (rho-D immune globulin)
Recurrent episodes of thrombocytopenia and bleeding	Splenectomy
No response to splenectomy	▪ Romiplostim (fusion protein equivalent to thrombopoietin) ▪ Eltrombopag (small molecule agonist of the c-mpl receptor, which is physiological target of thrombopoietin)

IVIg administration in patients with ITP has no direct activity against platelets. It is administered to prevent the action of macrophages against platelets. Drug stops up all the Fc receptors on the macrophages, IVIg leaves no room for the antibodies on the platelets. Thus, it prevents platelets destruction.

- Mothers who have been sensitized to platelet antigens by blood transfusion during previous pregnancy may develop antibodies, that cross the placenta and coat fetal and neonatal platelets resulting in platelet destruction by reticuloendothelial system.
- Neonates with platelet alloantibodies may also become thrombocytopenic following blood transfusion (post-transfusion thrombocytopenic purpura). Neonates present with unexplained bruising and purpura.

QUALITATIVE INHERITED PLATELET DISORDERS

Qualitative inherited platelet disorders are characterized by abnormal platelet function in the presence of normal platelet count due to various inherited thrombocytopenias. Defective platelet function disorders present with spontaneous skin purpura, mucosal bleeding and prolonged bleeding after trauma; as a result of defective hemostatic plug. Diseases of the platelet storage pool occur due to deficiencies in either the α-granules or dense granules resulting in poor secondary platelet aggregation. A neonate or small infant with bleeding manifestations must be evaluated for inherited bleeding disorders (inherited platelet disorders, hemophilia and von Willebrand disease).

PLATELET PHOSPHOLIPID PLASMA MEMBRANE DEFECTS

Plasma membrane of activated platelets provide a catalytic phospholipid surface on which factor IXa–factor VIIIa complex and the 'prothrombokinase' complex (factor Xa–factor–Va) can be assembled. The formation of a procoagulant platelet surface involves the exposure of anionic phospholipids, e.g. phosphatidylserine and is associated with shedding of microvesicles from the membranes of activated platelets. Moreover, tissue factor, which initiates extrinsic coagulation pathway, requires the presence of phospholipids for optional biological activity. Platelet phospholipid plasma membrane defects are linked to many platelet disorders.

GLANZMANN DISEASE (THROMBASTHENIA)

Glanzmann disease (thrombasthenia) is an autosomal recessive disorder due to inherited deficiency of glycoprotein GpIIb–IIIa (GpIIb, GpIIIa) resulting in impaired binding of platelets to fibrinogen, hence impaired platelet aggregation.

- Normally, GpIIb–IIIa act as receptor for fibrinogen bridging between adjacent platelets resulting in platelet aggregation.
- Patient presents with mild to severe bleeding (i.e. epistaxis, petechial hemorrhages, gum bleeding).
- Platelet count and morphology are normal.
- Diagnostic test includes aggregometry (platelet aggregation impaired with platelet agonists, i.e. deficient ADP, collagen and arachidonic acid; but normal platelet aggregation with ristocetin).

BERNARD-SOULIER SYNDROME

Bernard-Soulier syndrome is characterized by GpIBA; GpIBB; GpIX gene mutations, impaired binding of platelets to von Willebrand factor (vWF) resulting in impaired platelet aggregation and mild to severe bleeding.

Laboratory Diagnosis of Bernard-Soulier Syndrome

- Peripheral blood smear examination shows mild thrombocytopenia and giant platelets.
- Diagnostic tests include aggregometry (impaired agglutination only with ristocetin; but normal platelet aggregation with ADP, epinephrine, collagen and arachidonic acid) and defective blood clot retraction.

SCOT SYNDROME

Scot syndrome is an autosomal recessive disorder characterized by hematopoietic stem cell ANO6 gene mutation and defect in phosphatidylserine translocating to the platelet membrane resulting in impaired thrombin formation; and episode of bleeding improved by platelet transfusion. Coagulation studies are done.

PLATELET STORAGE POOL DEFECTS

Platelet storage pool defects are linked to coagulopathy resulting from defects in the granules in platelets particularly a lack of granular non-metabolic adenosine diphosphate.

HERMANSKY-PUDLAK SYNDROME

Hermansky-Pudlak syndrome is an autosomal recessive disorder due to mutations in HPSA1; AP3B1; HPS3; HPS4; HPS5; HPS6; DTNBP1 genes resulting in impaired platelet activation due to dense platelet granule defects.

Clinical Features

Patient presents with mucocutaneous bleeding, neutropenia, pulmonary fibrosis, and albinism. Electron microscopy demonstrates absence of dense platelet granules.

CHÉDIAK-HIGASHI SYNDROME

Chédiak-Higashi syndrome is an autosomal recessive disorder characterized by CHS1/LYST gene mutation, impaired activation of platelets due to platelet dense granule defects, mucocutaneous bleeding, neutropenia, recurrent pyogenic infections, peripheral neuropathy, and albinism. Electron microscopy demonstrates absence of platelet dense granules.

WISKOTT-ALDRICH SYNDROME

Wiskott-Aldrich syndrome is X-linked disorder with defects of both B and T cells, which occurs due to WASp gene mutation located on chromosome Xp11, 23 resulting in impaired activation of platelets due to platelet dense granule defects. WASp gene encodes WASp protein expressed on hematopoietic stem system. Platelets and T cells show deficient surface glycoproteins (CD43), which are ligand for intercellular adhesion molecule 1 (ICAM-1). Half-life of platelet is reduced. Electron microscopy demonstrates absence of dense platelet granules.

Clinical Features

Patient presents with mucocutaneous bleeding, neutropenia, recurrent infections, immune deficiency, and bloody diarrhea with fatal outcome due to bleeding tendencies, recurrent infections and development of lymphoma before attaining age of six years. Patient is treated by stem cell transplantation.

PLATELET STORAGE POOL DEFICIENCY (PLATELET DENSE GRANULES DEFECT)

Platelet storage pool deficiency dense granules defect is an autosomal dominant disorder due to lack of dense granular nonmetabolic ADP resulting in impaired platelet aggregation and prolonged bleeding after surgery and anemia. Diagnostic tests include flow cytometry, bleeding time analysis and decrease in mean platelet volume.

GRAY PLATELET SYNDROME (PLATELET α-GRANULES DEFECT)

Normal platelet contains α-granules and dense granules. The α-granules contain fibrinogen, platelet factors IV and V, thrombospondin. Dense granules contain ATP, ADP, calcium and serotonin.

- Gray platelet syndrome (platelet α-granule defect) is an autosomal recessive disorder due to NBEAL2 gene mutation. The term gray platelet syndrome is derived from gray appearance of platelets in the peripheral blood. Number of megakaryocytes is normal in bone marrow. But megakaryocytes are unable to produce mature platelets resulting in thrombocytopenia.
- Platelet survival is reduced due to deficient platelet α-granule mutation resulting in impaired platelet activation, thrombocytopenia and large platelets.
- Electron microscopy demonstrates absence of platelet α-granules.
- Patient meets all the criteria for idiopathic thrombocytopenic purpura (ITP). Peripheral blood smear examination shows large gray platelets demonstrated in gray platelet syndrome.
- Patient presents with epistaxis, bruising, ecchymosis, and splenomegaly resulting in fatal outcome.

PARIS-TROUSSEAU SYNDROME (PLATELET α-GRANULE DEFECT)

Paris-Trousseau syndrome (platelet α-granule defect) occurs due to deletion of chromosome 11q23 involving

FLI-1 gene resulting in impaired platelet function. Patient presents with mild bleeding tendencies due to chronic thrombocytopenia. Peripheral blood smear shows abnormal platelets with giant granules confirmed on electron microscopy.

JACOBSEN SYNDROME (PLATELET α-GRANULE DEFECT)

Jacobsen syndrome occurs due to loss of genetic material of chromosome 11q resulting in impaired platelet function due to platelet α-granule defect. Patient presents with bleeding manifestations, distinctive facial features with delayed development of motor skills and speech; and cognitive impairment. Cytogenetic testing is done to demonstrate broken chromosome.

QUEBEC PLATELET SYNDROME (PLATELET α-GRANULE DEFECT)

Quebec platelet syndrome (α-granule defect disorder) is an autosomal dominant disorder due to **PLAU gene** mutation leads to overexpression of urokinase plasminogen activator. Impaired platelet function due to α-granule defect disorder with overexpression of urokinase plasminogen activator resulting in platelet dependence fibrinolysis. Patient presents with bleeding manifestations. Genetic testing is done to demonstrate PLAU gene mutation.

X-LINKED MACROTHROMBOCYTOPENIA WITH DYSERYTHROPOIESIS

X-linked macrothrombocytopenia with dyserythropoiesis is X-linked recessive disorder due to GATA1 gene mutation. Patient presents with bleeding manifestations. Genetic testing is done to demonstrate GATA1 gene mutation.

THROMBOCYTOPENIA WITH ABSENT RADII BONE (TAR SYNDROME)

Thrombocytopenia with absent radii bone (TAR syndrome) is platelet dense granules disorder due to deletion of genes on chromosome 1q21.1. Patient presents with absence of radius bone bleeding manifestations due to thrombocytopenia. Cytogenetic testing is done to demonstrate chromosome breakage.

CONGENITAL AMEGAKARYOCYTIC THROMBOCYTOPENIA

Congenital amegakaryocytic thrombocytopenia occurs due to **cMPL gene mutation** located on chromosome 31b34 resulting in insensitivity to thrombopoietin. Patient presents with bleeding manifestations, and severe thrombocytopenia from birth. Diagnostic tests include bone marrow aspirate, elevated TPO (thrombopoietin) and genetic testing.

PLATELET DISORDERS OF RECEPTORS AND SIGNAL TRANSDUCTION

CYCLOOXYGENASE ENZYME DEFICIENCY DISORDER

Cyclooxygenase enzyme deficiency occurs due to PTGS1 or PTGS2 gene mutation. Quantitative deficiency of cyclooxygenase deficiency results in thromboxane A_2 synthesis. Thromboxane A_2 has prothrombotic properties, as it stimulates the activation of platelets and platelet aggregation. It also acts as vasoconstrictor in inflammation. Patient presents with thrombotic and bleeding phenotype. Bleeding and thrombosis work up should be done.

THROMBOXANE A_2 RECEPTOR DEFECT DISORDER

Thromboxane A_2 receptor defect is an autosomal recessive disorder due to **TBXA2R gene mutation**. TXA_2 functional activity is impaired with loss of prothrombotic property. Patient presents with mucocutaneous gastrointestinal tract or surgical bleeding. Diagnostic tests include platelet aggregometry, impaired platelet aggregation to low dose adenosine diphosphate (ADP), collagen and arachidonic acid) and normal platelet aggregation to thrombin.

ADP RECEPTORS OF PLATELETS AND THEIR INHIBITION DISORDER

Adenosine diphosphate (ADP) receptors of platelets and their inhibition is an autosomal recessive disorder due to P2YC12 gene mutation located on chromosome 3q24–q25 resulting in impairment of platelet aggregation. Patient presents with bleeding manifestations. Diagnostic test includes selective impairment of platelet aggregation responses to ADP.

BLEEDING DIATHESIS DUE TO GLYCOPROTEIN VI DEFICIENCY DISORDER

Bleeding diathesis due to glycoprotein VI deficiency is an autosomal recessive disorder due to GpVI gene mutation. Patient presents with bleeding manifestations. Genetic testing is done to demonstrate glycoprotein VI deficiency.

PLATELET-TYPE VON WILLEBRAND DISEASE

Platelet-type von Willebrand disease is an autosomal recessive disorder due to GpXA2R gene mutation. Patient presents with bleeding manifestations. Genetic testing is done to demonstrate GpXA2R gene mutation.

QUALITATIVE ACQUIRED PLATELET DISORDERS

Qualitative acquired platelet disorders are characterized by abnormal platelet function in the presence of normal platelet count in various acquired thrombocytopenias. Patient presents with spontaneous skin purpura, mucosal bleeding and prolonged bleeding after trauma; as a result of defective primary hemostatic plug.

POST-TRANSFUSION PURPURA

Post-transfusion purpura is a rare complication of blood transfusion presenting with severe thrombocytopenia 7–10 days after blood transfusion. Patients are usually multiparous women, who are negative for HPA1a (human platelet antigen Ia). Antibodies to HPA1a results in immune-mediated destruction of the patient's own platelets.

DRUG-INDUCED THROMBOCYTOPENIA

Drugs cause thrombocytopenia by either inhibiting bone marrow production or immune mechanism.

- The most common drug-induced immune mediated thrombocytopenia occurs when the drug (quinine, benzylpenicillin, heparin, chlorothiazide) or its metabolite combines with antibody (IgG) in plasma and forms antigen–antibody complex resulting in adsorption on the platelet surface.
- Antigen–antibody complex adsorbed on the platelet surface gives rise to highly immunogenic epitope resulting in platelet destruction by reticuloendothelial system.
- Administration of aspirin prevents platelet aggregation by inhibiting thromboxane A_2 and prostaglandin E_2 lasting for 3–4 days. Plasma expander dextran interferes with platelet function probably by coating of platelets.

HEPARIN-INDUCED THROMBOCYTOPENIA

Heparin-induced thrombocytopenia (HIT) is a complication of heparin therapy. There are two types of HIT. Features of heparin-induced thrombocytopenia are given in Table 10.12.

- Best initial diagnostic tests are analysis of platelet factor IV antibodies or heparin-induced antiplatelet antibodies in type 2 HIT.
- Best initial therapy is to stop heparin and use fondaparinux.

HEPARIN-INDUCED THROMBOCYTOPENIA TYPE 1

Heparin-induced thrombocytopenia (HIT) type 1 is nonimmune-mediated disorder. Patient presents within mild to moderate drop in platelets within first two days after exposure to heparin. The platelet count normalizes with the continued heparin therapy. It is not immune-mediated disorder, that results from the direct effect of heparin on platelet activation. It is treated by administration of low-molecular weight heparin preparations.

HEPARIN-INDUCED THROMBOCYTOPENIA TYPE 2

Heparin-induced thrombocytopenia (HIT) type 2 is immune-mediated disorder, that typically occurs 4–10 days after exposure to heparin. The most common drug-induced immune-mediated thrombocytopenia occurs when the drug (heparin) or its metabolite combines with antibody (IgG) in plasma and forms antigen–antibody complex resulting in adsorption on the platelet surface.

- Antigen–antibody adsorbed on the platelet surface gives rise to highly immunogenic epitope resulting in platelet destruction by reticuloendothelial system.
- Patient presents with thrombocytopenia and thrombosis in limbs coexisting. Once type 2 HIT is diagnosed, further administration of heparin is contraindicated.

POOLING OF PLATELETS IN MASSIVE SPLENOMEGALY

Spleen normally pools about one-third of the platelet mass, but in massive splenomegaly this can increase

Table 10.12 Features of heparin-induced thrombocytopenia (HIT)

Features of HIT	Comments
Platelet count in thrombocytopenia	▪ Fall of platelets >50% (20×10^9/L) ▪ Fall of platelets <30% ($<10 \times 10^9$/L) ▪ Fall of platelets 30–50% ($10–19 \times 10^9$/L)
Time of decrease in platelet count	Platelet count is decreased within 5–10 days after start of heparin administration
Type of heparin administered	Thrombocytopenia is more common with unfractioned heparin than low molecular weight heparin
Type of patient and sex predilection	Thrombocytopenia is more common in women than in men
Thrombosis frequency in blood vessels	Venous thrombosis is more common than arterial thrombosis in cases of HIT

up to 90% of platelet mass resulting in thrombocytopenia.

- Increased splenic platelet sequestration can occur in various disorders that cause splenomegaly. The platelet count is $>30 \times 10^9$/L unless the disorder causing splenomegaly also impairs platelet production (e.g. myelofibrosis with myeloid metaplasia).
- Sequestrated platelets are released from the spleen at the time of stress. Therefore, thrombocytopenia caused by splenic sequestration rarely causes bleeding manifestations.
- In patient with normal liver function, splenectomy corrects thrombocytopenia. Splenectomy is not indicated unless severe thrombocytopenia due to simultaneous bone marrow failure is present.

HYPOPLASIA OF MEGAKARYOCYTES

Hypoplasia of megakaryocytes refers to decrease in megakaryocytes in bone marrow due to drugs, chemical agents and following viral infection, thrombocytopenia with absent radii (TAR), May-Hegglin anomaly, Wiskott Aldrich syndrome and Bernard-Soulier syndrome. Bone marrow is deficient in megakaryocytes. However, bone marrow shows normal erythropoiesis and myelopoiesis.

DISSEMINATED INTRAVASCULAR COAGULATION

Disseminated intravascular coagulation (**DIC**) is a reflection of an underlying systemic disorder, which affects the coagulation system simultaneously resulting in procoagulant activation, fibrinolytic activation, and consumption coagulopathy resulting deposition of fibrin clots in the small- and medium-sized blood vessels of organs dysfunction with fatal outcome. Widespread consumption of platelets results in thrombocytopenia. Though septicemia is the most common cause of DIC, yet several conditions (acute promyelocytic leukemia, prostatic carcinoma) may cause disseminated intravascular coagulation. Low molecular weight heparin is administered in these patients. Platelet count is low. Prothrombin time and international normalzed ratio (PT–INR) and activated partial thromboplastin time (APTT) are prolonged.

THROMBOTIC THROMBOCYTOPENIC PURPURA

Thrombotic thrombocytopenic purpura (TTP) is a rapidly fulminating blood disorder caused by deficiency of a plasma metalloprotease ADAMTS13, that cleaves von Willebrand factor when exposed to high shear stress in the microcirculation.

- The von Willebrand factor and platelets form widespread thrombi in the small blood vessels (arterioles and capillaries) throughout the body resulting in low platelet count and dysfunction of kidneys, heart and brain due to ischemia.
- Erythrocyte fragmentation and hemolysis are believed to result from mechanical injury induced by abnormally high shear stress in the microvasculature.
- Acquired thrombotic thrombocytopenic purpura is demonstrated in 90% of adults may be associated with systemic lupus erythematosus, pregnancy and infections.
- Patient presents with fever, bleeding manifestations (large bruises), pain abdomen, hematuria, weakness, shortness of breath, headache, seizures and confusion.

Laboratory Diagnosis of Thrombotic Thrombocytopenic Purpura (TTP)

- Peripheral blood smear shows schistocytes and low platelet count.
- Platelet count ranges between 5×10^9/L and 100×10^9/L.
- Reticulocyte count is increased.
- Coombs' test is negative
- Urine analysis shows hematuria and proteinuria due to flea bitten kidneys
- Blood urea and serum creatinine may be elevated.

- Treatment of TTP includes plasma exchange using fresh frozen plasma as the replacement fluid. Antiplatelet drugs (aspirin or dipyridamole), corticosteroids, splenectomy, rituximab and vincristine should be administered. Response to treatment may be monitored by analysis of hemoglobin level, reticulocyte count, platelet count, plasma bilirubin and presence of von Willebrand factor multimers in plasma.

HEMOLYTIC UREMIC SYNDROME

Hemolytic uremic syndrome is a clinical syndrome characterized by the triad of thrombotic microangiopathy, thrombocytopenia and acute renal injury. It is one of the most common causes of acute renal damage in children.

- Clinical findings in hemolytic uremic syndrome occurs as a result of thrombotic microangiopathy. Shiga toxin producing *Escherichia coli* is the most common cause of hemolytic uremic syndrome. Shiga toxin-induced vascular endothelial injury of arterioles and capillaries leads to thrombi formation in the microvasculature. The pathologic lesion is thickening of small blood vessels, vascular endothelial swelling

Table 10.13 Comparison between thrombotic thrombocytopenic purpura and hemolytic uremic syndrome

Features	Thrombotic Thrombocytopenic Purpura	Hemolytic Uremic Syndrome
Age group	20–40 years age group	Children <5 years of age
Etiology	Severe deficiency of ADAMTS13 protein due to gene mutation	Shiga toxin producing *Escherichia coli*
Thrombocytopenia	Present	Present
Microangiopathic hemolytic anemia	Present	Present
Renal injury induced manifestations	▪ Uncommon ▪ Mild renal dysfunction	▪ Common ▪ Rapidly progressive renal failure
Neurological manifestations	▪ Common manifestations ▪ Severe CNS symptoms	▪ Uncommon ▪ Mild CNS symptoms
Fever	Present	Absent
Bloody diarrhea	Absent	Present
Coagulation tests	Normal studies	Normal studies
Management	Plasma exchange	Suppurative

and detachment of arterioles and capillaries resulting in obstruction in the vascular lumen.

- Patient presents with diarrhea and rapidly progressive renal failure. In HUS, treatment for seizures, hypertension and renal failure is required. Comparison between thrombotic thrombocytopenic purpura and hemolytic uremic syndrome is given in Table 10.13.

DENGUE HEMORRHAGIC FEVER

Dengue hemorrhagic fever is caused by flavivirus transmitted by Aedes mosquito. Patient presents with fever, bleeding manifestations due to thrombocytopenia, hypotension and shock. There is impaired thrombopoiesis and increased platelet destruction.

Laboratory Diagnosis of Dengue Hemorrhagic Fever

- Peripheral blood smear examination shows many transformed lymphocytes and giant platelets.
- Platelet count ranges between 5×10^9/L and 80×10^9/L.
- Diagnosis is established by dengue hemagglutination inhibition antibody titer.

- Dengue hemorrhagic fever is treated by platelet transfusion, intravenous fluids and hydrotherapy. Immature platelet fraction (IPF), that contains RNA particles are analyzed by automatic counters.
- Immature platelet fraction is decreased in hypoplastic bone marrow. Immature platelet fraction (IPF) percentage can be used to evaluate the recovery of platelets in these patients. It can be used as a guide for platelet transfusion.

PARAPROTEINEMIA-INDUCED PLATELET DYSFUNCTION

Paraprotein synthesized in multiple myeloma and Waldenström's macroglobulinemia coat the platelets resulting in dysfunctional platelets.

UREMIA-INDUCED PLATELET DYSFUNCTION

Uremia by itself prevents platelets to perform normal functions. Platelets fail to degranulate.

DIAGNOSTIC APPROACH AND TREATMENT OF PLATELET DISORDERS

It is essential to evaluate patients with platelet disorders by taking clinical history, examination of patients, laboratory hematologic investigations and management.

- Abnormal bleeding associated with thrombocytopenia or abnormal platelet function is characterized by spontaneous skin petechiae and ecchymoses, bleeding from mucous membrane, menorrhagia, nose bleed in children. In thrombocytopenic patients, severe spontaneous bleeding is unusual with a platelet count of $\geq 20 \times 10^9$/L.
- Laboratory investigation of a suspected platelet disorder depends on the clinical presentation and history in each patient (e.g. complete blood count, peripheral blood smear examination, platelet function tests, coagulation studies and biochemical investigations).

PLATELET DISORDERS TREATMENT

All serious bleeding due to platelet disorders requires hematological assessment and treatment. Transient post-viral or aspirin-induced thrombocytopenia needs no active treatment.

INHERITED PLATELET DISORDERS TREATMENT

Newborn and small infants with bleeding should be evaluated for inherited bleeding disorders (inherited platelet disorders, hemophilia, von Willebrand disease). Congenital platelet disorders are treated by platelet transfusions (leukodepleted, HLA compatible and irradiated), DDAVP, tranexamic acid (antifibrinolytics), recombinant factor VII and bone marrow transplantation. It is worth mentioning that bone marrow transplantation can potentially cure many inherited platelet disorders.

ACQUIRED PLATELET DISORDERS TREATMENT

Bone marrow failure is treated by administration of platelet transfusions, if platelet count is $<10 \times 10^9$/L.

- Autoimmune thrombocytopenic purpura in adults is treated by prednisolone, intravenous immunoglobulin and splenectomy.
- Splenectomy is indicated in those patients not responding to therapy.
- Post-transfusion purpura is managed by administration of intravenous immunoglobulin and plasma exchange.
- In patient with heparin-induced thrombocytopenia type II, heparin should be discontinued, and another anticoagulant should be administered.
- Thrombotic thrombocytopenic purpura is treated by plasma exchange and aspirin when platelet count is $>30 \times 10^9$/L.
- Disseminated intravascular coagulation (DIC) is managed by treating underlying cause, fresh frozen plasma and platelet transfusion.
- Splenectomy is indicated in severe cases of hypersplenism causing thrombocytopenia. Platelet function disorders are treated by administration of platelet transfusion. Desmopressin acetate (DDAVP) is administered in patient with uremia.

SECONDARY THROMBOCYTOSIS AND ESSENTIAL THROMBOCYTHEMIA

In secondary thrombocytosis and essential thrombocythemia (ET), the platelet count is elevated above the upper limit of normal. The platelet count is $>450 \times 10^9$/L in secondary thrombocytosis.

- ET is blood clotting disorder in which bone marrow produces excess of platelet count is $>450 \times 10^9$/L.
- Patients with reactive thrombocytosis are normally asymptomatic. Patients with essential thrombocythemia present with excessive spontaneous bleeding.
- Antiplatelet drugs can be helpful to prevent thrombosis in high-risk patients in postoperative cases. Causes of increased platelet count are given in Table 10.14.

Table 10.14 Causes of increased platelet count

Primary/Clonal Thrombocytosis	
▪ Essential thrombocythemia	▪ POEMS syndrome
▪ Myeloproliferative disorders (e.g. chronic myelogenous leukemia, polycythemia vera, primary myelofibrosis)	▪ Familial thrombocytosis (thrombopoietin overproduction, MPL gene mutation)
▪ Myelodysplastic syndrome with isolated del 5q	▪ 5q– syndrome
Secondary/Reactive Thrombocytosis	
▪ Iron deficiency anemia	▪ Hyposplenism/splenectomy
▪ Acute hemorrhage or hemolytic anemia	▪ Drugs (gemcitabine, corticosteroids, adrenaline, thrombopoietin, following immunosuppressive therapy)
▪ Severe hemolysis	▪ 'Rebound' (correction of megaloblastic anemia, post-ethanol abuse, alcohol-induced thrombocytopenia, chemotherapeutic drugs)
▪ Traumatic injury, postoperative state	
▪ Infection, inflammation	
▪ Malignancy	
Spurious Thrombocytosis	
▪ Cryoglobulinemia	▪ Cytoplasmic fragments of malignant cells
▪ Schistocytes	▪ Bacterial infection
▪ Microspherocytes	
Transient Thrombocytosis	
▪ Vigorous exercise	▪ Epinephrine
▪ Childbirth	

SECONDARY THROMBOCYTOSIS

In secondary thrombocytosis, platelet count is elevated above the upper limit of normal. The platelet count is >450 × 10^9/L.

- Secondary thrombocytosis is caused by infection, malignant disorders, acute and chronic inflammatory diseases, pregnancy, post-splenectomy, hemorrhage and iron deficiency anemia. Patient is usually asymptomatic.
- Secondary thrombocytosis is the most common cause of thrombocytosis in adults (88–97%) and children (100%).
  - In adults, secondary thrombocytosis is attributed to chronic inflammation and malignancy in 75% of cases.
  - In children, secondary thrombocytosis is attributed to thalassemia. JAK2V617F, MPLW515L/L or CALR gene mutations are not demonstrated in secondary thrombocytosis.
- Platelets are produced by megakaryocytes in the bone marrow. Under physiologic state, about 2 × 10^9/L platelets are produced per day. Normal life span of platelets is 3–7 days.
- Thrombopoietin synthesized by liver regulates thrombopoiesis. Thrombopoietin binds to its receptor MPL on megakaryocytes and activates the signal transduction pathways that regulate megakaryocyte proliferation, maturation and platelet production.
  - Normally, thrombopoietin is mostly bound to MPL, with very low detectable free form in the serum.
  - Thrombopoietin levels are elevated in secondary thrombocytosis.
  - Besides thrombopoietin, cytokines (IL-1, IL-4, IL-6, IL-11, TNF) also participate in regulation of thrombopoiesis. C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are usually elevated in reactive thrombocytosis.

Laboratory Diagnosis of Secondary Thrombocytosis

Peripheral Blood Smear Examination

- Peripheral blood smear shows increased platelets mainly of small granular mature forms.
- Microcytic hypochromic picture suggests iron deficiency anemia.
- Neutrophils containing toxic granules are suggestive of infectious or inflammatory etiology.

Bone Marrow Trephine Biopsy Examination

- Bone marrow trephine biopsy shows normal cellularity.
- Megakaryocytes show normal morphology. These can be increased and usually without clustering.
- Reticulin stain and trichrome stain are usually normal without significant fibrosis.

ESSENTIAL THROMBOCYTHEMIA

Essential thrombocythemia is a BCR-ALBL1 negative myeloproliferative neoplasm of bone marrow. It is characterized by abnormal morphology in the megakaryocytic lineage and sustained thrombocytosis over 450 × 10^9/L (Table 10.15).

- **Molecular genetics: JAK2V617F** (59–60%), **CALR** (calreticulin in 25–30%) and **MPLW515L/K** (3–5%) gene mutations are demonstrated in essential thrombocythemia.
- **Clinical features:** Patient presents with excessive spontaneous bleeding, headache, blurred vision, dizziness, erythromelalgia (red burning, pain of distal ends of toes and fingers). Thrombosis in small- and medium-sized arteries results in cerebral stroke, myocardial infarction, occlusion of deep vein and peripheral vessels. Bleeding is less common than thrombosis. Patient presents with bleeding tendencies, if platelet count is >1000 × 10^9/L. If there is palpable splenomegaly, a raised platelet count is much more likely to be due to primary thrombocythemia than to reactive thrombocytosis. The risk of occlusive vascular lesions is high in essential thrombocythemia but very small in reactive thrombocytosis.
- Bone marrow trephine biopsy examination: The bone marrow trephine biopsy from a patient with essential thrombocythemia shows clusters of megakaryocytes. Patient with advanced idiopathic myelofibrosis shows marked reticulin staining.

Comparison between secondary thrombocytosis and essential thrombocythemia is given in Table 10.16.

Table 10.15 2024 World Health Organization diagnostic criteria for essential thrombocythemia (diagnostic criteria include either all four major criteria or the first three major criteria and one minor criterion)

Major Diagnostic Criteria
▪ Platelet count ≥400 × 10^9/L
▪ Bone marrow: Normal cellularity, normal erythropoiesis and granulopoiesis; enlarged megakaryocytes including staghorn appearance and clustering or scattered focally; and minimal increase in reticulin fibrosis (up to grade I)
▪ Not meeting the WHO criteria for BCR-ABL1 positive for chronic myelogenous leukemia, polycythemia vera, primary myelofibrosis, myelodysplastic syndrome or other myelodysplastic neoplasms
▪ Presence of JAK2V617F (59–60%), CALR (25–30%) and MPLW515L/K (3–5%) gene mutations
Minor Diagnostic Criteria
Presence of a clonal marker or absence of evidence of reactive thrombocytosis

Table 10.16 Comparison between secondary thrombocytosis and essential thrombocythemia

Clinicopathologic Features	Secondary Thrombocytosis	Essential Thrombocythemia
Platelet count increase	Transient	Persistent
History of bleeding/thrombotic episode/ vasomotor symptoms	Present in 1–2%	Present
Hepatomegaly	Absent	Present
History of infections/inflammatory stimuli or nonmyelogenous malignancy	Present	Absent
Elevated acute phase reactants (C-reactive protein) and ESR	Present	Absent
Megakaryocyte morphology in the bone marrow	Megakaryocytes with normal morphology can be increased and usually without clustering	Enlarged megakaryocytes including staghorn appearance and clustering
Clonal molecular abnormality	Absent	Usually present (JAK2V617F, MPLW515L/L or CALR gene mutations)

Laboratory Diagnosis of Essential Thrombocythemia

Peripheral Blood Smear Examination

- Peripheral blood smear shows marked thrombocytosis with anisocytosis of platelets. Mean platelet volume is usually lower than normal. Leucocytes are within normal range.
- Red blood cells are normocytic normochromic except in patients with hemorrhage or iron deficiency anemia.
- Leukoerythroblastic picture raises the possibility of myelofibrosis.

Bone Marrow Trephine Biopsy Examination

- Bone marrow trephine biopsy is essential to differentiate essential thrombocythemia from polycythemia vera, primary myelofibrosis and myeloproliferative neoplasm.
- Bone marrow trephine biopsy in essential thrombocythemia shows normal cellularity, normal erythropoiesis and granulopoiesis; enlarged megakaryocytes including staghorn appearance and clustering or scattered focally.

BLEEDING ASSOCIATED WITH VASCULAR DISORDERS

Bleeding from vascular disorders is sometimes referred to as nonthrombocytopenic purpura.

- Vascular disorders may occur due to weakening of blood vessel wall by inherited or acquired disorders.
- Most of vascular disorders are characterized by easy bruising and spontaneous petechiae and purpura of the skin and mucous membranes.
- In patients with bleeding associated with vascular disorders, the platelet count, prothrombin time and international normalized ratio (PT–INR) and activated partial thromboplastin time (APTT) are normal.
- Inherited vascular disorders are characterized by thin-walled arterioles and capillaries.
- Scurvy caused due to vitamin C deficiency results in poor collagen synthesis and failure of the endothelial cells to be cemented together properly, which causes a fragile vascular wall.
- Senile purpura is caused by impaired collagen synthesis in the aging process resulting in bruising.
- Vascular defects also occur in the course of disseminated intravascular coagulation or as a result of microthrombi and corticosteroid therapy. It is triggered by tissue injury, endothelial cell injury, or combination of both of these processes. Due to failure of the normal mechanism controlling hemostasis, tissue factor expression, factor XII activation and platelet activation and thrombin generation; intravascular coagulation occurs.
- Characteristics of inherited connective disorders causing purpura are given in Table 10.17. Bleeding (purpura) associated with vascular disorders are given in Table 10.18.

SCURVY PURPURA

Vitamin C participates in the synthesis of collagen and reticulin fibrils from mucopolysaccharide ground substance in wound healing. Deficiency of vitamin C is known as scurvy, in which there is defective formation of mesenchymal tissue and osteoid matrix.

- Patient presents with hemorrhages in mucocutaneous and muscles along fascial planes due to capillary fragility. Insufficient production of osteoid matrix results in cartilaginous overgrowth, widening of epiphysis, bowing of the long bones and chest deformity.

Table 10.17 Characteristics of inherited connective disorders causing purpura

Disorder	Gene	Chromosome Locus	Pathological Effects
Hereditary hemorrhagic telangiectasia (Osler-Rendu disease)	▪ Endoglin/ENG-1 (HHT-1) ▪ ACVRL HHT-2	▪ 9q (HHT-1) ▪ 12q (HHT-2)	Arteriovenous malformations in skin, mucous membrane, lungs, liver, brain
Ehlers-Danlos syndrome	▪ COL1A1 ▪ COL1A2 ▪ COL3A1 ▪ COL5A1 ▪ COL5A2	Multiple chromosomes	Fragile blood vessels (bruises), flexible joints
Marfan's syndrome	Fibrillin 1 (FBN-1)	Chromosome 15	Decreased strength of blood vessels, overgrowth of long bones, arachnodactyly
Osteogenesis imperfecta	▪ COLA1 ▪ COLA2	Chromosome 2	Patchy defective bone matrix, brittle bones prone to fracture
Pseudoxanthoma elasticum	ABCC6	Chromosome 16	Degeneration and calcification of elastic fibers

Table 10.18 Bleeding (purpura) associated with vascular disorders

Purpura Associated with Vasculitis
▪ Henoch-Schönlein purpura ▪ Drug-induced purpura ▪ Infections (bacteria, viruses, rickettsia) ▪ Food allergen-induced purpura
Purpura Associated with Dysparaproteinemia
▪ Paraproteins (cryoglobulinemia, cryofibrinogenemia) ▪ Amyloidosis
Purpura Resulting from Decreased Connective Tissue
▪ Scurvy purpura (vitamin C deficiency) ▪ Senile purpura ▪ Cushing syndrome ▪ Glucocorticoid therapy
Miscellaneous Causes of Purpura
▪ Simple easy bruising syndrome ▪ Fat embolism ▪ Mechanical purpura ▪ Purpura fulminans ▪ Artificially induced purpura

- Osteoporosis is seen especially at the metaphyseal ends of bone. Other findings are gingival swelling and periodontal infection, impaired wound healing, impaired localization of focal infections and anemia.

HENOCH-SCHÖNLEIN PURPURA

Henoch-Schönlein purpura is the most common type of childhood immune complex-mediated vasculitis in the age group 4–11 years. The glomerular lesion is identical with that of IgA nephropathy. The disorder may be precipitated by exogenous antigens such as drugs, foods, or upper respiratory infections. IgA deposition occurs in blood vessel of skin often similar to IgA nephropathy in >50% cases.

- Patient presents with nephritic syndrome and skin rashes on extensor surfaces of the arms, legs, and buttocks, painful joints, gastrointestinal tract involvement (colicky abdominal pain with malena).
- Most patients do well, but a few progress to end stage renal disease. In adults, a rapidly progressive (crescentic) glomerulonephritis can occur.

SENILE PURPURA

Senile purpura occurs in elderly persons. There is atrophy of collagen fibers in the dermis with advancing age. Patient presents with purpura, ecchymosis on the back of neck, extensor aspect of forearms and legs.

SIMPLE BRUISING

Simple bruising is most often demonstrated in the women on arms and legs, which resolves rapidly. Hemostatic tests are within normal range.

HEREDITARY HEMORRHAGIC TELANGIECTASIA

Hereditary hemorrhagic telangiectasia may form blood vessels without the capillaries (tiny blood vessels that pass blood from arteries to veins) that are usually between arteries and veins. The classic clinical

triad of hereditary hemorrhagic telangiectasia (HHT) disorder includes recurrent epistaxis, mucocutaneous telangiectasias, and one affected first-degree relative. Hereditary hemorrhagic telangiectasia type 1 is caused by mutations in the ENG gene. Type 2 is caused by mutations in the ACVRL1 gene. A person with diagnosis, HHT can be diagnosed by performing genetic testing.

- Patient presents with epistaxis, bright red spots on the face, lips, nose, flexor surface of forearms and conjunctiva.
- Bleeding from these malformed blood vessels in gastric mucosa may cause gastrointestinal hemorrhages resulting in iron deficiency anemia.

MARFAN'S SYNDROME

Marfan's syndrome is connective tissue disorder caused by missense mutations of the fibrillin gene 1 (FGN-1) mapped on chromosome 15, which encodes fibrillin protein essential for synthesis of elastic fibers in connective tissue. Fibrillin belongs to family of connective tissue proteins analogous to the collagen fibers. Fibrillin protein also affects another protein transforming growth factor β (TGF-β) in the body, which helps regulate how cells function throughout the body.

- **Molecular genetic alterations:** Missense mutations of fibrillin gene 1 (FGN-1) change the amount of fibrillin protein inadequate for normal cellular functions, which cause changes in the strength and performance of connective tissues of cardiovascular system (cardiac valve floppiness, aortic aneurysm, and aortic dissection), ligaments, tendons (hyperflexibility of joints), eyes (dislocation of the ocular lens in both eyes), lungs, and skin. Overgrowth of bones make them longer than usual.
- **Inheritance pattern:** In most cases, Marfan's syndrome is inherited as an autosomal dominant disorder meaning it occurs equally in men, women and children and can be inherited one normal copy of the FBN1 gene from one parent and one abnormal copy from another parent. Persons who have 50% chance of transmitting along the disorder to each of their children. Marfan's syndrome is also referred to as a 'variable expression' genetic disorder.
- **Physical appearance:** Physical features in Marfan's syndrome include: long narrow face, tall and thin body built, arms, legs, fingers, and toes seem too long for the rest of body, scoliosis, sternum caves in or sticks out, weak joints easily undergo dislocation, flat feet, highly arced roof of the mouth and crowding of teeth and loose joints.
- **Cardiovascular system manifestations:** About 90% of persons with Marfan's syndrome develop changes in their heart and blood vessels. Patient presents with life-threatening complications such as aortic aneurysm, aortic dissection, brain aneurysms, cardiac valve floppiness, mitral valve prolapse, cardiomyopathy, and cardiac arrhythmia. Tests to evaluate changes in the cardiovascular system may include: X-ray chest, magnetic resonance imaging, computed tomography scan, electrocardiogram, and echocardiogram.
- **Ocular manifestations:** More than 50% of persons with Marfan's syndrome develop nearsightedness, lens subluxation, cataracts, difference in the shape of eye, retinal detachment, and glaucoma.
- **Lung's manifestations:** The changes in the lung tissue that occur emphysema, chronic obstructive pulmonary disease, bronchitis, and pneumothorax.

EHLERS-DANLOS SYNDROME

Ehlers-Danlos syndrome (EDS) is a genetic disorder (autosomal dominant, autosomal recessive or sex-linked recessive inheritance) affecting generalized defect in collagen fibers synthesis, molecular structure, and function. Ehlers-Danlos syndrome affects virtually every organ system (skin, joint and blood vessels), which can result in significant morbidity and mortality. Complications of this disorder include arterial rupture, bleeding diathesis due to fragile skin, organ rupture, hypermobility of joints and dislocation, chronic pain, and fatigue.

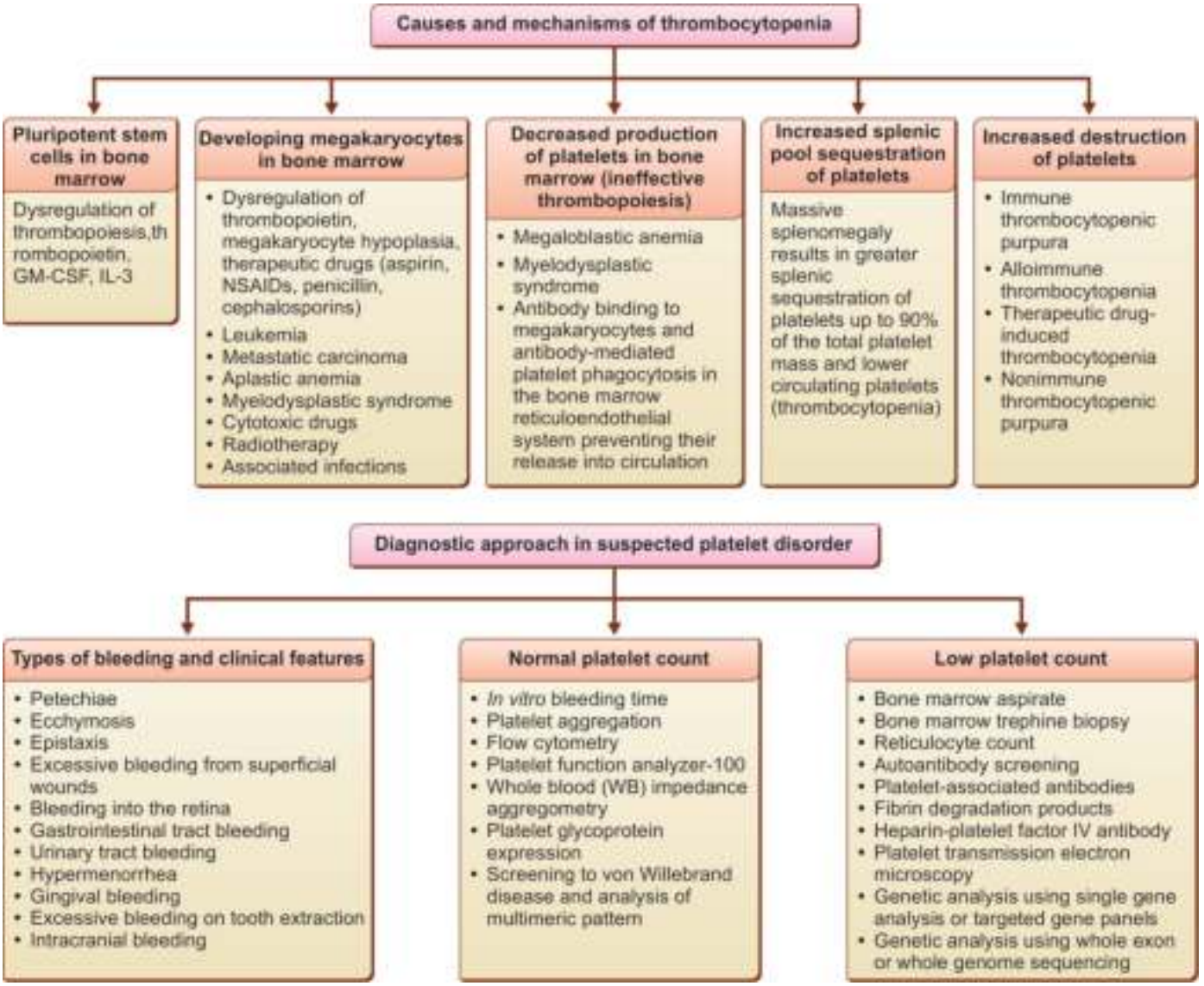
- Ehlers-Danlos syndrome (EDS) is an autosomal dominant disorder of collagen and extracellular matrix with wide array of phenotypic expressions due to *de novo* gene mutations. Most clinically significant variants of EDS include classical EDS, classical-like EDS, cardiac valvular EDS, vascular EDS, and hypermobile EDS.
  - **Classical Ehlers-Danlos syndrome:** Classical EDS is an autosomal dominant disorder associated with COL5A and COL1A1 gene mutations encoding type V and type I collagen fibers respectively. Patient presents with atrophic scarring, skin hyperflexibility, and generalized joint hypermobility.
  - **Classical-like Ehlers-Danlos syndrome:** Classical EDS is an autosomal recessive disorder associated with TNXB gene mutation encoding tenascin XB. Patient presents with skin hyperextensibility without atrophic scarring, and easy bruising.
  - **Vascular Ehlers-Danlos syndrome:** EDS is an autosomal dominant disorder associated with

COL3A1 and/or COL1A1 gene mutations encoding type III and type I collagen fibers respectively. Patient presents with arterial rupture at young age, uterine rupture during third trimester, carotid cavernous sinus fistula formation without trauma, and family history established by genetic testing. Congenital hip dislocation and spontaneous pneumothorax are also observed.

- **Hypermobile Ehlers-Danlos syndrome:** Hypermobile EDS is an autosomal dominant disorder associated with unknown gene mutations.

- **Physical examination:** The skin becomes white in color, soft to the touch, easily hyperextensible, apparent blood vessels. Fragility of dermal skin with frequent bruises and lacerations is common. Smaller, deep, and movable subcutaneous nodules in the arms and over tibia are observed and demonstrated on radiograph. The joints are hyperextensible, but the degree of involvement varies. The digital joints are most often involved, but alterations can be present in all the joints.
- **Laboratory diagnosis:** The initiation workup of Ehlers-Danlos syndrome should focus on clinical history, extent of the involvement of body systems, imaging techniques (echocardiography, CT scan, MRI), coagulation studies, and genetic testing.

Annexure



CHAPTER 11

Coagulation Disorders and Diagnostic Approach of Bleeding Diathesis

Vinay Kamal, Anubhav and Vigyat

LEARNING OBJECTIVES

BLOOD COAGULATION SYSTEM
- Synthesis of coagulation factors
- Regulation of coagulation system
- Coagulation system disorders

INHERITED COAGULATION SYSTEM DISORDERS
- Hemophilia A
- Hemophilia B (Christmas disease)
- von Willebrand disease
- Factor I (fibrinogen) deficiency
- Factor II (prothrombin) deficiency
- Factor V (labile factor) deficiency
- Factor VII (stable factor) deficiency
- Factor X (Stuart-Prower factor) deficiency
- Factor XI (plasma thromboplastin antecedent) deficiency
- Factor XIII (fibrin stabilizing factor) deficiency

ACQUIRED COAGULATION SYSTEM DISORDERS
- Disseminated intravascular coagulation
- Hemorrhagic disease of newborn
- Coagulation system disorders in hepatic disease

THROMBOPHILIA (HYPERCOAGULABLE STATE)
- Hereditary thrombophilia (hypercoagulable state)
  - Antithrombin III deficiency
  - Protein C deficiency
  - Protein S deficiency
  - Factor V Leiden (rs6025)
  - Prothrombin G20210A gene mutation
  - Hyperhomocysteinemia
- Acquired thrombophilia (hypercoagulable state)
  - Antiphospholipid syndrome

BLEEDING DIATHESIS: DIAGNOSTIC APPROACH
- Clinical history
- Physical examination
- Hematologic investigations
  - Platelet count
  - Platelet function tests
  - Bleeding time
  - Coagulation profile
    - Activated partial thromboplastin time
    - Thrombin time
    - Blood clot retraction test
    - Euglobulin lysis time
    - Fibrinogen degradation products and D-dimer tests

BLOOD COAGULATION SYSTEM

Hemostasis is regulated by procoagulant and anticoagulation factors synthesized by vascular endothelium.

- The vascular endothelium forms a barrier between platelets and plasma coagulation factors and subendothelial connective tissues (basement membrane, collagen, microfibrils, mucopolysaccharides and fibronectin).
- Vascular endothelial cells synthesize procoagulant factor (e.g. tissue factor, prostacyclin, nitric oxide, von Willebrand factor) and anticoagulation factors (e.g. antithrombin III, tissue factor pathway inhibitor, protein S, protein C, tissue plasminogen activator).
- Hemostasis is initiated by vascular endothelial cell injury resulting in exposure of collagen and tissue thromboplastin.
- Coagulation factors and enzymes are activated and get assembled on the platelet surface resulting in formation of thrombin, that converts soluble fibrinogen to insoluble fibrin strands.
- The insoluble fibrin strands create a meshwork that cements platelets and other blood components to form the blood clot.
- Abnormalities of the coagulation system occur either due to deficiency of one or more coagulation factors or inappropriate activation of coagulation factors.

SYNTHESIS OF COAGULATION FACTORS

Majority of coagulation factors are proteins synthesized in the liver and present in inactive form. Vitamin K is essential for the synthesis of coagulation factors II, VII, IX, X, prothrombin and protein C.

- Liver enzyme reductase converts inactive coagulation factors to their active form. Liver reductase enzyme is inhibited by oral anticoagulants.

Table 11.1 Coagulation factors

Coagulation Factor	Synonyms	Site of Synthesis	Inheritance	Deficiency Affects	Therapy and Half-life of Infused Factor
I	Fibrinogen	Liver	Autosomal recessive (rare)	Both sexes	Cryoprecipitate, fresh frozen plasma, fibrinogen (96–144 hours)
II	Prothrombin	Liver (vitamin K dependent)	Autosomal recessive (rare)	Both sexes	Fresh frozen plasma, factors II, VII, IX, X concentrate (50–80 hours)
III	Tissue extract (thromboplastin)	Tissue	NA	NA	NA
IV	Calcium	–	–	–	–
V	Labile factor	Liver	Autosomal recessive	Both sexes	Fresh frozen plasma (24 hours)
VI	Not assigned	NA	NA	NA	NA
VII	Stable factor	Liver (vitamin K dependent)	Autosomal recessive	Both sexes	Factors II, VII, IX, X concentrate in plasma (4–6 hours)
VIII	Antihemophilic globulin A	Liver, spleen, kidneys	Sex-linked recessive	Male affected (1 in 3000) (female career)	Factor VIII concentrates, cryoprecipitate, fresh frozen plasma (12 hours)
IX	Christmas factor (anti-hemophilic factor B)	Liver (vitamin K dependent)	Sex-linked recessive (1 in 20,000)	Male affected (female career)	Factors II, VII, IX, X concentrate (20–30 hours)
X	Stuart-Prower factor	Liver (vitamin K dependent)	Autosomal recessive (rare)	Both sexes	Factors II, VII, IX, X concentrate in plasma
XI	Plasma thromboplastin antecedent (anti-hemophilic factor C)	Unknown	Autosomal dominant (rare)	Both sexes	Plasma (40–84 hours)
XII	Hageman factor	Unknown	Autosomal recessive (rare)	Both sexes	Treatment not required
XIII	Fibrin stabilizing factor	Unknown	Autosomal recessive (rare)	Both sexes	Plasma (150 hours)
–	von Willebrand factor (factor vWF antigen assay)	Endothelial cells	Autosomal dominant	Both sexes	DDAVP, cryoprecipitate, fresh frozen plasma, whole blood (24 hours)

Prekallikrein: Fletcher factor, Kininogen (high MW): Fitzerald factor, NA: Not applicable

- On the other hand, liver enzyme epoxide has opposite function by converting active coagulation factors into their inactive form. Vitamin K deficiency and hepatocellular failure lead to deficient synthesis of prothrombin resulting in bleeding manifestations.
- Each of the coagulation factors performs specific function in the coagulation system cascade. Majority of the inactive procoagulant factors are present in the blood at all times, the multistep process ensures inhibition of intravascular coagulation (Table 11.1).

REGULATION OF COAGULATION SYSTEM

Each step of coagulation system is regulated at two levels: (a) activation of the zymogen to an active enzyme; and (b) the presence of exquisite cofactor. Since some cofactors such as thromboplastin (tissue factor) and thrombomodulin are integral membrane proteins, so their functions are limited to the cells and tissues, that express these proteins.

- Presence of active enzyme and cofactor accelerate the activity of a coagulation factors as much as 1000-fold. In the absence of the cofactor, the enzyme has limited activity. Inactive precursor coagulation factor contains glutamyl residue. Inactive coagulation factor becomes active/functional coagulation factor by the addition of carboxyl from HCO_3 with the help of carboxylase enzyme. Calcium then binds to glutamyl residue.
- Binding of calcium to vitamin K-dependent coagulation factors is essential for normal coagulation system cascade at every step except first two steps. Calcium, factor X, factor V, platelet phospholipids combine to form **prothrombin activator**, which then converts prothrombin to thrombin. This interaction

causes conversion of fibrinogen into fibrin strands stabilized by fibrin stabilizing factor (factor XIII) that creates the insoluble blood clot, with platelets and red blood cells resulting in stoppage of bleeding. Without calcium, the coagulation factors fail to bind to platelet phospholipids resulting in reduction of rate of activation of coagulation system cascade.

- The extrinsic pathway of coagulation is initiated by a cellular lipoprotein known as tissue factor on the cell surface. Coagulation system is initiated by intrinsic pathway by activation of circulating factor XII.
- Both extrinisic and intrinsic pathways of coagulation system lead to the activation of factor X. When plasma comes in contact with tissue

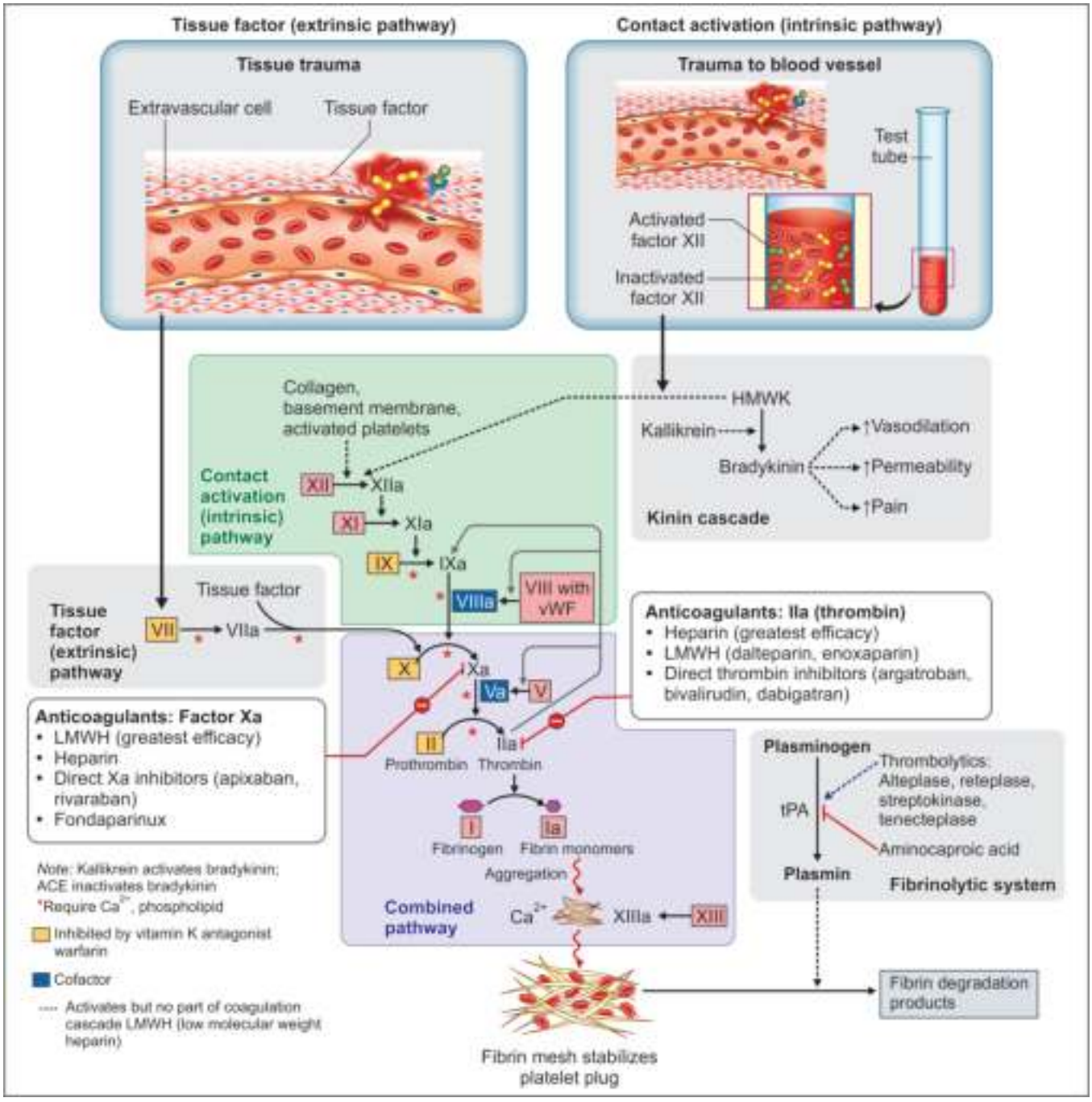


Fig. 11.1: Intrinsic, extrinsic and common pathways coagulation system; fibrinolysis and coagulation inhibition. Secondary hemostasis involves activation of the coagulation system and coagulation factors to produce cross-linked fibrinogen (fibrin) to stabilize the platelet plug. Fibrinolysis represents the process by which clot generated by coagulation system is degraded by plasmin to permit tissue repair. Anticoagulants are administered in clinical practice to prevent intravascular coagulation in various pathological disorders.

factor (TF), factor VII binds to the tissue factor resulting in formation of tissue factor/VIIa complex, which activates factors X and IX. Tissue factor pathway inhibitor (TFPI) is an important inhibitor of TF/VIIa complex. The VIIIa–IXa complex greatly amplifies Xa generated from factor X. The generation of thrombin from prothrombin by the action of Xa–Va complex leads to fibrin formation (Fig. 11.1).

- Thrombin is a central regulator of hemostasis and thrombosis, which has both procoagulant and anticoagulant properties. Thrombin promotes blood coagulation system by activation of factors V and X; conversion of fibrinogen into fibrin and platelet activation and platelet aggregation. Thrombin has anticoagulant properties, which activates protein C via thrombomodulin. Thrombin also induces thrombin activatable fibrinolysis inhibitor (TAFI), which protects the fibrin clot against lysis.
- Cell-based model of coagulation in which coagulation is regulated by properties of cell surfaces in three overlapping stages of initiation, amplification, and propagation, rather than the traditional cascade. Thrombin is important in each step. Cell-based model of coagulation system cascade is shown in Fig. 11.2A to C. Thrombin is a central regulator of hemostasis and thrombosis as shown in Fig. 11.3.

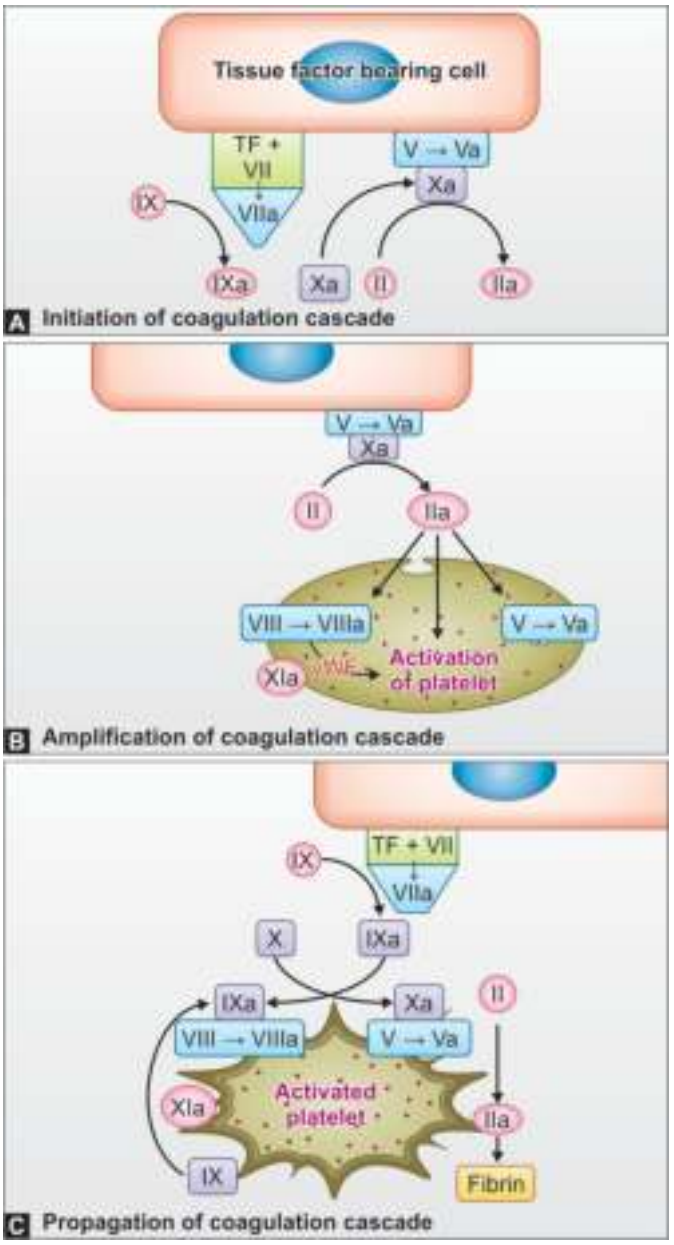


Fig. 11.2A to C: Cell-based model of coagulation system cascade is regulated by properties of cell surfaces in three overlapping stages of initiation, amplification and propagation rather than the traditional coagulation cascade. Thrombin is important in each step.

COAGULATION SYSTEM DISORDERS

INHERITED COAGULATION SYSTEM DISORDERS

Inherited coagulation system disorders occur due to deficient or reduced level of coagulation factors. Clinical manifestations vary according to the type of clotting factors. Common inherited coagulation disorders include von Willebrand disease (most common), hemophilia A (factor VIII deficiency), Christmas disease (factor IX deficiency). Rare inherited coagulation disorders include deficiency of clotting factors I, II, V, VII, X, XI and XIII.

ACQUIRED COAGULATION SYSTEM DISORDERS

Major causes of acquired coagulation system disorders include vitamin K deficiency, liver disease, disseminated intravascular coagulation and development of circulating anticoagulants. Severe liver diseases (cirrhosis, fulminant hepatitis, acute fatty liver of pregnancy) may disturb hemostasis by impaired synthesis of coagulation factors.

- Because, all the coagulation factors are synthesized by liver and vascular endothelium; thus, both prothrombin time and international normalized ratio (PT–INR) and activated partial thromboplastin time (APTT) are prolonged.
- Occasionally, decompensated liver disease also causes excessive fibrinolysis and bleeding manifestations due to decreased hepatic synthesis of antiplasmin.
- Disseminated intravascular coagulation (DIC) and hemorrhagic disease of newborn are acquired important causes of bleeding diathesis.
- Causes of inherited and acquired abnormalities of hemostasis are given in Table 11.2A to C.

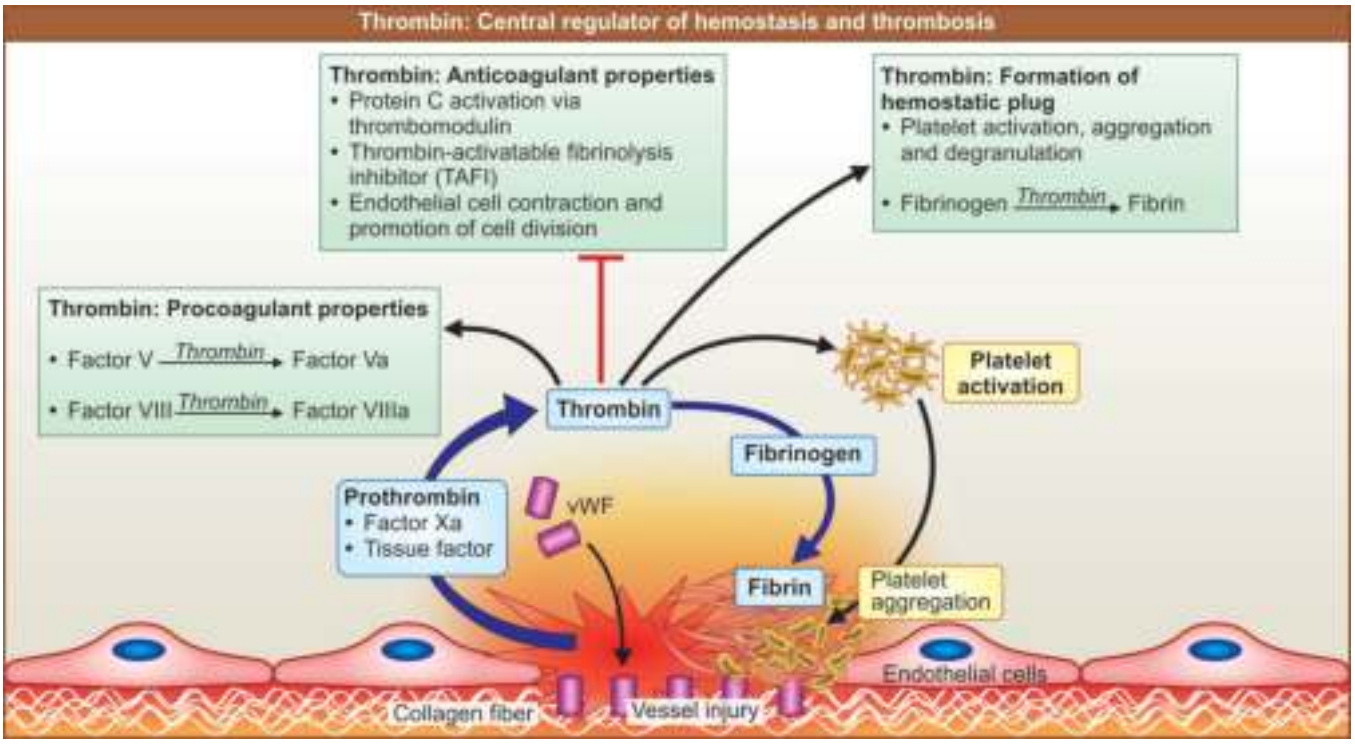


Fig. 11.3: Thrombin is a central regulator of hemostasis and thrombosis. Thrombin has both procoagulant and anticoagulant properties. Procoagulant properties include activation of factors V and VIII, conversion of fibrinogen to fibrin and activation and aggregation of platelets. Anticoagulant properties include activation of protein C via thrombomodulin and activation of TAFI. Endothelial effects of thrombin also include contraction of endothelial cells and acquisition of prothrombotic properties and promotion of cell division.

Table 11.2 Causes of inherited and acquired abnormalities of hemostasis

Inherited Abnormalities of Hemostasis	
Hemophilia A	▪ X-linked disorder ▪ Decreased or depletion of coagulation factor VIII deficiency ▪ Activated partial thromboplastin time (APTT) prolonged ▪ Severity and risk of bleeding depend on severity of deficiency of coagulation factor VIII ▪ About 5–20% of patients develop inhibitors to coagulation factor VIII due to treatment
Hemophilia B	▪ X-linked recessive disorder ▪ Decreased or depletion of coagulation factor IX deficiency ▪ Activated partial thromboplastin time (APTT) prolonged ▪ About 1–3% of patients develop inhibitors to coagulation factor IX due to treatment
Hemophilia C	▪ Decreased or depletion of coagulation factor XI deficiency ▪ Autosomal recessive disorder (heterozygotes, homozygotes) ▪ Common in Ashkenazi Jews
Afibrinogenemia/hypofibrinogenemia	▪ Patient has fibrinogen level 50% of normal level—heterozygous gene mutation with mild bleeding ▪ Prothrombin time and international normalized ratio (PT–INR) prolonged ▪ Activated partial thromboplastin time (APTT) prolonged ▪ Thrombin time prolonged
Dysfibrinogenemia—structural abnormality	▪ Structural abnormality of fibrinogen—usually heterozygous ▪ About 50% of patients have no clinical bleeding, 25% of cases with clinical bleeding; and 25% of cases develop thrombosis ▪ Prothrombin time and international normalized ratio (PT–INR) prolonged ▪ Activated partial thromboplastin time (APTT) prolonged ▪ Thrombin time (TT) prolonged ▪ Fibrinogen levels variable
von Willebrand disease (some cases)	Decreased vWF leads to decreased stability and function of factor VIII

Contd...

Table 11.2 Causes of inherited and acquired abnormalities of hemostasis (*Contd...*)

Acquired Abnormalities of Hemostasis	
Vitamin K deficiency	Impaired synthesis of coagulation factors II, VII, IX and X; and natural anticoagulant protein C and protein S
Liver disease	Impaired synthesis of coagulation factors I, II, V, VIII, IX, X, XI; and natural anticoagulant protein C, protein S and antithrombin III
Disseminated intravascular coagulation (DIC)	Multiple coagulation factor deficiencies especially fibrinogen depletion; as well as thrombocytopenia
Nephrotic syndrome	Loss of antithrombin III and antiplasmin in urine along with massive proteinuria
Multiple myeloma and amyloidosis	Consumption of coagulation factor X
Direct oral administration of anticoagulants for therapeutic purpose	Oral anticoagulants antagonize thrombin

INHERITED COAGULATION SYSTEM DISORDERS

Inherited coagulation system disorders occur due to deficient or reduced level of coagulation factors. Clinical manifestations vary according to the type of clotting factors. Common inherited coagulation system disorders include von Willebrand disease (most common), hemophilia A (factor VIII deficiency), Christmas disease (factor IX deficiency). Rare inherited coagulation disorders include deficiency or reduced levels of clotting factors I, II, V, VII, X, X1 and XIII.

HEMOPHILIA A

Normal factor VIII is essential for blood clotting. Gene encoding factor VIII is located on tip of the long arm of the X chromosome. Hemophilia A is an X-linked recessive disorder of blood clotting, which results from mutation in the gene encoding factor VIII. Patient presents with spontaneous bleeding into joints, muscles, and internal organs.

MODE OF INHERITANCE

As males have only one X chromosome, hence, defective VIII gene leads to decreased synthesis of factor VIII resulting in hemophilia.

- Heterozygous females are carriers, which transmit hemophilia A disease to the male progeny, which is clinically indistinguishable from hemophilia B (factor IX deficiency). The family pedigree of Queen Victoria shows a number of hemophilic descendents as she has been carrier of the disease. In females, the other normal X chromosome corrects the abnormality, but females can be asymptomatic carriers of hemophilia A.
- In males, hemophilia A disease is expressed because there is no normal X chromosome to correct the abnormality. Family trees in a case of hemophilia A are shown in Figs 11.4 and 11.5.

FACTOR VIII (ANTIHEMOPHILIC GLOBULIN)

Factor VIII gene encoding factor VIII glycoprotein is located on X chromosome, which comprises A1, A2, B, A3, C1 and C2 domains. First 19 amino acids of protein sequence comprise secretory leader peptide of precursor factor VIII. Factor B domain of factor VIII is cleaved off during its activation. Most of the 23 cysteine residues of mature factor VIII are located in A and C domains.

POSSIBLE SYNTHESIS SITES OF FACTOR VIII

Possible sites of synthesis of factor VIII are liver, spleen, bone marrow and kidney. Factor VIII concentration in plasma is 0.1 mg/dl with half-life of 5–12 hours.

- On starch electrophoresis, factor VIII migrates as α-globulin. Factor VIII solvents of high ionic strength such as NaCl and $CaCl_2$ form high MW fractions (VIIIAg and vWF) and low MW (VIIIc).
- Normal plasma adsorbed with barium salts removes factor IX and not VIII, which will correct activated partial thromboplastin time (APTT). Serum contains factor IX but not VIII.
- Comparison of subunits of factor VIII (VIIIc and vWF) is given in Table 11.3.

SEVERITY OF HEMOPHILIA A

Depending on the concentration of factor VIII in comparison to its normal activity. Hemophilia A cases are categorized as severe deficiency of factor VIII

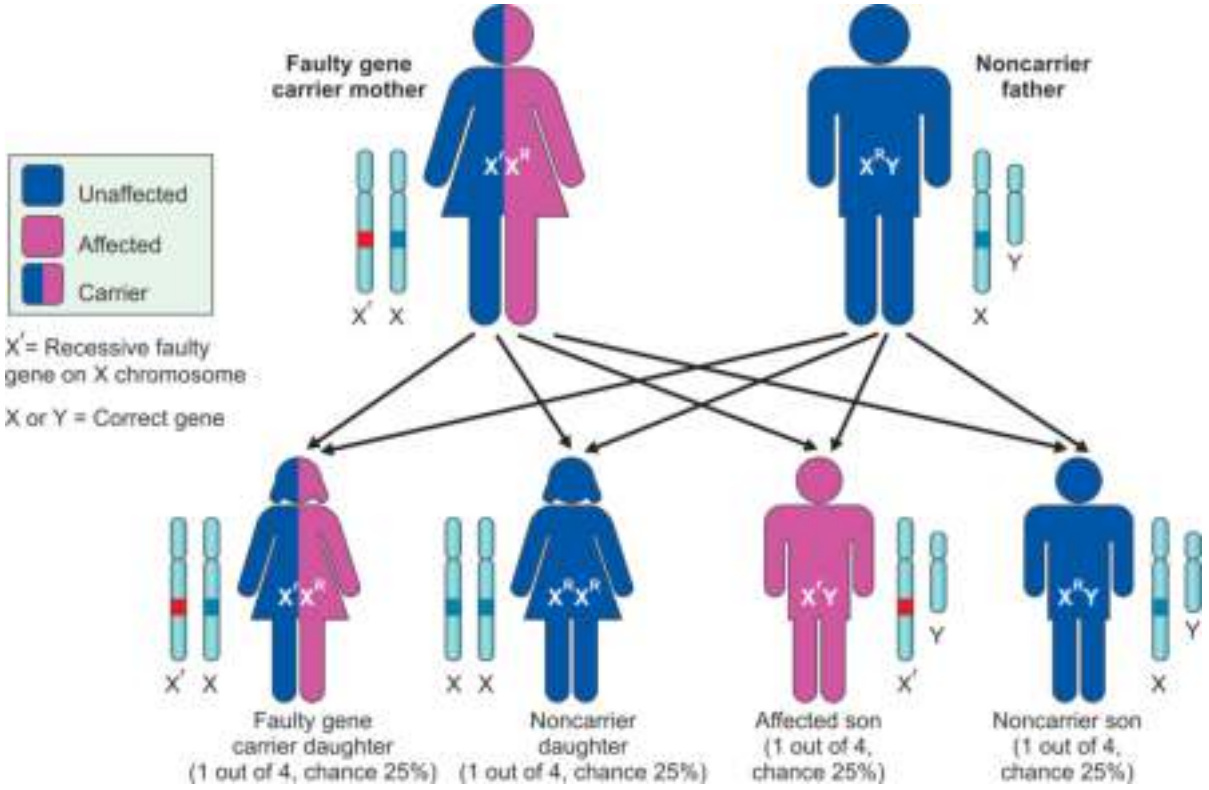


Fig. 11.4: Family tree in a case of hemophilia A, where mother is faulty gene carrier and noncarrier father. Chances of hemophilia in their children are shown.

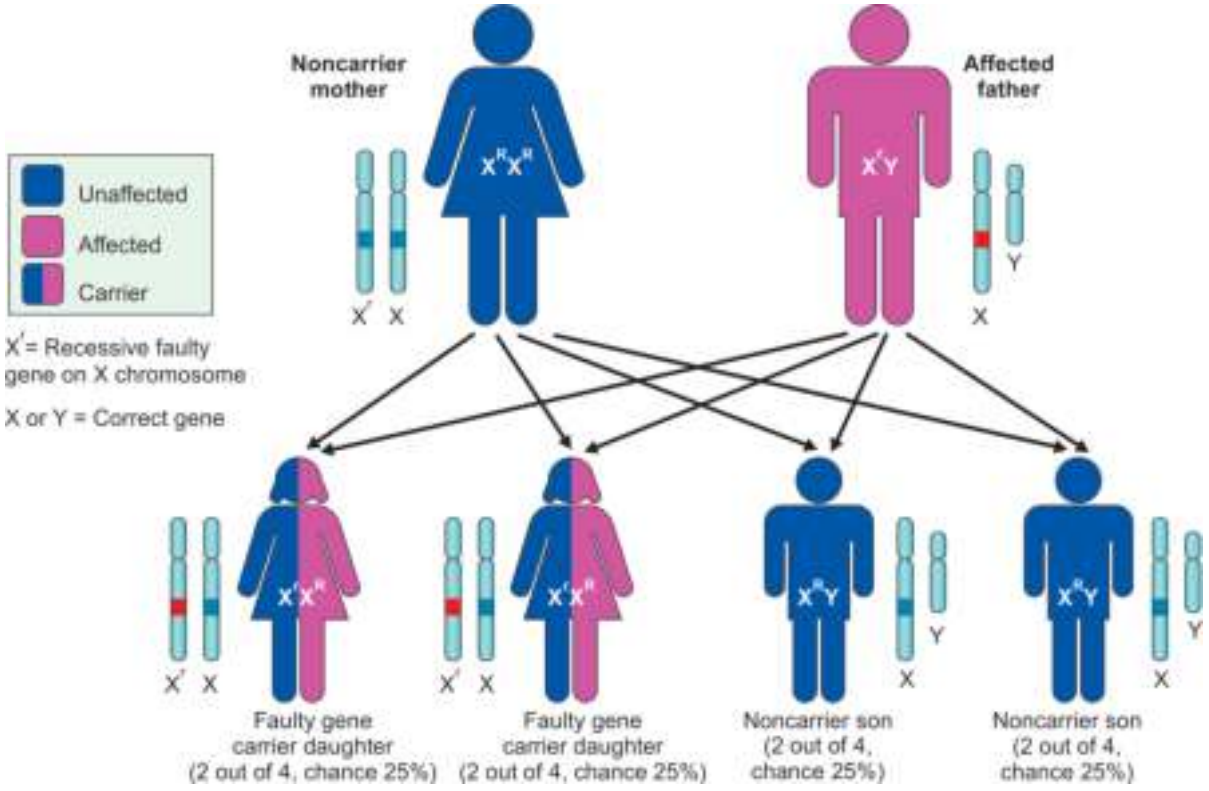


Fig. 11.5: Family tree in a case of hemophilia A, where mother is noncarrier and father with faulty gene carrier. Chances of hemophilia in their children are shown.

Table 11.3 Comparison of subunits of factor VIII (VIIIc and vWF)

Parameters	Factor VIIIc	von Willebrand Factor (vWF)
Possible sites of synthesis	Liver (most common site), spleen, bone marrow and kidney	Endothelial cells and megakaryocytes
Gene control	X-linked gene	Chromosome 12
Function	Participates in intrinsic coagulation pathway resulting in formation of hemostatic plug	Platelet adhesion (aggregation by ristocetin *in vitro*)
Deficiency	Hemophilia	von Willebrand disease

Table 11.4 Clinical manifestations in deficiency of factor VIII (hemophilia) and factor IX (Christmas disease)

Features	Severe Deficiency of Factors VIII and IX	Moderate Deficiency of Factors VIII and IX	Mild Deficiency of Factors VIII and IX
Factor VIII or IX level, units/dl	▪ <1 unit/dl	▪ 1–5 unit/dl	▪ 6–30 unit/dl
Frequency	▪ 15%	▪ 15%	▪ 70%
Detection of disorder	▪ Newborn	▪ Children	▪ Adults
Clinical features	▪ Frequent spontaneous hemarthrosis with crippling disease ▪ Frequent severe, spontaneous hemorrhage in the intracranial and intramuscular regions	▪ Severe bleeding from major/minor surgery or trauma ▪ Bleeding after circumcision site ▪ Infrequent spontaneous joint and tissue bleeding	▪ Rare spontaneous bleeding ▪ Excessive bleeding after surgery or trauma ▪ Disorder might not be discovered until bleeding episode occurs

level (<1%), moderate deficiency of factor VIII level (1–5%) and mild deficiency of factor VIII level (6–25%). Clinical manifestations in deficiency of factor VIII (hemophilia) and factor IX (Christmas disease) are given in Table 11.4.

CLINICAL FEATURES

Patient presents with hemorrhage from minor wounds and trauma, bleeding from oral mucosa, hematuria, and bleeding into joints. Recurrent hemarthroses can lead to progressive crippling deformities. Clinical manifestations of hemophilia A are shown in Fig. 11.6.

Hemarthrosis

Hemarthrosis is the most common event, which can be spontaneous or with trauma. Joints involved in hemophilia include knee, elbow, ankle, wrist, shoulder, hip and temporomandibular joints.

- Repeated bouts of bleeding lead to the joint destruction resulting in deformities in weight-bearing joints.
- Joints are swollen, tender and painful. Approximately 400 units of factor VIII are administered by intravenous route.

Soft Tissue Bleeding

Soft tissue bleeding is the second most bleeding manifestation occurring between fascial planes such as retropharyngeal region, oral cavity and between two heads of gastrocnemius muscles. To avoid pain, the patient hyperextends the ankle to shorten the gastrocnemius muscles.

- If these hemophilia A patients are not treated, gastrocnemius muscle deformities become chronic and the patient walks on the toes of the affected legs.
- The classic equine gait is often the first orthopedics event due to bleeding into gastrocnemius muscle. Approximately 300–400 units of factor VIII are administered by intravenous route.

Genitourinary System Bleeding

Nontraumatic spontaneous genitourinary bleeding without a structural lesion is encountered in hemophilics. Epsilon aminocaproic acid (**EACA**) is usually administered in hemophilia A patients to prevent lysis of blood clots. But this drug is contraindicated in patients with genitourinary bleeding for the fear of causing obstructive uropathy from blood clots.

Retroperitoneal Hemorrhage

Retroperitoneal hemorrhage should always be considered in hemophilia A patients, who presents with hypotension, tachycardia and mild abdominal pain. Surgical exploration is not indicated.

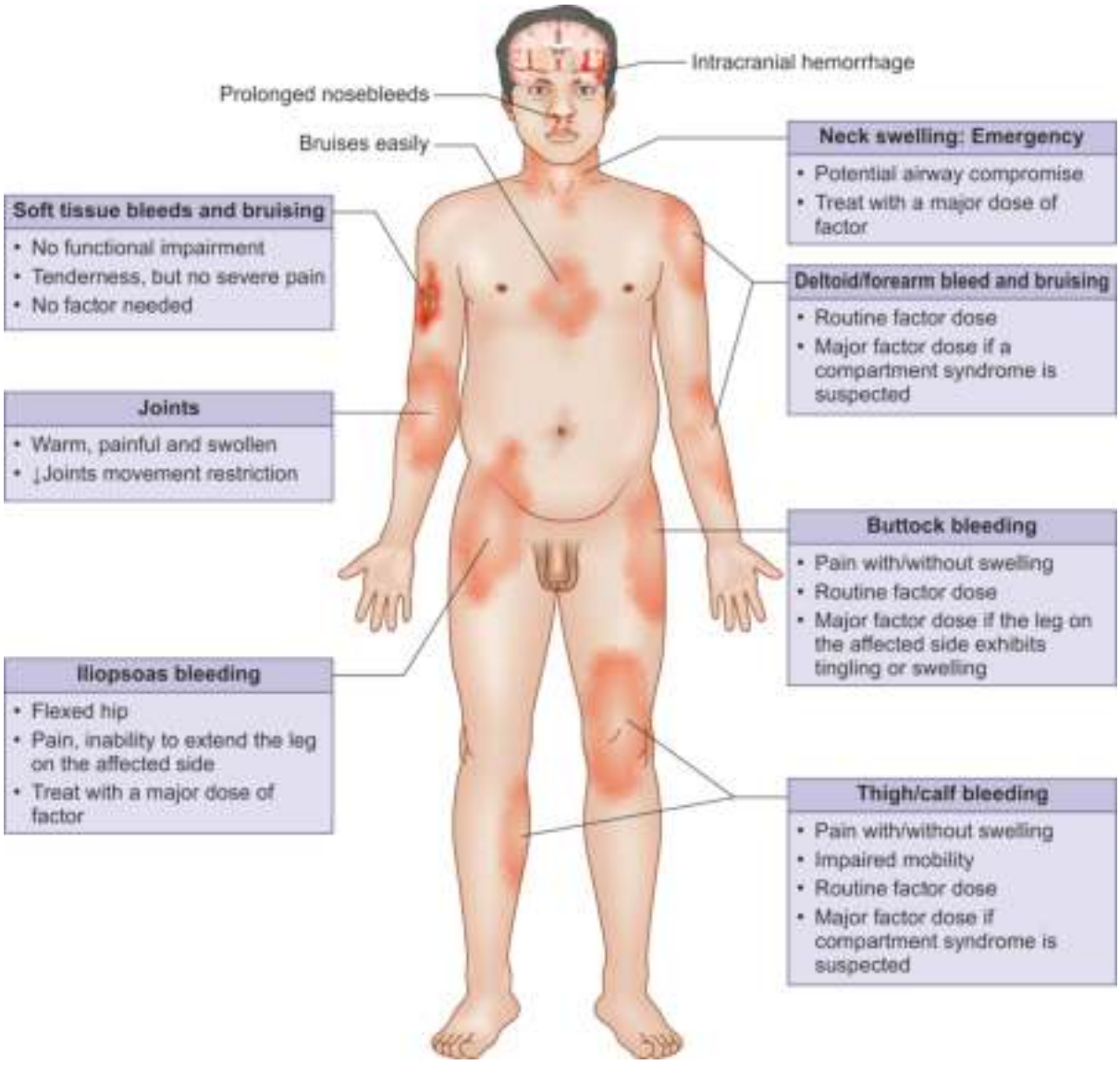


Fig. 11.6: Clinical manifestations of hemophilia A.

Gastrointestinal Tract Bleeding

Gastrointestinal tract bleeding in hemophilia A patients is associated with a structural lesion in contrast to genitourinary bleeding. Traumatic bleeding occurs following tonsillectomy and tooth extraction. Approximately 300–400 units of factor VIII are administered by intravenous route to stop bleeding in these patients.

Delayed Wound Healing

Delayed wound healing occurs due to intermittent bleeding and infection in hemophilia A patients.

Bleeding-related to Dental Procedures

In normal persons, bleeding may persist for 12 hours after dental procedures. But bleeding persists for 3 days in hemophilic patients. Factor VIII should be administered to control bleeding.

Laboratory Diagnosis of Hemophilia A
Bleeding Time and Prothrombin Time
▪ Bleeding time and prothrombin time and international normalized ratio (**PT–INR**) are within normal range. ▪ Platelet count and functions are normal.
Clotting Time
Clotting time is prolonged.
Activated Partial Thromboplastin Time
▪ Activated partial thromboplastin time (**APTT**) is prolonged. Mixing of a patient's blood with that of a normal donor normalizes the APTT. Normal APTT is 30–35 seconds.

- APTT measures intrinsic pathway of coagulation system cascade, coagulation factors XI, IX, VIII, X, V, prothrombin and fibrinogen. Phospholipid is added to plasma and clotting is observed. Phospholipid is a part of thromboplastin. Therefore, test is known as activated partial thromboplastin time.

Factor VIII Assay

Factor VIII assay is analyzed to assess severity of hemophilia A disease.

DIFFERENTIAL DIAGNOSIS

Hemophilia A should be differentiated from hemophilia B (factor IX deficiency) and severe von Willebrand disease. Comparison between hemophilia A and von Willebrand disease is given in Table 11.5.

DETECTION OF CARRIER

Most of the carrier women of hemophilia A are asymptomatic. Only a few females with very low factor VIII levels may present with menorrhagia. Accurate family tree study is carried out in hemophilia A patients.

- Coagulation profile should be estimated on three occasions by analyzing factors VIII, IX and VWAg to diagnose hemophilia in 75% of cases.
- Exact detection of hemophilia carriers is carried out by DNA analysis. Heterozygous women carriers are diagnosed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or Southern blotting to detect intron 12 inversion.

TREATMENT

Approximately 6–12% hemophilia A patients develop antibodies against exogenous administered fresh frozen plasma (factor VIII).

- Factor VIII inhibitors develop in patients with no detectable factor in their plasma. Therefore, recombinant factor VIII is being administered in hemophilia A patients by intravenous route, which has a half-life of 8–12 hours.
- Fresh frozen plasma is prepared from fresh blood <12 hours old. It is stored at –20°C for one year. It is administered in the dose of 7–15 ml/kg body weight for 30–60 minutes.
- Plasma products/recombinant factor VIII concentrates used in hemophilia are given in Table 11.6.

DDAVP Therapy

DDAVP (D-amino-delta-D-arginine vasopressin) hormone preparation is administered to enhance activity of factor VIII in mild hemophilia A patients.

Epsilon Aminocaproic Acid (EACA)

Epsilon aminocaproic acid is usually administered in hemophilia A patients to prevent lysis of blood clots in patients undergoing dental procedures.

Table 11.5 Comparison between hemophilia A and von Willebrand disease

Features	Hemophilia A	von Willebrand Disease
Sex predilection	Males	Females > males
Skin and mucous membrane bleeding	Rare bleeding	Common, persistent and profuse bleeding
Superficial ecchymoses	Common; large and solitary	Small and multiple
Deep hematoma	Common	Rare
Hemarthroses	Common	Rare
Delayed bleeding	Common	Rare
Gastrointestinal bleeding	Present with GIT lesions	Present
Hematuria	Present	Present
Epistaxis	Present	Present
Gingival bleeding	Present	Present
Excessive bleeding after tooth extraction	Present	Present
Intracranial bleeding	Present	Present
Bleeding time	Normal	Increased
Clotting time	Increased	Increased
Prothrombin time	Normal	Normal
APTT	Increased	Increased
Factor VIII assay	Decreased	Normal or decreased
vWF ristocetin cofactor assay	Normal	Decreased

Table 11.6 Plasma products/recombinant factor VIII concentrates used in hemophilia

Plasma Products/Recombinant Factor VIII Concentrates	Indications for Use
Human factor VIII	Hemophilia A, von Willebrand disease
Human factor VIII (high purity)	Hemophilia A especially with HIV infection
Recombinant factor VIII	Hemophilia A
Porcine factor VIII	Factor VIII inhibitors
Activated prothrombin complex concentrates	Factor VIII inhibitors

HEMOPHILIA B (CHRISTMAS DISEASE)

Hemophilia B is also known as Christmas disease. Hemophilia B is an X-linked recessive disorder caused by mutations in the F9 gene encoding factor IX, which affects 1 in 40,000 males and accounts for 15% of hemophilia cases. One-third of all cases represents new gene mutations. Hemophilia B (factor IX deficiency) is clinically indistinguishable from hemophilia A (factor VIII deficiency).

CLINICAL FEATURES

Patient presents with mild clinical manifestations such as hemarthrosis during 5–15 years of age.

Laboratory Diagnosis of Hemophilia B

- In both forms of hemophilia A and B, the activated partial thromboplastin time (APTT) is prolonged. Mixing of a patient's blood with that of a normal donor normalizes the APTT.
- Prothrombin time and international normalized ratio (PT–INR) is within normal range.
- Activated plasma thromboplastin time (APTT) is increased (normal 30–40 seconds).
- Thromboplastin generation time is performed to differentiate factor VIII deficiency (hemophilia A) from factor IX deficiency (Christmas disease).

TREATMENT

Patients with factor IX deficiency (Christmas disease) are treated with recombinant factor IX (Benefix), prothrombin complex concentrates (e.g. Konya, Proplex), factor IX concentrates (ergonovine) and gene therapy (under research phase). Differentiating features of hemophilia A, hemophilia B and hemophilia C are given in Table 11.7.

VON WILLEBRAND DISEASE

The von Willebrand disease is a common bleeding disorder. Most cases present with mild mucocutaneous bleeding. It occurs due to a reduction or structural

Table 11.7 Differentiating features of hemophilia A, hemophilia B and hemophilia C

Features	Hemophilia A (Factor VIII Deficiency)	Hemophilia B (Factor IX Deficiency)	Hemophilia C (Factor XI Deficiency)
Mode of inheritance	X-linked disorder	X-linked disorder	Autosomal recessive
Frequency	1:5000	1:30.000	▪ Common in Ashkenazi Jews ▪ 1:8 Heterozygotes ▪ 1:90 Homozygotes
Classification of hemophilia according to factor concentration	▪ Severe deficiency of factor VIII <1% of normal ▪ Moderate deficiency of factor VIII ≥1% and <5% of normal ▪ Mild deficiency of factor VIII >5% of normal	▪ Severe deficiency of factor IX <1% of normal ▪ Moderate deficiency (factor IX ≥1% and <5% of normal) ▪ Mild deficiency of factor IX >5% of normal	▪ Severe deficiency of factor XI <20 IU/L ▪ Not application ▪ Mild deficiency of factor XI ≥20 IU/L
Clinical features	▪ Hemarthrosis ▪ Intramuscular hematoma ▪ Genitourinary bleeding ▪ Retroperitoneal hemorrhage ▪ Equine gait ▪ Delayed wound healing	▪ Hemarthrosis ▪ Intramuscular hematoma	Mucocutaneous bleeding in mouth, nose and genitourinary regions (areas of fibrinolysis)

Contd...

Table 11.7 Differentiating features of hemophilia A, hemophilia B and hemophilia C (*Contd...*)

Features	Hemophilia A (Factor VIII Deficiency)	Hemophilia B (Factor IX Deficiency)	Hemophilia C (Factor XI Deficiency)
Laboratory findings	▪ Prothrombin time: Normal ▪ APTT: Increased ▪ Platelet aggregation with ristocetin: Normal	▪ Prothrombin time: Normal ▪ APTT: Increased ▪ Platelet aggregation with ristocetin: Normal	▪ Prothrombin time: Normal ▪ APTT: Increased ▪ Platelet aggregation with ristocetin: Normal
Treatment	▪ Plasma derived factor VIII concentrates ▪ Recombinant factor VIII concentrates ▪ Gene therapy under clinical trials	▪ Plasma derived factor IX concentrates ▪ Recombinant factor IX concentrates ▪ Gene therapy under clinical trials	▪ Plasma derived factor XI concentrates ▪ Recombinant factor XI concentrates ▪ Antifibrinolytic agents
Incidence of development of inhibitors against coagulation factor	33% of patients with severe deficiency of factor VIII	3% of patients with severe deficiency of factor IX	33% of patients with null gene mutations (Glu117 stop gene mutation)

abnormality of large multimeric glycoprotein von Willebrand factor.

- Monomers of von Willebrand factor (vWF) glycoprotein undergo N-glycosylation to form dimers, which get arranged to give multimers. Binding of von Willebrand factor with factor VIII is the main function of von Willebrand factor.
- The von Willebrand disease occurs inherited and acquired forms.
- Inherited von Willebrand disease is of three types: type 1 (most common about 70%), type 2 (2A, 2B, 2M, 2N), and type 3 (most severe disease). Type 2 has similar symptoms of hemophilia A and B.
- The pathophysiology of each type of inherited of von Willebrand disease depends on the qualitative or quantitative defects in vWF antigen, vWF activity and factor VIII coagulant activity. The von Willebrand disease, subtypes and characteristics are given in Table 11.8.

Table 11.8 von Willebrand disease, subtypes and characteristics

Features	vWD Type 1	vWD Type 3	vWD Type 2A	vWD Type 2B	vWD Type 2M	vWD Type 2N
Inheritance						
Mode of inheritance	Autosomal dominant	Homozygous or heterozygous	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal recessive
Incidence of von Willebrand disease, subtypes and functional abnormalities						
Frequency %	70–80%	Very rare	10–15%	<5%	Rare	Rare
Functional abnormalities	Partial quantitative deficiency with normal function of the multimers	Qualitative absence of vWF in platelets and plasma	▪ Qualitative disorder with functionally abnormal vWF (multimer assembly defect) ▪ Decreased platelet adhesion; absence of largest multimers	▪ Qualitative disorder with functionally abnormal vWF (multimer assembly defect) ▪ Increased affinity for platelets GpIb or absence of largest multimers	▪ Qualitative disorder with functionally abnormal vWF (multimer assembly defect) ▪ Decreased platelet adhesion not due to absence of largest multimers	▪ Qualitative disorder with functionally abnormal vWF (multimer assembly defect) ▪ Decreased affinity for factor VIII
Clinical manifestations						
Clinical history	Mild to moderate bleeding	Severe bleeding	Mild to moderate bleeding	Mild to moderate bleeding	Mild to moderate bleeding	Mild to moderate bleeding

Contd...

Table 11.8 von Willebrand disease, subtypes and characteristics (*Contd...*)

Features	vWD Type 1	vWD Type 3	vWD Type 2A	vWD Type 2B	vWD Type 2M	vWD Type 2N
Screening tests						
Platelet count	Normal	Normal	Normal	Decreased	Normal	Normal
Bleeding time	Normal or increased	Increased	Increased	Increased	Increased	Normal
Diagnostic tests						
vWF: Antigen assay	Decreased	Absent	Normal or decreased	Normal or decreased	Normal or decreased	Normal
Factor VIII assay	Normal or decreased	Severely decreased	Normal or decreased	Normal or decreased	Normal or decreased	Decreased
vWF: Ristocetin cofactor activity	Decreased	Absent	Decreased relative to vWF: antigen	Normal or decreased	Decreased relative to vWF: antigen	Normal
Tests to determine von Willebrand disease						
RIPA (ristocetin induced platelet agglutination)	Normal or decreased	Absent	Decreased relative to vWF: antigen	Increased	Decreased relative to vWF: antigen	Normal
Multimer analysis	Normal multimers	Absence of multimers	Absence of large and intermediate multimers	Absence of large multimers	Normal multimers	Normal multimers

VON WILLEBRAND FACTOR AND FACTOR VIII SYNTHESIS

The von Willebrand factor (vWF) is stored in Weibel-Palade bodies of vascular endothelial cells. The vWF is secreted from activated vascular endothelial cells into the subendothelial space. vWF is also synthesized by α-granules of platelets. von Willebrand factor (vWF) is involved in hemostasis.

- After vascular endothelial injury, vWF binds to platelet glycoprotein (Gp) receptors, GpIb-α and promotes platelet adherence and protects factor VIII. Released vWF stabilizes plate adhesion to the damaged blood vessel wall and promotes platelet–fibrin interactions. The vWF also binds GpIIb/IIa on the activated platelet surface to promote platelet aggregation. ADAMTS13 is the protease that cleaves ultra-large multimers of vWF.
- Factor VIII is an essential blood coagulation protein, also known as antihemophilic factor (AHF), synthesized in liver sinusoidal cells and vascular endothelial cells outside liver throughout the body. Factor VIII protein circulates in the blood in an inactive form bound to another molecule called von Willebrand factor.
- Human, factor VIII is encoded by **FB gene.** Defects in FB gene result in hemophilia A, recessive X-linked coagulation disorder.

vWF and Factor VIII Complex

Both factor VIII (10%) and vWF (90%) form complex and circulate in the plasma to promote blood clotting and the interaction with vascular endothelium and platelets resulting in primary hemostatic plug. vWF serves as a carrier for factor VIII. vWF stabilizes half-life of factor VIII in the circulation for 12 hours. If patient has deficient vWF, half-life of factor VIII is reduced to 2–4 hours (Fig. 11.7).

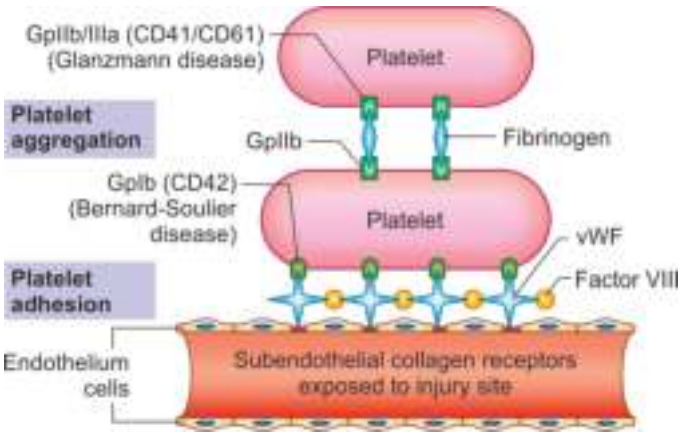


Fig. 11.7: vWF and factor VIII complex shows platelet aggregation on exposed subendothelial tissue.

CLINICAL FEATURES

Patient with von Willebrand disease presents with mild to severe spontaneous bleeding from mucous membranes, excessive bleeding from wounds and menorrhagia. It is worth mentioning that hemarthroses are rare in contrast to hemophilia A. Unlike other forms of hemophilia, von Willebrand disease is not X-linked disorder, so men and women are equally affected. Clinical manifestations of von Willebrand disease worsen following intake of aspirin and NSAID.

LABORATORY DIAGNOSIS

Factor VIII and vWF levels are decreased in von Willebrand disease. Tourniquet test (Hess test) is positive. Platelet aggregation test is normal with ADP, collagen and epinephrine (absent with ristocetin). Activated partial thromboplastin time (APTT) is normal or decreased. Template bleeding time, platelets count, Prothrombin time and international normalized ratio (PT–INR), thrombin time (TT) and fibrinogen lysis tests are within normal range (Table 11.9).

Table 11.9 Laboratory findings in von Willebrand disease

Parameters	Results
Tourniquet test (Hess test)	Positive
Template bleeding time	Normal
Platelet count	Normal
Platelet aggregation test	Normal with ADP, collagen and epinephrine (absent with ristocetin)
Factor VIII assay	Decreased
vWF assay	Decreased
Prothrombin time and international normalized ratio (PT–INR)	Normal
Thrombin time (TT)	Normal
Activated partial thromboplastin time (APTT)	Normal or increased
Fibrinolysis test	Normal

TREATMENT

The von Willebrand disease is treated by administration of desmopressin or DDAVP to stop bleeding. This therapeutic agent will release subendothelial stores of vWF and factor VIII. If DDAVP is not effective, patient is treated by factor VIII replacement. If DDAVP and factor VIII administration are not effective, and recombinant of von Willebrand factor (vWF) is administered.

FACTOR I (FIBRINOGEN) DEFICIENCY

Fibrinogen is synthesized in the liver, which plays an important role in primary hemostasis due to normal platelet aggregation. Due to proteolytic cleavage of fibrinogen by thrombin, fibrin is produced. Inherited abnormality of fibrinogen results in abnormal hemostasis (Table 11.10).

Table 11.10 Inherited abnormalities of fibrinogen associated with abnormal hemostasis

Parameters	Afibrinogenemia	Hypofibrinogenemia	Dysfibrinogenemia
Definition	Total absence of fibrinogen measured by antigenic assay	Decreased level of fibrinogen	Structural abnormality of fibrinogen molecule with altered functional properties
Clinical manifestations	▪ Umbilical stump bleeding ▪ Mucosal bleeding ▪ Central nervous system bleeding ▪ Musculoskeletal bleeding ▪ Intra-abdominal bleeding ▪ Recurrent miscarriage ▪ Antepartum and postpartum bleeding	▪ Umbilical stump bleeding ▪ Mucosal bleeding ▪ Central nervous system bleeding ▪ Musculoskeletal bleeding ▪ Intra-abdominal bleeding ▪ Recurrent miscarriage ▪ Antepartum and postpartum bleeding	▪ Umbilical stump bleeding ▪ Central nervous system bleeding ▪ Soft tissues bleeding ▪ Bleeding in pregnant women following vaginal delivery, cesarean section ▪ Postpartum bleeding ▪ Bleeding following dental extraction and surgical procedures ▪ Delayed wound healing
Clinical course	Clinical course variable in severity	Clinical course variable in mild	Clinical course variable in severity
Laboratory diagnosis	▪ ↑Prothrombin time ▪ ↑Activated partial thromboplastin time ▪ ↑Thrombin time ▪ ↑Bleeding time ▪ Fibrinogen levels undetectable by antigen assay ▪ Coagulation tests are markedly prolonged	▪ ↑Prothrombin time ▪ ↑Activated partial thromboplastin time ▪ ↑Thrombin time ▪ ↑Bleeding time ▪ Fibrinogen level and function reduced ▪ Coagulation tests abnormal depending on fibrinogen level	▪ ↑Thrombin time (most sensitive) ▪ ↑Prothrombin time (may be prolonged) ▪ ↑Activated partial thromboplastin time (may be prolonged) ▪ Reptilase time variable (increased or normal) ▪ Diagnosed by demonstration of fibrinogen molecule defect

- Afibrinogenemia is total absence of fibrinogen analyzed by an antigenic assay.
- Hypofibrinogenemia refers to decreased level of fibrinogen.
- Dysfibrinogenemia refers to structural abnormality of fibrinogen molecule leading to altered functional properties.

CLINICAL FEATURES

Clinical manifestations depend on concentration of factor I (fibrinogen) in the blood.

- Patient with afibrinogenemia presents with life-threatening bleeding diathesis, e.g. mucosal bleeding, umbilical stump bleeding, muscle hematoma, hemarthrosis, intracranial bleeding, recurrent miscarriage with antepartum and postpartum hemorrhages.
- Patient suffering from dysfibrinogenemia presents with delayed wound healing, umbilical stump bleeding, intracranial bleeding, muscle hematoma, and bleeding after tooth extraction.

MOLECULAR GENETIC ALTERATIONS

Three subunits of fibrinogen (Aα, Bβ and γ) are encoded by three different genes located on chromosome 4. Subunit Aα represents 99% of RNA. Synthesis of Bβ chain is the rare-limiting step in the production of mature molecule of fibrinogen. The γ-chain is produced by alternative splicing. Fibrinogen constitutes 15% and contains binding site for thrombin and factor XII.

LABORATORY DIAGNOSIS

Fibrinogen antigen assay is done to measure fibrinogen protein concentration in blood.

- **Afibrinogenemia:** In afibrinogenemia, there is marked prolongation of prothrombin time and international normalized ratio (**PT–INR**), activated partial thromboplastin time (**APTT**), thrombin time (**TT**) and bleeding time (**BT**). Fibrinogen is undetectable by analyzing functional and antigenic assays.
- **Hypofibrinogenemia:** In hypofibrinogenemia, there is prolongation of PT–INR, APTT and TT. Thrombin time is most sensitive test. Fibrinogen level is detected by analysis of functional and antigen assays.
- **Dysfibrinogenemia:** In dysfibrinogenemia, there is prolongation of TT. Prothrombin time and international normalized ratio (PT–INR), APTT may be prolonged.

FACTOR II (PROTHROMBIN) DEFICIENCY

Prothrombin (factor II) is synthesized by liver. It is one of the vitamin K-dependent coagulation factors that functions in the blood coagulation cascade. Prothrombin is encoded by a gene located on chromosome 11.

- Prothrombin requires post-translational carboxylation to become functionally active. In the presence of factor V and calcium, factor Xa activates prothrombin.
- Prothrombin deficiency is an autosomal recessive disorder characterized by hypoprothrombinemia (type I prothrombin deficiency with reduced antigen and activity) and dysprothrombinemia (type II prothrombin deficiency with normal activity but normal antigen level).

CLINICAL FEATURES

Patient may present with hemarthrosis, muscle hematomas, and intracranial bleeding.

Laboratory Diagnosis of Factor II (Prothrombin) Deficiency

- Prothrombin time and international normalized ratio (PT–INR) is prolonged.
- Activated partial thromboplastin time (APTT) is also prolonged.
- Bleeding time (BT) is within normal reference range.
- Diagnosis of mild prothrombin deficiency may be difficult in premature neonates.

TREATMENT

Prothrombin deficiency is treated by administration of prothrombin concentrate. Factor III concentrate contains factors II, IX and X. Prophylactic administration of prothrombin concentrate prevents development of chronic arthropathy in children.

FACTOR V (LABILE FACTOR) DEFICIENCY

Coagulation factor V (labile factor) is a large glycoprotein encoded by a gene located on chromosome 1. Factor V is synthesized by liver and megakaryocytes.

- Platelets contain 20% of total circulating factor V.
- Thrombin activates factor V resulting in formation of factor Va, which acts as a cofactor for factor Xa involved in the conversion of prothrombin to thrombin.
- Factor V deficiency is an inherited autosomal recessive disorder, also known as **Owren's disease** or parahemophilia that results in poor coagulation after an injury or surgical procedures.
- Factor V deficiency should not be confused with factor V Leiden mutation that causes excessive blood coagulation.

CLINICAL FEATURES

Homozygous deficiency of factor V results in moderate to severe bleeding tendencies, e.g. epistaxis and oral

mucosal bleeding. Hematuria and gastrointestinal bleeding are rare.

Laboratory Diagnosis of Factor V (Labile Factor) Deficiency

- Factor V deficiency is associated with prolongation of prothrombin time and international normalized ratio (PT–INR) as well as activated partial thromboplastin time (APTT).
- Thrombin time (TT) is within normal range.
- Immunological assay of factor V is essential in establishing its deficiency.
- Factor VIII immunological assay should also be performed to exclude combined deficiency of factors V and VIII deficiency.

TREATMENT

Fresh frozen plasma (FFP) containing factor V should be administered to achieve hemostasis. Close monitoring of factor V deficiency is essential in pregnant women.

FACTOR VII (STABLE FACTOR) DEFICIENCY

Factor VII (stable factor) is a vitamin K-dependent glycoprotein encoded by factor VII gene located on chromosome 13. Factor VII deficiency is rare inherited autosomal recessive coagulation disorder.

CLINICAL FEATURES

Patient presents with epistaxis, mucosal bleeding in gums, menorrhagia, chronic iron deficiency anemia and bleeding into central nervous system.

LABORATORY DIAGNOSIS

Factor VII deficiency results in prolonged prothrombin time and international normalized ratio (PT–INR). It is essential to exclude vitamin K deficiency and other acquired clotting factor disorders. Immunological analysis of factor VII and factor VII antigen (VIIa) is performed by using enzyme-linked immunosorbent assay and immunoradiometric assay.

TREATMENT

Therapeutic agents, e.g. fibrinolytic inhibitors, factor VII concentrates are administered to control bleeding during surgical procedures and postoperative period. Prophylactic administration of factor VII is essential to prevent intracranial bleeding in neonates.

FACTOR X (STUART-PROWER FACTOR) DEFICIENCY

Factor X (Stuart-Prower factor) is a vitamin K-dependent protein encoded by gene located on chromosome 13. Factor X is synthesized by liver and activated by tissue factor, factor VIIa, calcium ions and phospholipid membrane. Severe deficiency of factor X is an autosomal recessive disorder.

CLINICAL FEATURES

Patient with severe deficiency of factor X presents with mucosal bleeding, umbilical stump bleeding, recurrent hemarthrosis, menorrhagia and postoperative bleeding. Moderate deficiency of factor X results in bleeding due to trauma or surgical procedures.

LABORATORY DIAGNOSIS

Factor X deficiency results in prolonged prothrombin time and international normalized ratio (PT–INR) and activated partial thromboplastin time (APTT). Diagnosis is confirmed by analyzing plasma factor X levels using PT–INR and APTT-based assays or Russell viper venom assay or chromogenic assay. It is essential to exclude vitamin K deficiency or other acquired causes of coagulation disorder.

TREATMENT

Patient suffering from factor X deficiency is treated by therapeutic administration of fibrinolytic inhibitors and plasma to control or prevent bleeding in neonates and during surgical procedures.

FACTOR XI (PLASMA THROMBOPLASTIN ANTECEDENT) DEFICIENCY

Factor XI is a serine protease encoded by factor XI gene. Factor XI is activated by thrombin resulting in initiation of intrinsic pathway of coagulation. Factor XI deficiency is an autosomal recessive disorder that may be associated with bleeding provoked by injury or surgery. Other terms used for factor XI deficiency disorder include plasma thromboplastin deficiency, hemophilia C and Rosenthal syndrome.

CLINICAL FEATURES

Patient presents with bleeding provoked by injury or surgery from nose, mouth and genitourinary tract. Women are at risk of bleeding during childbirth and menorrhagia.

LABORATORY DIAGNOSIS

Factor XI deficiency results in prolongation of activated partial thromboplastin time (APTT).

TREATMENT

Patient with factor XI deficiency is administered oral tranexamic acid with or without a virtually inactivated fresh frozen plasma (**FFP**). Factor XI concentrate is

administered during major surgical procedures and labor. Neonatal intracranial bleeding does not occur due to deficiency of factor XI.

FACTOR XIII (FIBRIN STABILIZING FACTOR) DEFICIENCY

Factor XIII (fibrin stabilizing factor) is involved in stabilizing of blood clot, which also participates in wound repair and healing. Factor XIII deficiency is an autosomal recessive disorder characterized by potentially life-threatening hemorrhagic diathesis. Paradoxically, alterations in factor XIII may predispose to thrombosis. Congenital deficiency of factor XIII occurs due to defects in the catalytic A subunits of factor XIII. Approximately >100 mutations in the factor XIII gene have been demonstrated.

CLINICAL FEATURES

Clinical features of factor XIII deficiency depend on concentration of factor XIII in blood. Patient with severe deficiency of factor XIII presents with severe bruising, muscle hematomas, hemarthrosis, delay in wound healing, intracranial bleeding, miscarriages and bleeding following trauma and postoperative surgical procedures.

Laboratory Diagnosis of Factor XIII Deficiency

- All routine coagulation tests, e.g. bleeding time, prothrombin time (PT–INR) and activated partial thromboplastin time (APTT) are normal in factor XIII deficiency, which complicates the diagnosis of this disorder.
- Factor XIII activity, antigen assays and molecular studies confirm factor XIII deficiency.
- Clot solubility test is a qualitative screening test.

ACQUIRED COAGULATION SYSTEM DISORDERS

Major causes of acquired coagulation system disorders are vitamin K deficiency, liver disease, disseminated intravascular coagulation (DIC) and development of circulating anticoagulants.

- Severe liver diseases (cirrhosis, fulminant hepatitis, acute fatty liver of pregnancy) may disturb hemostasis by impaired synthesis of coagulation factors by diseased liver.
- Because all the coagulation factors are synthesized by liver and vascular endothelium; thus, both prothrombin time and international normalized ratio (PT–INR) and activated partial thromboplastin time (APTT) are prolonged.
- Occasionally, decompensated liver disease also causes excessive fibrinolysis and bleeding manifestations due to decreased hepatic synthesis of antiplasmin.
- Disseminated intravascular coagulation and hemorrhagic disease of newborn are important acquired causes of bleeding diathesis.

DISSEMINATED INTRAVASCULAR COAGULATION

Disseminated intravascular coagulation (DIC) is characterized by widespread coagulation due to consumption of platelets and coagulation factors, especially factors II, V, and VIII, and fibrinogen. DIC occurs as a result of release of tissue thromboplastin (tissue factor) or activation of the intrinsic pathway of coagulation system, and fibrinolytic system. The history of DIC is typical of amniotic fluid embolism, one of the major obstetric causes of disseminated intravascular coagulation, also known as consumptive coagulopathy.

PATHOGENESIS

Neoplastic causes of disseminated intravascular coagulation (DIC) include malignancies of the lung, pancreas, prostate, and stomach, and FAB-AML-3 acute myelogenous (promyelocytic hypergranular) leukemia.

- Tissue damage can result from lung surgery, hemolysis or hemolytic transfusion reactions, gram-negative sepsis and immune complex disease. Pathophysiology of disseminated intravascular coagulation is shown in Fig. 11.8.
- Activation of both intrinsic and extrinsic pathways of coagulation system lead to wide systemic thrombi formation in small vessels. Entrapment of platelets in the thrombi causes thrombocytopenia, in addition due to marked reduction of coagulation factors in consumptive coagulopathy.
- Tissue factor inhibitor mechanism having anticoagulant property is disabled in DIC. Proteins C and S having anticoagulant properties are reduced due to consumption of coagulation factors in DIC. Causes of disseminated intravascular coagulation are given in Table 11.11.

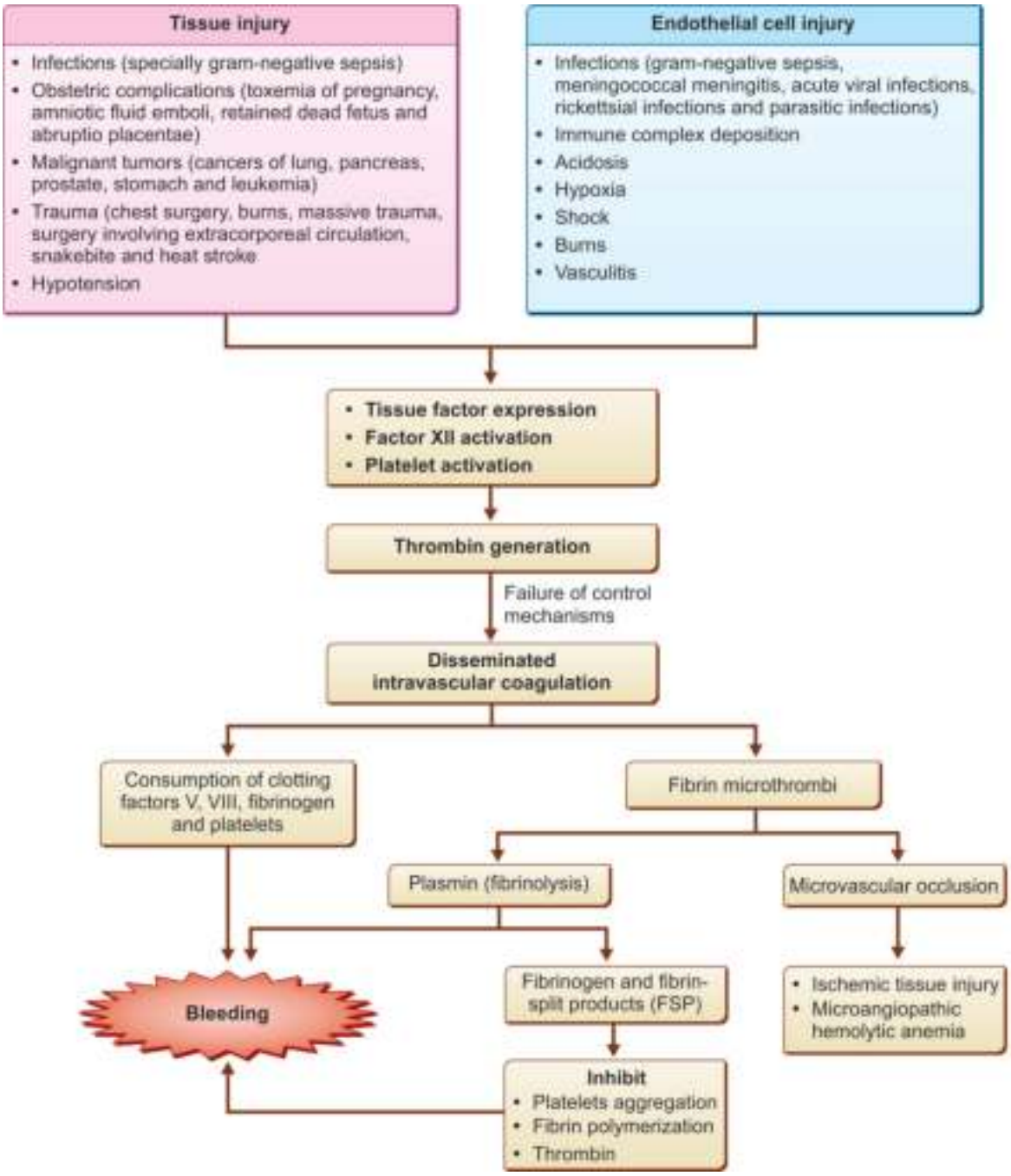


Fig. 11.8: Pathophysiology of disseminated intravascular coagulation. It is triggered by tissue injury, vascular endothelium injury or combination of both these processes. Intravascular coagulation occurs as a result of normal mechanisms controlling hemostasis.

CLINICAL FEATURES

Clinical manifestations of disseminated intravascular coagulation are thrombotic phenomena in small blood vessels of multiple organs and hemorrhages. Patient presents with abrupt onset of bleeding in the form of petechiae or ecchymosis. There may be bleeding from gums. Patient develops hypotensive shock, intracranial bleeding and pulmonary hemorrhages.

Clinical features of disseminated intravascular coagulation are given in Table 11.12.

Laboratory Diagnosis of Disseminated Intravascular Coagulation

- Peripheral blood smear examination shows microangiopathic hemolytic anemia with fragmented red blood cells (schistocytes) and low platelet count.
- Fibrin and fibrinogen degradation products (FDPs) are increased. FDP-dimer test is specific diagnostic test for disseminated intravascular coagulation.
- Bleeding time (BT) is prolonged.

- Prothrombin time and international normalized ratio (PT–INR) is increased.
- Activated partial thromboplastin time (APTT) is increased.
- Thrombin time (TT) is increased.
- Euglin lysis time measures fibrinolytic activity in disseminated intravascular coagulation.
- D-dimer assay can be measured by commercially available latex agglutination. It can be analyzed by ELISA or immunodilution-based D-dimer assay kits.
- Hematologic findings in disseminated intravascular coagulation are given in Table 11.13.

TREATMENT

Patient with disseminated intravascular coagulation (DIC) manifestations is treated by heparin therapy, platelets transfusion, replacement therapy with coagulation factors and fresh frozen plasma (FFP).

Table 11.11 Causes of disseminated intravascular coagulation

Infections	
▪ Gram-negative infections	▪ Endotoxic shock
Obstetric Causes	
▪ Placental abruption ▪ Amniotic fluid embolism ▪ Intrauterine death	▪ Severe pre-eclampsia or eclampsia
Malignant Tumors	
▪ Prostatic carcinoma ▪ Lung carcinoma ▪ Pancreatic carcinoma ▪ Ovarian carcinoma	▪ Gastric carcinoma ▪ Colon carcinoma ▪ Acute promyelocytic leukemia (FAB-AML-3)
Trauma	
▪ Head injury	▪ Burns
Vascular Causes	
▪ Aortic aneurysms	▪ Giant hemangioma

Table 11.12 Clinical features of disseminated intravascular coagulation

Coagulation Manifestations
Venous thromboembolism, acute renal failure due to renal cortical ischemia, skin necrosis or skin gangrene, coma due to cerebral infarction, hepatocellular failure due to hypotension and infection.
Hypotensive Shock
Hypotensive shock occurs due to underlying disease together with disseminated intravascular coagulation
Central Nervous System Manifestations
▪ Transient neurological symptoms ▪ Signs of coma and delirium
Respiratory System Manifestations
Transient hypoxemia due to impairment of lung functions, pulmonary hemorrhage, adult respiratory distress syndrome (ARDS).

Table 11.13 Hematologic findings in disseminated intravascular coagulation (DIC)

Laboratory Investigations	Results
Peripheral blood smear	Anemia and thrombocytopenia
Platelet count	Low
Fibrinogen	Low
Prothrombin time and international normalized ratio (PT–INR)	Increased
Activated partial thromboplastin time (APTT)	Increased
Thrombin time (TT)	Increased
Fibrin and fibrinogen degradation products (FDPs)	Increased
Antithrombin III, protein C and protein S levels	Low
Prothrombin time and INR	Increased

Euglin lysis time measures fibrinolytic activity in DIC. FDP-dimer test is specific diagnostic test for DIC. Other laboratory investigations in patient with suspected DIC include blood urea, serum creatinine, liver function tests, blood culture and pulse oximetry (oxygen saturation). INR: International normalized ratio.

HEMORRHAGIC DISEASE OF NEWBORN

Vitamin K is needed for production of vitamin K-dependent coagulation factors in the liver. Vitamin K is a cofactor for hepatic carboxylation of prothrombin, factors VII, IX, and X, protein C and protein S. Vitamin K deficiency leads to serious life-threatening hemorrhagic disease of the newborn.

PATHOGENESIS

Vitamin K deficiency is exclusively in breastfed and premature babies, because human milk is low in vitamin K, and their gut is not yet colonized with bacteria. Vitamin K deficiency is uncommon in adults, except in those with severe liver disease and an oral anticoagulant. There is excessive bleeding from umbilical stump or gastrointestinal tract (malena) in the newborn. Intracranial bleeding is life-threatening in the newborns. Hemorrhagic disease of newborn is given in Table 11.14.

LABORATORY DIAGNOSIS

Prothrombin time and international normalized ratio (PT–INR) and activated partial thromboplastin time (APTT) are prolonged.

Table 11.14 Hemorrhagic disease of newborn

Type	Clinical Signs	Causes
Early hemorrhagic disease of newborn	Severe bleeding often internal within early 24 hours	Mother receiving drugs affecting vitamin K, e.g. anticonvulsant (phenytoin), warfarin, antitubercular drugs (isoniazid, rifampicin)
Classic hemorrhagic disease of newborn	Classic bruising or bleeding from gastrointestinal tract or after circumcision within 2–5 days	Breastfeeding full term
Delayed hemorrhagic disease of newborn	Intracranial hemorrhage common up to one month	Prolonged breastfeeding without prophylaxis; chronic diarrhea, malabsorption syndrome or oral antibiotics

TREATMENT

Liver of the newborn is not mature to synthesize vitamin K-dependent coagulation factors. Routine vitamin K is administered prophylactically. Coagulation factors are required to activate both intrinsic and extrinsic pathways of coagulation.

COAGULATION SYSTEM DISORDERS IN HEPATIC DISEASE

Vitamin K is needed for synthesis of vitamin K-dependent coagulation factors (II, VII, IX and X) and protein C and protein S in the liver. Hepatic disease is unable to synthesis of vitamin-K dependent coagulation factors (II,VII, IX, X).

CLINICAL FEATURES

Patient with hepatic disease presents with bleeding from gastrointestinal tract; a few patients develop spontaneous superficial hemorrhage.

LABORATORY DIAGNOSIS

Prothrombin time and international normalized ratio (PT–INR) and activated partial thromboplastin time (APTT) are prolonged. PT–INR is increased, which test measures for the blood to clot. PT–INR estimation helps to monitor anticoagulant therapeutic response. It can also be used to check blood coagulation system disorder. Laboratory findings in various coagulation system disorders and anticoagulant therapy are given in Table 11.15. Drugs affecting coagulation system are given in Table 11.16.

Table 11.15 Laboratory findings in various coagulation disorders and anticoagulant therapy

Disorder	Platelet Count	Prothrombin Time and INR (PT–INR)	Activated Partial Thromboplastin Time (APTT)	Thrombin Time (TT)	Fibrinogen Assay	Other Findings
Hemophilia A	Normal	Normal	Increased	Normal	Normal	Decreased factor VIII assay
Hemophilia B	Normal	Normal	Increased	Normal	Normal	Decreased factor IX assay
von Willebrand disease	Normal	Normal	Increased	Normal	Normal	▪ Decreased factor VIII and vWF ▪ Increased bleeding time
Liver disease	Normal	Increased	Increased	Normal	Normal	Decreased synthesis of coagulation factors II, VII, IX and X
Massive blood transfusions	Decreased	Increased	Increased	Normal	Normal or decreased	Normal FDP (fibrinogen degradation products)
Warfarin drug	Normal	Increased	Increased	Normal	Normal	Decreased synthesis of factors II, VII, IX and X
Heparin drug	Normal	Normal	Increased	Increased	Normal	Increased anti-Xa

Table 11.16 Drugs affecting coagulation system

Hemostatic Process Affected	Class of Therapeutic Drug	Specific Therapeutic Drug
Platelet plug formation	Antiplatelet plug drug	Nonsteroidal anti-inflammatory drugs (NSAIDs)
Coagulation cascade	Intravenous and oral anticoagulant drugs	Heparin and warfarin
Fibrinolysis	Fibrinolytic agents	Streptokinase and urokinase

THROMBOPHILIA (HYPERCOAGULABLE STATE)

Hereditary thrombophilia is a prothrombotic familial syndrome caused by deficiency of a number of anti-thrombotic proteins, including antithrombin III, protein C, and protein S.

- Hereditary hemophilia is characterized by recurrent venous thrombosis and thromboembolism, which most often occurs in adolescents or young women. Patient has history of thrombosis at young age, unexplained recurrent episodes of thromboembolic phenomenon. Strong family history of thrombosis in the first degree relatives, recurrent abortions and pregnancy associated thrombi in women should be investigated.
- Secondary (acquired) hypercoagulable thrombophilia is due to underlying systemic diseases or clinical conditions and cannot be identified until thrombosis occurs.
- Most common causes of hereditary and acquired thrombophilia are given in Table 11.17. Laboratory diagnosis of thrombophilia is given in Table 11.18.

Table 11.17 Most common causes of hereditary and acquired thrombophilia

Inherited Thrombophilia	Acquired Thrombophilia
Risk of clinical thromboembolic disease	
Excessive risk patients ▪ Homozygous or double heterozygous antithrombin III deficiency ▪ Homozygous or double heterozygous protein C (PC) deficiency ▪ Homozygous or double heterozygous protein S (PS) deficiency	**Excessive risk patients** Mucin secreting adenocarcinoma
High risk patients ▪ Double heterozygotes activated protein C resistance (APCR) ▪ Homozygous prothrombin gene G20210 A mutation ▪ Homozygous factor V Leiden mutation	**High risk patients** ▪ Hip fracture ▪ Total hip or knee replacement ▪ Acute promyelocytic leukemia
Moderate risk patients ▪ Heterozygous antithrombin III deficiency ▪ Heterozygous protein C deficiency ▪ Heterozygous protein S deficiency	**Moderate risk patients** ▪ Sepsis ▪ Malignancy ▪ Antiphospholipid syndrome (lupus anticoagulant causing pregnancy loss) ▪ Prolonged immobilization and post-surgical state ▪ Paroxysmal nocturnal hemoglobinuria ▪ Major surgery/major trauma
Low risk patients ▪ Heterozygous activated protein C resistance (APCR) ▪ Heterozygous heparin cofactor II (HC II) ▪ Tissue factor pathway inhibitor variant ▪ Dysfibrinogenemia/hyperfibrinogenemia ▪ Methylene tetrahydrofolate reductase gene mutation (MTHFRC677T) ▪ Elevated factor VIII ▪ Factor XII deficiency ▪ Plasminogen activator deficiency ▪ Elevated plasminogen activator inhibitor 1 (PAI-1) ▪ Sickle cell anemia	**Low risk patients** ▪ General surgery ▪ Oral contraceptives use ▪ Pregnancy ▪ Elevated factor VIII ▪ Elevated lipoprotein A, i.e. Lp(a) ▪ Heparin-induced thrombocytopenia ▪ Nephrotic syndrome (loss of antithrombin III and antiplasmin in urine) ▪ Congestive heart failure ▪ Complicated atheromatous plaque
Family history of thrombophilia	
Present of family member with known thrombophilia	Absence of family member with known thrombophilia
Venous thromboembolism	
Unprovoked venous thromboembolism or with minor risk factor	Unprovoked venous thromboembolism
Site of thrombosis	
Recurrent venous thromboembolism in <50 years	Thrombosis at unusual sites especially in patient >50 years (e.g. splanchnic region)
Clinical manifestations	
Skin necrosis associated with vitamin K antagonists	Late or recurrent pregnancy loss

Patient with history of deep vein thrombosis or pulmonary embolism should receive prophylactic LMW heparin.

Table 11.18 Laboratory diagnosis of thrombophilia

Laboratory Test	First Diagnostic Step	Comments
Factor V Leiden (FVL)	Deficient factor V Leiden (FVL) coagulation-based functional assay	DNA analysis for factor V Leiden
Prothrombin G20210A mutation (FII G20210A)	DNA analysis	Measurement of prothrombin (F-II) activity in plasma should not be used to screen thrombophilic patients for this mutation due to its inability to clearly distinguish carriers from non-carriers of the mutation
Antithrombin (AT) deficiency	Functional coagulation-based antigenic chromogenic assay	Measurement of antithrombin antigen by an immunoassay in order to classify the type of deficiency as type I or II
Protein C (PC) deficiency	Functional coagulation-based chromogenic assay	Measurement of protein C antigen by an immunoassay in order to classify the type of deficiency as type I or II or III
Protein S (PS) deficiency	Functional coagulation-based antigenic assay	Measurement of protein S antigen by an immunoassay in order to classify the type of deficiency as type I or II or III
Activated protein C resistance (APCR)	APTT (activated partial thromboplastin time) or RVVT (Russell's viper venom time)-based assays	Work up for congenital or acquired thrombophilia, e.g. APCR (activated protein C resistance) and FVL (factor V Leiden)
Antiphospholipid antibodies (aPLAs): LA, ACL and anti-β_2-GP1	▪ LA: A panel of screening two or more assays and at least one confirmatory assay ▪ ACL and anti-β_2-GP1: Enzyme immunosorbent assays for both IgM and IgG isotopes	Repeat testing for a positive test result with at least 12 weeks apart in order to confirm a positive test result
Increased factor VIII level (F VIII >159%)	Coagulation or chromogenic functional assay	Repeat testing 3–6 months after initial testing
Hyperhomocysteinemia (HHC)	Plasma level of homocysteine	Repeat testing in case of questionable or borderline test result or to confirm a positive test result
Dysfibrinogenemia	Screening assays: Thrombin time (TT) and reptilase time (RT), functional (Clauss) fibrinogen level	Parallel analysis of functional and immunoreactive fibrinogen as confirmatory assays

HEREDITARY THROMBOPHILIA (HYPERCOAGULABLE STATE)

Hereditary thrombophilia has autosomal dominant, autosomal recessive or X-linked inheritance, which has tendency to form blood clots in arteries and veins. Hereditary thrombophilia and its characteristics of most common defects are given in Table 11.19.

ANTITHROMBIN III DEFICIENCY

Antithrombin III is synthesized by liver, which prevents thrombus formation by inhibiting the activity of thrombin, Xa, IXa and XIa.

- Gene coding for antithrombin III is located on chromosome 1, band q23.1–23.9. Hereditary deficiency of antithrombin III is an autosomal dominant disorder.
- Approximately 50% reduction in the plasma concentration of antithrombin III results in venous thrombosis and recurrent thromboembolic pulmonary phenomenon in adolescents or early life.

Clinical Aspects

In clinical practice, heparin is most often administered to prevent thrombus formation. Heparin combines with antithrombin III.

- Patient with hereditary deficiency of antithrombin III, antithrombotic action of heparin is markedly reduced.
- Acquired deficiency of antithrombin III occurs in nephrotic syndrome due to its loss in urine along with massive proteinuria.

Assessment of Antithrombin III

Assessment of antithrombin III is performed by chromogenic assay and coagulation assay.

PROTEIN C DEFICIENCY

Vitamin K is required to synthesize protein C, which prevents thrombus formation. Thrombin, factor Xa and protein C activating enzyme convert protein C into activated form. Normally, activated protein C along with protein S degrade factors Va and VIIIa. Protein C enhances clot lysis.

Clinical Features

Hereditary deficiency of protein C is an autosomal dominant disorder.

- Approximately, 50% reduction in the plasma concentration of protein C results in venous thrombosis and recurrent thromboembolic pulmonary phenomenon in adolescents or early life.

Table 11.19 Inherited thrombophilia and its characteristics of most common defects

Features	Factor V Leiden Deficiency	Prothrombin Deficiency	Protein C Deficiency	Protein S Deficiency	Antithrombin Deficiency
Frequency and physiologic effects					
General population frequency	4–10%	2–4%	0.2–0.4%	0.07–2.3%	0.02%
First deep vein thrombosis	20%	7%	3%	2–3%	1–2%
Thrombophilic families	40%	18%	6%	4%	4%
Physiologic effects	Factor Va is resistant to cleavage by activated protein C	Increased formation of prothrombin and thrombin	Decreased inactivation of factor Va, factor VIIIa and upregulation of coagulation	Decreased cofactor for activated protein C and upregulation of coagulation	Inhibition of factor Xa, thrombin and upregulation of coagulation
Clinical manifestations					
Homozygous state	Increased risk of venous thromboembolism (80 times)	Increased risk of venous thromboembolism and possible arterial thromboembolism	Purpura fulminans	Purpura fulminans	Lethal *in utero*
Heterozygous state	Increased risk of venous thromboembolism (4–7 times)	Increased risk of venous thromboembolism (3–4 times)	Increased risk of deep vein thrombosis (4–8 times)	Increased risk of deep vein thrombosis (4–8 times)	Increased risk of venous thromboembolism (10 times)

Family history of pulmonary embolism is most important cause of death due to inherited defect. Patient with history of deep vein thrombosis or pulmonary embolism should receive prophylactic LMW heparin.

- A total lack of protein C is usually associated with death *in utero*.
- Repeated infusions of prothrombin complex help in keeping these children alive. Deficiency of protein C may also occur in liver disease.

PROTEIN S DEFICIENCY

Vitamin K is required to synthesize protein S, which prevents thrombus formation. Normally, activated protein C along with protein S degrade coagulation factors Va and VIIIa.

- Hereditary deficiency of protein S is an autosomal dominant disorder due to mutation of PROS1 gene. Hereditary deficiency of protein S is associated with arterial and venous thrombi.
- Protein C activity is measured by enzyme-linked immunosorbent assay (ELISA), functional and antigenic assays.

Laboratory classification of protein S deficiency is given in Table 11.20.

Table 11.20 Laboratory classification of protein S deficiency

Features	Type I Protein S Deficiency	Type II Protein S Deficiency	Type III Protein S Deficiency
Total protein S	Decreased	Normal	Normal
Free protein S	Decreased	Normal	Decreased
Protein S activity	Decreased	Decreased	Decreased

FACTOR V LEIDEN (rs6025)

Factor V Leiden is a mutation of one of the coagulation factors in blood and has been named after the city in Netherlands. It is the most common cause of hereditary thrombophilia. Pregnancy and oral contraceptive intake increases risk of hypercoagulable state.

Molecular Genetic Alterations

Factor V Leiden protein is formed due to specific mutation by substitution of glutamine for arginine at position 506. It alters the cleavage site targeted by APC.

This abnormal factor V Leiden protein becomes resistant to cleavage by protein C. As a result, an important antithrombotic counter-regulatory mechanism is lost. There is increased generation of prothrombokinase–thrombin complex.

Clinical Features

Patient presents with cerebral and recurrent deep venous thrombosis in legs. Arterial thrombi are rare. There is increased risk of abortions during second trimester of pregnancy.

Laboratory Diagnosis

Laboratory tests for factor V Leiden mutation include activated protein C resistance assay and genetic analysis by polymerase chain reaction (PCR).

PROTHROMBIN G20210A GENE MUTATION

Prothrombin G20210A gene mutation is the second most common cause of hereditary thrombophilia. Mutation of prothrombin gene occurs due to a single nucleotide change (G to A) in the 3′-untranslated region at position 20210, which is associated with elevated plasma prothrombin levels. Patient has almost three times increased risk of arterial and venous thrombosis.

HYPERHOMOCYSTEINEMIA

Methylenetetrahydrofolate reductase gene mutation (MTHFRC677T) results in mild elevation of homocysteine levels in 5–15% of White and East Asia population.

- Elevated levels of homocysteine in blood inhibit antithrombin III and thrombomodulin leading to increased risk for thrombosis.
- The increased homocysteine in blood can be reduced by dietary supplementation with folic acid and vitamins B_6 (pyridoxine) and B_{12} (cobalamin).
- Hyperhomocysteinemia is also associated with an increased risk for neural tube defects and possibly a number of diverse neoplasms.

ACQUIRED THROMBOPHILIA (HYPERCOAGULABLE STATE)

ANTIPHOSPHOLIPID SYNDROME

Antiphospholipid antibody syndrome has underlying etiology of systemic lupus erythematosus, which is associated with high titers of circulating antibodies (IgG) directed against anionic phospholipids (cardiolipin) such as plasma protein epitopes prothrombin.

Pathogenesis

Lupus anticoagulant is an IgG or IgM, which binds to phospholipid used in activated partial thromboplastin time (APTT) resulting in prolonged activated partial thromboplastin time (normal 30–40 seconds). *In vivo*, these antibodies induce a hypercoagulable state by activating platelets, interfering in protein C activity and inhibiting synthesis of PGI_2 by vascular endothelial cells.

Clinical Features

Lupus anticoagulant is associated with recurrent venous thrombosis in deep veins of legs or arterial thrombi. But renal, hepatic, and retinal veins are also susceptible.

- There is history of repeated miscarriages due to inhibition of tissue plasminogen activator (tPA), which is required for trophoblastic invasion of uterus.
- There is increased risk of cardiac valvular vegetations or thrombocytopenia in systemic lupus erythematosus patients. Anticardiolipin antibodies are associated with arterial thrombi.

Laboratory Diagnosis

In vitro, these antibodies interfere with the assembly of phospholipid complexes and thus inhibit coagulation. Activated partial thromboplastin time (APTT) is prolonged. Prothrombin time and international normalized ratio (PT–INR), thrombin time and fibrinogen level are within normal range. Confirmatory tests include kaolin clotting time, platelet neutralizing test and Russell viper venom time test.

BLEEDING DIATHESIS: DIAGNOSTIC APPROACH

Bleeding diathesis occurs due to abnormalities in coagulation factors; platelet quantitative and qualitative disorders; disorders associated with thrombosis and miscellaneous acquired causes of bleeding diathesis, e.g. vitamin K deficiency, liver disease, disseminated intravascular coagulation (DIC) and heparin-induced thrombocytopenia (HIT) (Fig. 11.9).

- Investigations of hemostatic function are essential if patient has clinical history of excessive bleeding following injury or dental extractions, menorrhagia, hematuria, poor healing of superficial lacerations, easy bruising, hemarthrosis, incidental abnormal hemostatic test; and past or family history of excessive bleeding or bruising.

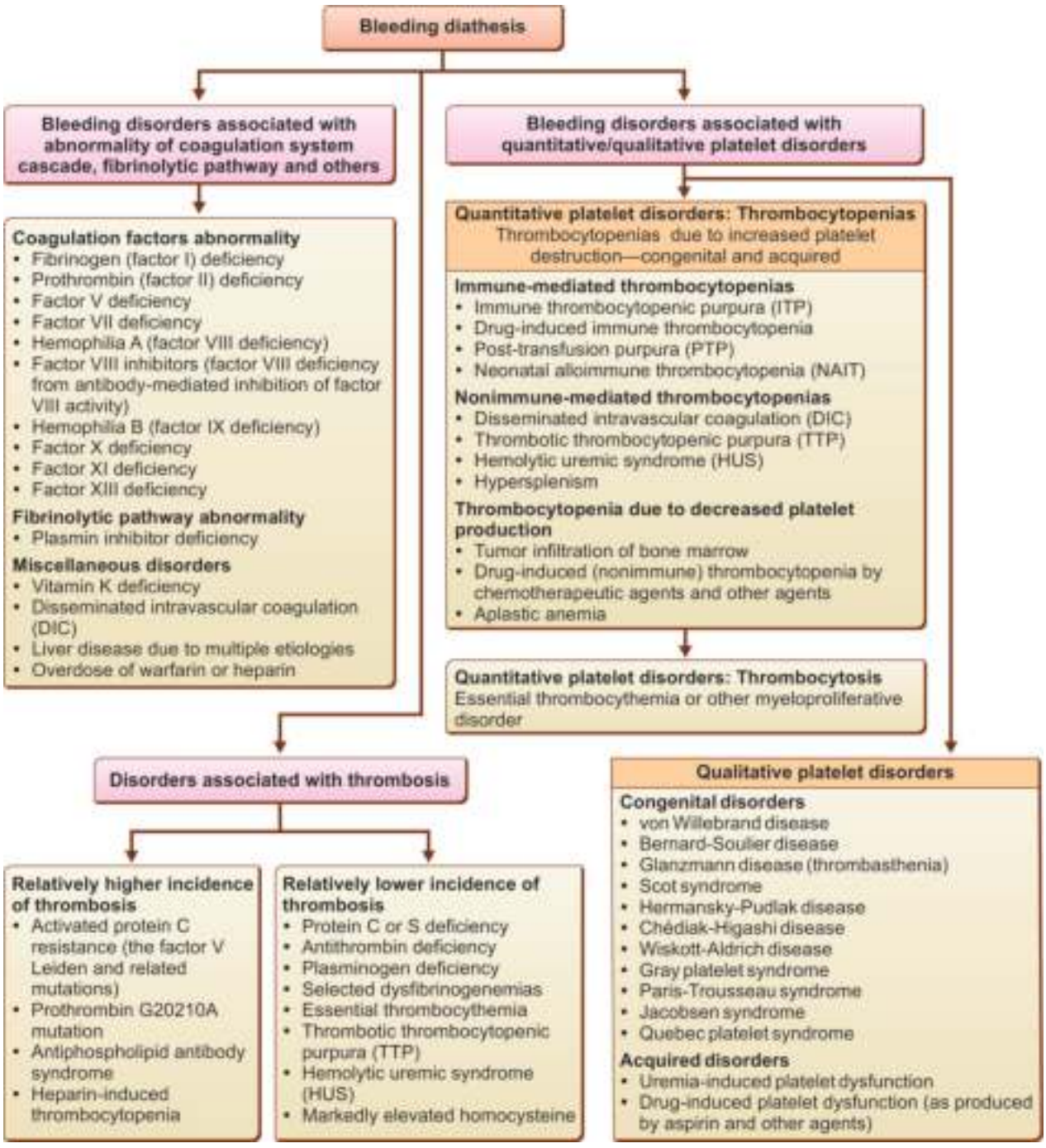


Fig. 11.9: Bleeding diathesis occurs due to abnormalities in coagulation factors; platelet quantitative and qualitative disorders; disorders associated with thrombosis; and miscellaneous acquired causes.

- Four steps of investigating hemostatic function include: complete history, physical examination, screening tests of hemostasis and special hematological tests such as PT–INR and APTT to assess the bleeding risk.
- Clinical manifestations in vascular, platelet and coagulation disorders are given in Table 11.21. Laboratory tests for primary and secondary hemostasis are given in Table 11.22.

CLINICAL HISTORY

Patient suffering from bleeding diathesis may present with easy bruising, purpura, epistaxis, heavy menstruation with passage of blood clots, excessive bleeding following dental procedures, passage of red-colored urine, painful joints or muscles and delayed wound healing and tissue repair. Exposure to drugs or toxins and family history of bleeding should be enquired.

Table 11.21 Clinical manifestations in vascular, platelet and coagulation disorders

Clinical Manifestations	Vascular Disorders	Platelet Disorders	Coagulation Disorders
Petechial hemorrhages	Common	Common	Rare
Bleeding superficial cuts and scratches	Persistent; often profuse bleeding	Persistent; often profuse bleeding	Minimal bleeding
Superficial ecchymosis	Small and multiple	Small and multiple	Common; large and solitary
Deep hematoma	Rare	Rare	Common
Hemarthrosis	Rare	Rare	Common
Delayed bleeding	Rare	Rare	Common
Gastrointestinal bleeding	Absent	Present	Present with GIT lesions
Hematuria	Absent	Present	Present
Epistaxis	Absent	Present	Present
Gingival bleeding	Absent	Present	Present
Excessive bleeding after tooth extraction	Absent	Present	Present
Intracranial bleeding	Absent	Present	Present
Sex predilection	More common in females	More common in females	80–90% of inherited forms occur only in male patients
Family history	Rare (except vWD and HHT)	Rare (except vWD and HHT)	Common

Table 11.22 Laboratory tests for primary and secondary hemostasis

Laboratory Test	Normal Range	Type of Assay	Application
Assessment of primary hemostasis			
Bleeding time (BT)	1–9 minutes	Small incision on forearm and time recorded for the platelets to stop bleeding	Bleeding time is affected by depth of the incision, platelet count; and pressure on the arm
Platelet function analyzer 100 (PFA-100)		Platelet function analyzed by passing whole blood through cartridge containing agonists (collagen/epinephrine and collagen/ADP) and time needed for platelets to occlude aperture is analyzed	Platelet function defect or thrombocytopenia
Assessment of secondary hemostasis			
Prothrombin time (PT)	10–14 seconds	Tissue factor and phospholipids are added to plasma and clotting time is recorded	▪ Measures extrinsic pathway of coagulation (tissue factor VII), or common pathway of coagulation (factors X, V, II, I), presence of inhibitor, or circulating anticoagulant. ▪ Prothrombin time is also prolonged in the presence of deficiency of vitamin K-dependent factors ▪ Most common causes (liver disease, warfarin therapy, disseminated intravascular coagulation)
Prothrombin time and international normalized ratio (PT–INR)	2.0 to 3.0	INR = PT/mean prothrombin time	Monitoring oral anticoagulant therapy

Contd...

Table 11.22 Laboratory tests for primary and secondary hemostasis (*Contd...*)

Laboratory Test	Normal Range	Type of Assay	Application
Activated partial thromboplastin time (APTT)	30–40 seconds	Activators such as micronized silica, phospholipids and calcium are added to plasma and clotting time recorded	▪ Measures intrinsic pathway (contact factors VIII, IX, XI, XII) or common pathway (factors X, V, II, I), presence of inhibitor or circulating anticoagulant ▪ Prolonged APTT is associated with abnormality of the above mentioned. ▪ Measuring unfractioned heparin ▪ Most common causes (liver disease, unfractioned heparin therapy, hemophilia A and hemophilia B; disseminated intravascular coagulation)
Fibrinogen assay (factor)	150–400 mg/dl	Functional fibrinogen assay or derived from another assay, e.g. prothrombin time	▪ Decreased fibrinogen occurs in disseminated intravascular coagulation (DIC), primary or secondary fibrinolysis, afibrinogenemia or dysfibrinogenemia and liver disease ▪ Increased fibrinogen occurs in acute phase reactants, pregnancy, hormone therapy ▪ Inverse relationship (longer clotting time = lower fibrinogen)
Thrombin time (TT)	15–19 seconds	Excess of thrombin is added and fibrinogen to fibrin conversion is measured	▪ Evaluates formation of fibrin ▪ Measurement of thrombin time is affected in afibrinogenemia, dysfibrinogenemia, presence of fibrin/fibrinogen degradation products and circulating heparin ▪ Most common causes (disseminated intravascular coagulation, heparin therapy, fibrinolysis)
Specific factor assays	5–150%	Assessed by PT or depending on factor suspected	Tests are functional-based assays
Factor XIII assay: Urea solubility test	Not applicable	Clot solubility is measured in the presence of urea or monochloroacetic acid in 24 hours	▪ Clot dissolution corresponds to factor XIII level of 1–2% ▪ Symptoms of deficiency are delayed bleeding or increased bruising
von Willebrand factor (vWF)	Not applicable	vWF measured by platelet aggregation test and using antibody against vWF by enzyme immunoassay, ELISA, immunoturbidometric	▪ Results assessed in conjunction with APTT, vWF multimers, ADAMS-13 ▪ Essential to assess functional and antigenic levels to accurately diagnose and treat disease
Assessment of fibrinolysis			
Fibrin/fibrinogen degradation products (FDPs)	<10 mg/dl	Patient blood sample is collected into special test tube containing thrombin and a fibrinolytic inhibitor (prevents *in vitro* fibrinolysis). Now it mixed with latex beads coated with antibodies for FDPs	Agglutination is positive result in liver disease, deep vein thrombosis, disseminated intravascular coagulation and kidney disease
D-dimers (DD)	<0.5 µg/ml	Automated immunological assay or semiquantitative latex assay	Breakdown products of fibrin, and monitoring clot dissolution in deep vein thrombosis (DVT), pulmonary embolism and disseminated intravascular coagulation (DIC)

Contd...

Table 11.22 Laboratory tests for primary and secondary hemostasis (*Contd...*)

Laboratory Test	Normal Range	Type of Assay	Application
Assessment of inhibitors			
Bethesda titer (factor inhibitors)	Not applicable	Mix patient plasma with normal plasma and incubate at 37°C for two hours and measure factor level	▪ Presence of inhibitor inactivates patient factor resulting in decreased activity ▪ Factor VIII administration is ineffective in hemophiliac patient in the presence of factor inhibitor
Lupus-like anticoagulant (LA)	Not applicable	Diagnosis is based on several tests, e.g. APTT, dilute Russell viper venom test	Criteria for lupus-like anticoagulant are prolongation of phospholipid-dependent coagulation reaction, demonstration that is an inhibitor against and not a factor deficiency, and demonstration of inhibitor against phospholipid inhibitor

When a blood sample is obtained for prothrombin time, activated partial thromboplastin time and thrombin time, sodium citrate is as the anticoagulant in the sampling tubes.

PHYSICAL EXAMINATION

Patient may have evidence of bleeding such as petechial hemorrhages, purpura and ecchymosis, bleeding from mucous membranes, hemarthroses, muscle hematoma, gastrointestinal bleeding, renal hematuria or epistaxis. One must look for hepatosplenomegaly, jaundice, fever and joint abnormalities.

Clinical summary of bleeding disorders is given in Table 11.23. Clinical findings and diagnostic possibilities in thrombotic disease in various age groups are given in Table 11.24.

Table 11.23 Clinical summary of bleeding disorders

Observations	Diagnostic Possibilities
History observations	
▪ History of bleeding/bruising since childhood ▪ Recent history of bleeding/bruising since childhood ▪ Sudden onset of bleeding/bruising, poorly healing cuts	▪ Congenital defect of platelet/coagulation factor ▪ Acquired defect of platelet/coagulation factor ▪ Acquired defect, probably drug induced
Physical examination	
▪ Small hemorrhages from skin, mucous membrane especially in females ▪ Persistent bleeding from superficial lesions ▪ Delayed bleeding, hemarthrosis, hematomas, joint deformities, bleeding following trauma, bleeding from GIT and renal hematuria, epistaxis in males with positive family history ▪ Bleeding from only one organ	▪ Abnormalities of the blood vessels or low platelet count or impaired platelet function ▪ Defects in coagulation factor ▪ Probably not caused by hemostatic defect

Table 11.24 Clinical findings and diagnostic possibilities in thrombotic disease in various age groups

Age Group	Clinical Findings	Possible Causes
Birth to 45 years	▪ Family history with migratory or recurrent thrombi ▪ Smoking and hormones (estrogen, progesterone and oral contraceptives)	▪ Hereditary hypercoagulable state (deficiency of antithrombin III, protein C and protein S) and homocystinuria ▪ Aggravate thrombotic disease
10–45 years	▪ Migratory or recurrent thrombi ▪ Multiple organs failure, massive purpura (severely ill patients)	▪ Acquired hypercoagulable state (lupus inhibitor) ▪ Disseminated intravascular coagulation and hemolytic uremic syndrome

Contd...

Table 11.24 Clinical findings and diagnostic possibilities in thrombotic disease in various age groups (*Contd...*)

Age Group	Clinical Findings	Possible Causes
>45 years	▪ Localized sudden onset thrombi without recurrence ▪ Arterial occlusion ▪ Weight loss, anorexia, weakness ▪ Fever, infection (localized or systemic) ▪ Severe or massive trauma ▪ Deep vein thrombosis or thrombophlebitis, emboli	▪ Acquired thrombosis associated with infection/inflammation, trauma, venous stasis and drug reaction ▪ Atherosclerosis, hyperlipidemia, diabetes mellitus and protein S deficiency ▪ Underlying malignancy (lung, pancreas, prostate, stomach and AML—acute myelomonocytic leukemia) ▪ AML (M3) is commonly associated with DIC due to release of thromboplastin-like substance ▪ Underlying infection or inflammation ▪ Disseminated intravascular coagulation ▪ Any thrombotic disease

HEMATOLOGIC INVESTIGATIONS

Complete blood counts, hemoglobin or packed cells volume, WBC count, platelet count, erythrocyte sedimentation rate and peripheral blood smear examination to rule out macrocytic anemia, aplastic anemia and acute leukemia for underlying cause of thrombocytopenia. Interpretation of routine blood tests in bleeding disorders is shown in Fig. 11.10. Comparison between platelets and coagulation system defects is given in Table 11.25.

PLATELET COUNT

A normal platelet count in adults ranges from 150,000 to 450,000 platelets per microliter of blood. Platelets normally clump in groups of 2–5. Normal value is 4–8 platelets per 100 RBCs. A reasonable estimate of platelet number can be obtained by counting platelets present in relation to 500 RBCs. If platelet count is low, one should rule out secondary causes of thrombocytopenia before establishing acute or chronic ITP. Giant platelets are demonstrated in Bernard-Soulier syndrome, myeloproliferative disorders and May-Hegglin anomaly.

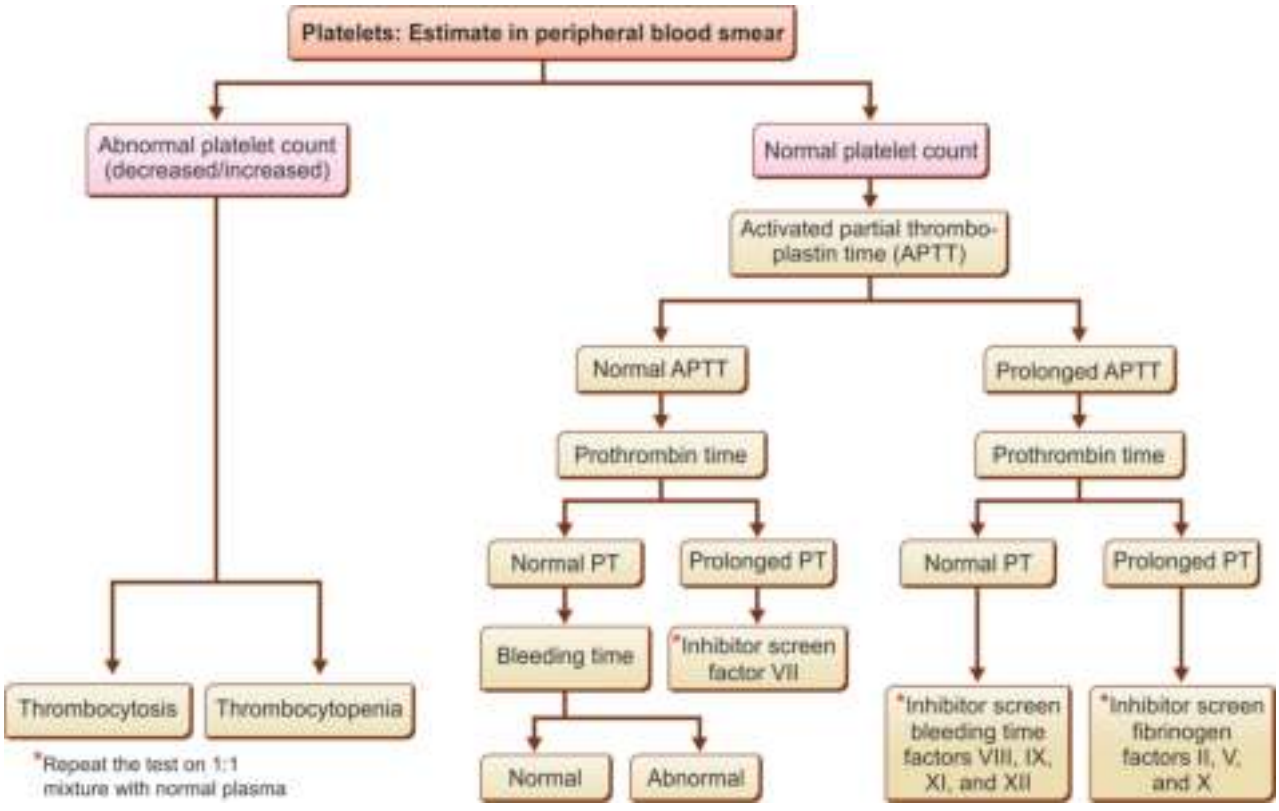


Fig. 11.10: Interpretation of routine blood tests in bleeding disorders.

Table 11.25 Comparison between platelet and coagulation system defects

Feature	Platelet Defects	Severe Coagulation System Defects
Purpura (petechiae)	Very common	Absent
Spontaneous bleeding	Common	Uncommon
Application of local pressure	Bleeding does not stop quickly	Bleeding stops quickly
Bleeding from superficial cut	Present	Absent
Family history	Absent	Possible
Mucosal bleeding from mouth and gut	Usually present	Relatively uncommon except urinary tract
Hemarthroses (bleeding in joints)	Absent	Very common in severe coagulation defect
Muscle hematomas	Present in response to trauma	Spontaneous even without trauma
Bleeding after trauma or surgery	Immediate for short time	Often delayed, after several more than 48 hours

PLATELET FUNCTION TESTS

Platelet aggregation test with ADP, collagen, epinephrine and ristocetin should be performed to rule out various platelet function disorders.

- In Bernard-Soulier syndrome, platelet aggregation is deficient with ADP, epinephrine, and collagen, but normal with ristocetin.
- In Glanzmann disease and von Willebrand disease, platelet aggregation is normal with ADP, epinephrine, and collagen, but deficient with ristocetin. Laboratory tests in bleeding diathesis are given in Table 11.26.

BLEEDING TIME

The bleeding time measures vascular and platelet integrity, which is estimated by Ivy method and template method. In general, 95% of all values are <4 minutes. If the bleeding time is >4 minutes, abnormality of platelets should be considered.

COAGULATION PROFILE

Prothrombin Time and International Normalized Ratio

Prothrombin time and international normalized ratio (PT–INR) is the time required for plasma to clot after tissue thromboplastin and optimum amount of calcium chloride is added. Incubate plasma at 37°C in a water bath for five minutes. Add 0.2 ml thromboplastin and 0.1 ml of calcium chloride to the test tube. Record the time for formation of blood clot every second. End point is identified by formation of fibrin strand attached to the wire hook.

Prothrombin time is measured to diagnose bleeding diathesis, monitoring patients on anticoagulant therapy and prior to liver biopsy. Normal range of prothrombin time is between 11 and 16 seconds. Procedure of one stage prothrombin time is shown in Fig. 11.11.

Prothrombin Time and International Normalized Ratio

- Prothrombin time measures extrinsic pathway of coagulation system.
- Prothrombin time is prolonged in disseminated intravascular coagulation, vitamin K deficiency, deficiency of one or more of clotting factors (fibrinogen, prothrombin, V, VII or X), liver disease and oral coagulant therapy.

Activated Partial Thromboplastin Time

Activated partial thromboplastin time (APTT) measures the intrinsic procoagulant activity of plasma. Partial thromboplastin is a substitute for platelet factor III. Contact activation is standardized by adding an activator (kaolin) to the reagent.

- Activated partial thromboplastin time test does not measure activities of factors VII and IX. APTT is prolonged in hemophilia A, hemophilia B, disseminated intravascular coagulation (DIC) and von Willebrand disease. Normal range of APTT is between 30 and 40 seconds.

Table 11.26 Laboratory tests in bleeding diathesis

Laboratory Test	Component Measured	Normal Values
Bleeding time (BT)	Platelet function vascular integrity	2–9 minutes
Prothrombin time (PT)	I, II, V, VII, IX and X	11–16 seconds
Activated partial thromboplastin time (APTT)	I, II, V, VIII, IX, X, XI and XII except VII and XIII	30–40 seconds
Thrombin time (TT)	I and II	15–19 seconds

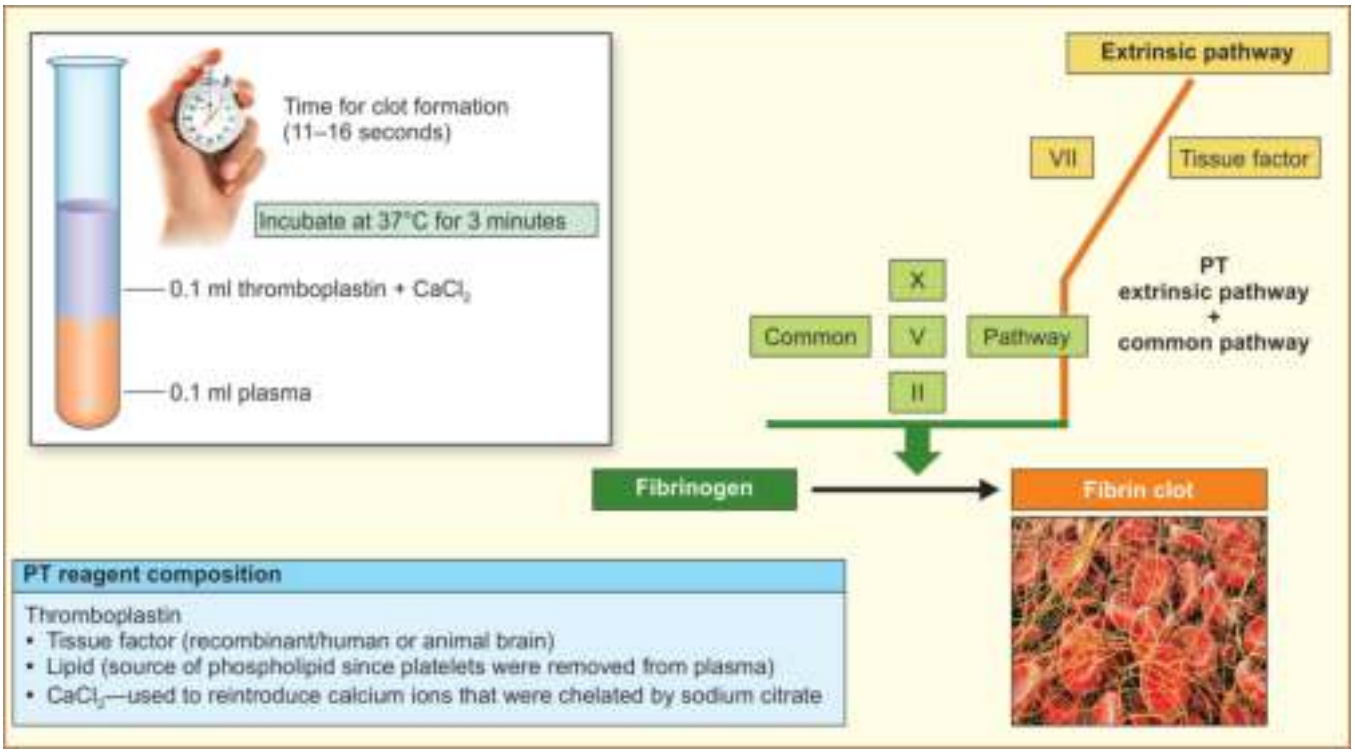


Fig. 11.11: Procedure of one stage prothrombin time.

- Procedure of activated partial thromboplastin time is shown in Fig. 11.12. Interpretation of tests of hemostasis function in normal and abnormal patients is given in Table 11.27.

Thrombin Time

Thrombin time (TT) measures fibrinogen concentration. Normal thrombin time is between 15 and 19 seconds.

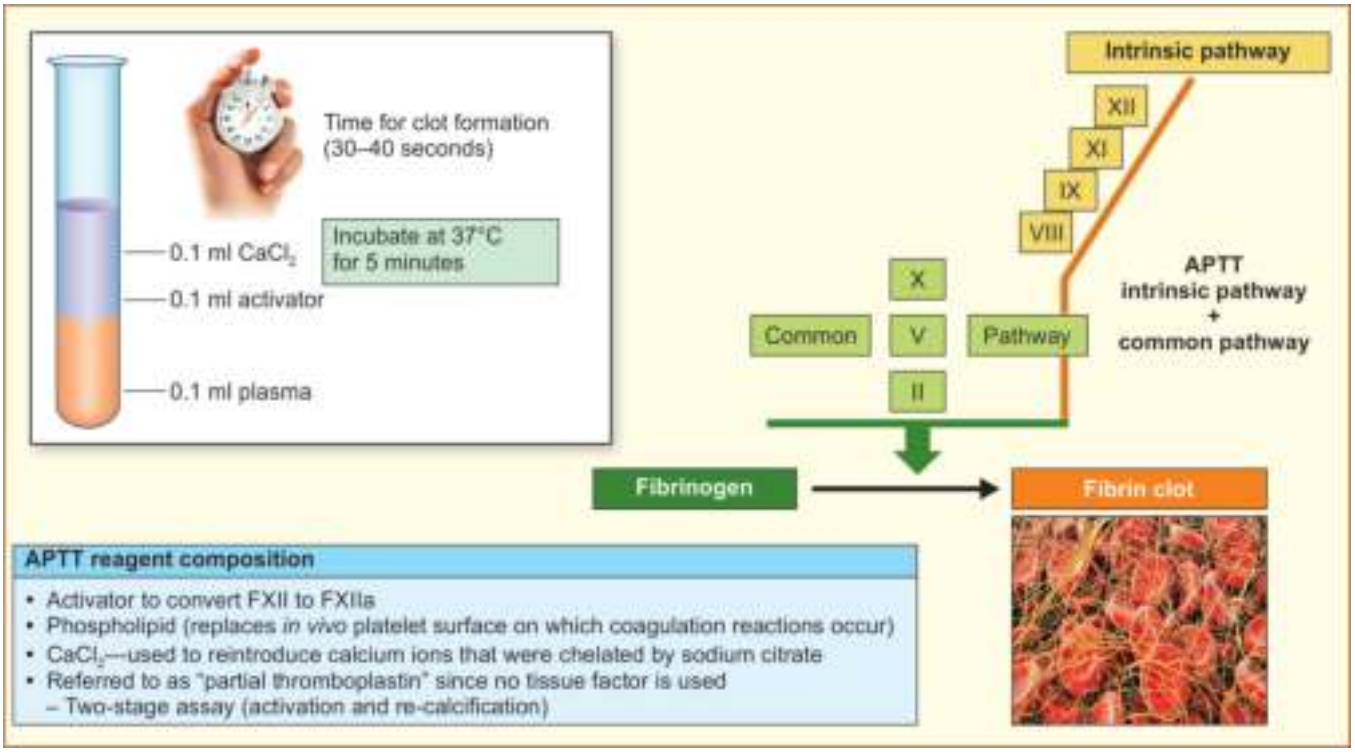


Fig. 11.12: Procedure of activated partial thromboplastin time (APTT).

Table 11.27 Interpretation of tests of hemostasis function in normal and abnormal patients

Tests and Results		Conclusion
Platelet count	Bleeding time	Interpretation
Normal platelet count	Normal	Normal result (platelets are related to bleeding disorders, so suspect coagulation defect in case of bleeding diathesis)
Low platelet count	Prolonged	Low platelet count (thrombocytopenia)
Normal platelet count	Prolonged	Abnormal platelet function
Prothrombin time (PT)	Activated partial thromboplastin time (APTT)	Interpretation
Normal PT	Normal APTT	Normal function of coagulation system (suspect platelet or vascular defect)
Abnormal PT	Abnormal APTT	Defects in common pathway of coagulation system
Normal PT	Abnormal APTT	Defects in the intrinsic pathway of coagulation system
Abnormal PT	Normal APTT	Defects in the extrinsic pathway of coagulation system

Thrombin time is prolonged in disseminated intravascular coagulation (DIC) and fibrinogen deficiency.

- Thrombin time is also prolonged in patient on anticoagulant therapeutic drugs, e.g. heparin, warfarin, hirudin, argatroban and hirulog.
- A prolonged thrombin time can be caused by the presence of paraproteins in a case of multiple myeloma.

Blood Clot Retraction Test

Blood clot retraction depends on the release of coagulation factors and platelets trapped in the fibrin mesh of the clot. Examination of blood clot found in a test tube provides information regarding concentration of fibrinogen, number and function of platelets; and activity of the fibrinolytic system. Test tube containing anticoagulant free blood is placed in water bath at 37°C for three hours. In normal persons, 30% of the total volume in the test tube should be blood clot.

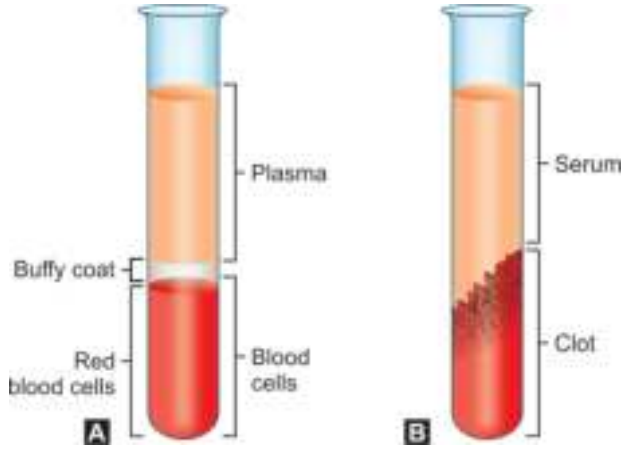


Fig. 11.13: (A) Unclotted whole blood with anticoagulant showing blood cells, plasma and buffy coat after centrifuge, (B) clotted whole blood without adding anticoagulant showing blood clot and serum.

Interpretation of blood clot retraction in various hematological disorders is shown in Figs 11.13, 11.14 and Table 11.28.

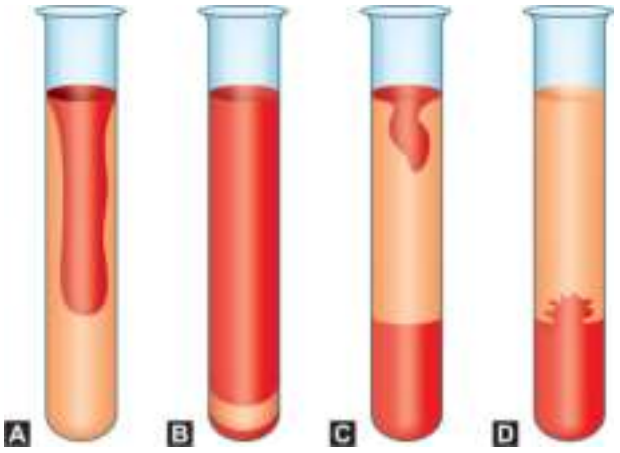


Fig. 11.14: Blood clots observed in normal persons and patients with coagulation disorders. (A) Blood clot in normal person, (B) blood clot in thrombocytopenia and thrombasthenia, (C) blood clot formed due to low fibrinogen concentration, (D) blood clot formed due to enhanced fibrinolysis in disseminated intravascular coagulation.

Table 11.28 Blood clots observed in various hematologic disorders

Blood Clot Structure	Disorders
Large blood clot with weak structure	▪ Thrombocytopenia ▪ Thrombasthenia
Severely affected blood clot retraction due to dysproteinemias	▪ Multiple myeloma ▪ Waldenström syndrome
Small clot with regular shape	Low fibrinogen concentration
Small irregular blood clot digested by enhanced fibrinolysis	▪ Afibrinogenemia ▪ Disseminated intravascular coagulation

Table 11.29 Interpretation of euglobulin lysis time

Time	Interpretation of Results
<2 minutes	Severe fibrinolysis, explaining severe bleeding
2–10 minutes	Moderate fibrinolysis, explaining postoperative or post-traumatic bleeding
10–30 minutes	Mild fibrinolysis, but not explaining bleeding
30 minutes to 2 hours	Physiological enhanced fibrinolysis
2–4 hours	Normal
>4 hours	Possibly defective fibrinolysis

Euglobulin Lysis Time

Euglobulin lysis time (ELT) is a blood test that measures how fast blood clots break down in the blood, which reflects the overall fibrinolytic activity of plasma. Euglobulins are proteins that precipitate when citrated platelet poor plasma is diluted in water. Precipitate contains plasmin, plasminogen and fibrinogen. Euglobulins are redissolved on fibrin gets clotted with thrombin.

- Activated thrombin activates plasminogen to plasmin.
- Time required for the plasmin to lyse fibrin is the euglobulin lysis time. Interpretation of euglobulin lysis time is given in Table 11.29.

Fibrinogen Degradation Products (FDPs) and D-Dimer Tests

Fibrinogen degradation products (FDPs) are formed as a result of breakdown of fibrinogen and fibrin.

- Increased fibrinogen degradation products are demonstrated in disseminated intravascular coagulation (DIC), severe liver disease, cirrhosis, eclampsia of pregnancy, thrombotic episodes, following fibrinolytic therapy, trauma and surgery.
- D-dimer is a protein fragment that is made when a blood clot dissolves in the body. Plasmin-mediated degradation of fibrinogen to fibrin degradation products is given in Fig. 11.15.

Fibrinogen Degradation Products (FDPs) and D-Dimer Tests: Interpretation

The fibrin degradation products (FDPs) test is a specific test that determines the amount of FDPs in blood.

- The tests, i.e. FDPs and D-dimer tests together are performed to evaluate suspected cases of primary fibrinogenolysis. In this, FDP test will be positive but D-dimer test remains normal.
- In disseminated intravascular coagulation (DIC), FDPs as well as D-dimer tests are positive. Fibrinogen degradation products are normally and rapidly cleared from blood circulation.
- Presence of large quantities of FDPs can interfere with the hemostatic process through several mechanisms.
- Forming soluble complexes with fibrin monomer from polymerizing antithrombin activity and by binding to platelets surface resulting in interference with platelet function.

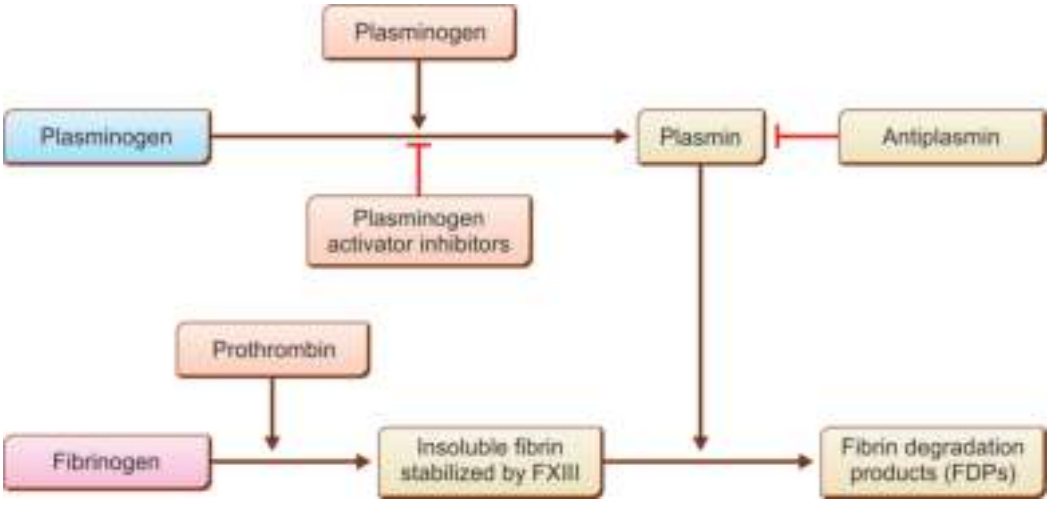


Fig. 11.15: Plasmin-mediated degradation of fibrinogen to fibrin degradation products. Fibrinolysis is the regulated dissolution of formed blood clot. Plasmin enzyme derived from plasminogen is involved in fibrinolysis. Plasmin degrades fibrin polymers and generate fibrin degradation products (FDPs) detected in plasma.

Annexure

Diagnostic workup of hemophilia A

Comprehensive relevant data

Hemophilia A is hereditary X-linked recessive disorder characterized by the deficiency of factor VIII resulting in bleeding and abnormal activated partial thromboplastin time (APTT). It is the most common hereditary bleeding disorder encountered in clinical practice affecting males, whereas females are usually carriers.

Clinical history data

- Clinical history is elicited with special emphasis on the following parameters: age, sex, ethnicity, demographic features, duration of bleeding diathesis, symptoms following injury or surgery, symptom profile, family history, number of previous blood transfusions and history of male relatives with hemophilia A such as maternal uncles, grandfathers, and cousins. The severity of hemophilia A depends on the amount of coagulation factor VIII and its activity in the blood.
- Patient with mild hemophilia A most often has bleeding only with major surgeries or tooth extractions. The patient may not be diagnosed until bleeding complications occur from surgical procedure. Patient with severe hemophilia A can experience bleeding with minimal activities of daily life or without known injury.
- Patient with severe hemophilia A may experience bruising under the skin causing hematoma, tendency to bleed from nose, mouth and gums with minor injury, bleeding while brushing teeth or dental extraction, and bleeding into the skeletal muscles induces swelling, pain, and redness.
  - Swelling from excessive blood in these areas can increase pressure on tissues and nerves. This can cause permanent damage and deformity.
  - The hallmark of hemophilia A is bleeding in the joints in ankles and elbows. Repeated bleeding in these joints can lead to chronic painful arthritis, deformity and crippling, and equine gait.
  - This bleeding is painful and leads to long-term inflammation and deterioration of the joint.
  - Bleeding in the brain from injury or spontaneously is the most common cause of death in children. Blood found in the urine or stool may also signal hemophilia A.

Recording reporting data

- All laboratory testing performed for the initial workup and diagnosis of patient with hemophilia and all related collection and evaluation of samples/specimens.

Collection and evaluation of samples/specimens

- Complete blood counts are normal in hemophilia A, however, if person with hemophilia A has unusually heavy bleeding for a long time, hemoglobin and red blood cell count can be low. Platelet number and functions are normal in hemophilia A.
- Activated partial thromboplastin time (APTT) test measures how long it takes for blood to clot.
  - APTT test evaluates the coagulation factors (XII, XI, IX and VIII) in the intrinsic pathway of coagulation system.
  - APTT test is prolonged among persons with hemophilia A.
- Prothrombin time and international normalized ratio (PT–INR) test also measures the time it takes for blood to clot. PT–INR evaluates primarily the clotting ability coagulation factors (I, II, V, VII and X) in the extrinsic pathway of coagulation system. If any of these coagulation factors are too low, it takes longer than normal time for the blood to clot. PT–INR also evaluates coagulation factors (X, V, II and I) in the common pathway of coagulation system.
  - The results of PT–INR test will be normal among persons with hemophilia A.
  - International normalized ratio (INR) is used to standardized prothrombin test results to adjust for the difference in thromboplastin reagents made by different manufacturers and used by different institutions.
- Fibrinogen (factor I) test also helps in assessment of patient's ability to form blood clot. Fibrinogen test is done either along with other blood coagulation tests or when patient has an abnormal PT–INR or APTT test result or both.
- Coagulation factor assays are required to diagnose bleeding diathesis such as hemophilia A.
  - Coagulation factor is a protein in the blood that helps stop bleeding.
  - Coagulation factor assays show the type of hemophilia A and the severity.
  - It is important to know severity of disease to create the best treatment plan.

- Babies should be tested for hemophilia A soon after birth: babies born to families with history of hemophilia A and whose mothers are carriers of hemophilia A and who have bleeding after symptoms at birth. Blood sample can be drawn from the umbilical cord immediately after birth and testing to determine the level of the factor VIII. Mild hemophilia A patient has FVIII level 6–40% of normal. Moderate hemophilia A patient has FVIII level 1–5% of normal. Severe hemophilia A patient has FVIII level <1% of normal.
- Acquired hemophilia A is characterized by the presence of autoantibodies against coagulation factor VIII, which can trigger bleeding diathesis. It has high morbidity and mortality, hence the importunate of establishing an early diagnosis and treatment.

CHAPTER 12

Blood Banking and Transfusion Practices

Vinay Kamal, Anubhav and Vigyat

LEARNING OBJECTIVES

ABO AND Hh BLOOD GROUP SYSTEMS

- ABO blood group genetics
  - Chromosomes and alleles
  - Amorphic 'O' gene
  - ABO gene products
  - Hh genes and ABO phenotypes
  - ABO and Hh blood group antigens
  - ABO and Hh blood group antibodies

Rh BLOOD GROUP SYSTEM

- Antigens of Rh blood group system
  - CDE (Fisher-Race) nomenclature system
  - Dr Alexander Wiener nomenclature system
  - Current genetic analysis nomenclature system
- Rh factor incompatibility during pregnancy
- Anti-D antibody (RgoGAM) administration

BOMBAY PHENOTYPE AND PARA-BOMBAY PHENOTYPE

- Bombay phenotype
- Para-Bombay phenotype

OTHER BLOOD GROUP SYSTEMS

BLOOD COLLECTION, PROCESSING AND STORAGE

- Donor selection criteria
- Blood donation process
- Anticoagulant preservatives used in blood bank
  - Acid-citrate-dextrose
  - Citrate-phosphate-dextrose
  - Citrate-phosphate-dextrose-adenine 1
- Preparation of blood components from whole blood
  - Whole blood composition
  - Red blood cell component
  - Plasma component
  - Platelet component
  - Granulocyte component
- Removal of blood components by apheresis
  - Collection of red blood cells by apheresis
  - Collection of plasma by apheresis
  - Collection of platelets by apheresis
  - Collection of granulocytes by apheresis
- Therapeutic apheresis
  - Therapeutic plasma exchange
  - Therapeutic leukapheresis
  - Therapeutic erythrocytapheresis
  - Therapeutic fluid replacement
  - Hematopoietic progenitor cells collection
  - Immunoadsorption/selective adsorption
  - Photopheresis
- Blood component labeling
- Changes in stored blood components
  - Red blood cell changes in stored blood
  - Platelet changes in stored blood
  - White blood cell changes in stored blood
  - Plasma protein component changes in stored blood
  - Bacterial contamination in stored blood
- Transportation of blood components
- Modification of blood components
  - Leukoreduction before blood storage
  - Inactivation of pathogens in stored blood
  - Aliquoting of blood components
  - Pooling of blood components
  - Volume reduction from blood components
  - Irradiation of blood components
  - Washing of blood components
  - Freezing, thawing and deglycerolizing red blood cells
  - Rejuvenation of red blood cells
- Blood plasma fractionation
- Recombinant plasma protein production

BLOOD TRANSFUSION AND BLOOD COMPONENT THERAPY

- Blood transfusion: goals
- Blood transfusion: indications
- Blood components transfusion
  - Whole blood transfusion
  - Red blood cell component transfusion
  - White blood cell component transfusion
  - Hematopoietic stem cells used for transplantation
  - Modification of cellular components
  - Transfusable plasma components
  - Platelet component transfusion
  - Fibrin glue and platelet gel
  - Plasma fractionation products
  - Pharmaceutical drugs used to reduce/prevent bleeding in hematologic disorders
  - Massive blood transfusion

ADVERSE BLOOD TRANSFUSION REACTIONS

- Blood transfusion-related immune hemolytic reactions
  - Blood transfusion-related acute hemolytic reactions
  - Blood transfusion-related delayed hemolytic reactions
- Blood transfusion-related febrile nonhemolytic reactions
  - Pathophysiology
  - Clinical features
- Blood transfusion-related allergic reactions
  - Clinical features
- Blood transfusion-related anaphylactic/anaphylactoid reactions
  - Pathophysiology
  - Clinical features
- Blood transfusion-related acute lung injury
  - Clinical features
- Blood transfusion-related circulatory overload
- Blood transfusion-related dyspnea
- Blood transfusion-related hypotension
  - Pathophysiology
  - Clinical features
- Blood transfusion-related graft-versus-host disease
  - Pathophysiology
  - Clinical features
- Blood transfusion-related sepsis
  - Pathophysiology
  - Prevention
- Blood transfusion-related hemosiderosis
  - Clinical features
- Blood transfusion-related purpura
  - Pathophysiology

- Rapid massive blood transfusion-related reactions
  - Citrate toxicity
  - Hypothermia
  - Dilutional coagulopathy
- Blood transfusion-related nonimmune hemolysis
- Blood transfusion-related hyperkalemia
- Blood transfusion-related hypokalemia
- Blood transfusion-related air embolism
- Blood transfusion-related thrombophlebitis

BLOOD GROUPING

- ABO blood grouping
  - Methods of ABO blood grouping
  - Methodology
- Rh blood grouping

BLOOD CROSS-MATCHING (COMPATIBILITY) TEST

- Immediate spin blood cross-matching
- Electronic based blood cross-matching
  - Electronic based major blood cross-match
  - Electronic based minor blood cross-match

ABO AND Hh BLOOD GROUP SYSTEMS

The ABO blood group system was first described by Karl Landsteiner in an article published in 1901. Landsteiner's rule states that normal, healthy persons possess ABO antibodies to ABO blood group antigens absent from their red blood cells. He obtained blood from his coworkers and then mixed with serum and red blood cells obtained from different persons. He observed three patterns of agglutination of red blood cells that he named A, B, and C. The 'C' pattern is now known as 'O'. Decastello and Sturli later describe AB blood group. Agglutination of red blood cells is still one of the standard detection methods being followed today in blood transfusion medicine.

- ABO and Rh blood group systems are most important in the blood transfusion in clinical practice. Red blood cell antigens (A, B and RhD) elicit strong humoral immune response. Alloantibodies formed can cause destruction of transfused red blood cells. ABO antigens are also significant in organ transplantation.
- A blood group may be defined as an inherited character of the red blood cell surface antigens detected by specific antibody. However, cell surface antigens on platelets and leukocytes might also be considered blood groups.
- Most blood groups are organized into blood group systems. Each blood group represents a single gene or cluster of two or more closely linked genes. Of the 347 red blood surface antigens classified by the International Society of Blood Transfusion, 303 red blood cell surface antigens belong to one of 36 blood group systems. Major red blood cell group systems containing 303 gene specificities are given in Table 12.1.

Table 12.1 Major red blood group system containing 303 gene specificities

Number	Name of RBC Blood Group System	Symbol	Gene Symbol(s)	Chromosome	RBC Membrane Associated with Antigen Expression
001	ABO blood group system	ABO	ABO	Chromosome 9	4
002	MNSs blood group system	MNS	GYPA, GYPB, GYPE	Chromosome 4	48
003	PIPK blood group system	PIPK	A3GALT	Chromosome 4	3
004	Rh blood group system	RH	RHD, RHCE	Chromosome 19	54
005	Lutheran blood group system	LU	BCAM	Chromosome 19	22
006	Kell blood group system	KEL	KEL	Chromosome 7	35
007	Lewis blood group system	LE	FUT3	Chromosome 19	6
008	Duffy blood group system	FY	DARC	Chromosome 1	5
009	Kidd blood group system	JK	SLC14A1	Chromosome 18	3
010	Diego blood group system	DI	SLC4A1	Chromosome 17	22
011	Yt blood group system	YT	ACHE	Chromosome 7	2
012	Xg blood group system	XG	XG, CD99	X/Y chromosome	2
013	Scianna blood group system	SC	ERMAP	Chromosome 1	7
014	Dombrock	DO	ART4	Chromosome 12	10
015	Colton blood group system	CO	AQP1	Chromosome 7	4
016	Landsteiner-Wiener blood group system	LW	ICAM4	Chromosome 19	3

Contd...

Table 12.1 Major red blood group system containing 303 gene specificities (*Contd...*)

Number	Name of RBC Blood Group System	Symbol	Gene Symbol(s)	Chromosome	RBC Membrane Associated with Antigen Expression
017	Chido/Rogers blood group system	CH/RG	C4A, C4B	Chromosome 6	9
018	H blood group system	H	FUT1	Chromosome 19	1
019	Kx blood group system	XK	XK	X chromosome	1
020	Gerbich blood group system	GE	GYPC	Chromosome 2	11
021	Cromer blood group system	CROM	CD55	Chromosome 1	18
022	Knops blood group system	KN	CR1	Chromosome 1	9
023	Indian blood group system	IN	CD44	Chromosome 11	4
024	Ok blood group system	Ok	BSG	Chromosome 19	3
025	Raph blood group system	RAPH	CD151	Chromosome 11	1
026	John Multon Hagen blood group system	JMH	SEMA7A	Chromosome 15	6
027	I blood group system	I	GCNT2	Chromosome 6	1
028	Globoside blood group system	GLOB	B3GALT3	Chromosome 3	2
029	Gill blood group system	GIL	AQP3	Chromosome 9	1
030	RHAG blood group system	RHAG	RHAG	Chromosome 6	4
031	Forssman blood group system	FORS	GBGT1	Chromosome 9	1
032	JR blood group system	JR	ABCG2	Chromosome 4	1
033	Lan blood group system	LAN	ABCB6	Chromosome 2	1
034	Vel blood group system	VEL	SMIM1	Chromosome 1	1
035	CD59 blood group system	CD59	ABCG2	Chromosome 11	1
036	Augustine blood group system	AUG	SLC29A1	Chromosome 6	2

- Most blood group antigens are proteins or glycoproteins, with the blood group specificity determined primarily by the amino acid sequence. Most of the blood group polymorphisms result from substitution of single amino acid with some exceptions. Some of these proteins cross the red blood cell membrane several times and attached by a glycosylphosphatidylinositol anchor.
- Some blood group antigens including the ABO, PIPK, Lewis, H and I blood group systems are carbohydrate structures on glycoproteins and glycolipids. These blood group antigens are not synthesized directly by the genes controlling their polymorphisms; rather by genes encoding transferase enzymes that catalyze the final biosynthetic stage of an oligosaccharide chain.

ABO BLOOD GROUP GENETICS

ABO blood group system follows Mendelian law. DNA constitutes for building blocks of life. Sequence of flow of genetic information includes DNA → RNA → synthesis of structural proteins and enzymes → cells → organs → human body.

CHROMOSOMES AND ALLELES

The gene for the ABO blood group system resides on chromosome 9. There are three alleles for A, B and O genes. Every individual has two copies of chromosome 9, hence every person has two ABO genes: one ABO gene inherited from father and one ABO gene inherited from mother. The particular alleles at a specific gene locus in a person constitute genotype, and their outward expression is known as phenotype. ABO blood group system consists of four major blood groups: A, B, AB and O identified based on presence or absence of A and/or B antigens on red blood cells.

- According to Landsteiner's law, anti-A and/or anti-B antibodies are always present in the plasma of persons, who lack corresponding antigen(s). ABO antibodies are mainly IgM type; however, there may be IgG, which have thermal activity at 37°C. IgM and IgG activate complement system and cause intravascular destruction of red blood cells, which can give rise to severe fatal hemolytic blood transfusion reactions. ABO antibodies seldom cause less severe hemolytic disease of the fetus and newborn. There are possible six combinations of ABO genotypes and corresponding phenotypes are given in Table 12.2.

Table 12.2 ABO genotypes and corresponding phenotypes

Genotype	Phenotype
▪ AA ▪ AO	A
▪ BB ▪ BO	B
O	O
AB	AB

- Most of the ABO genes are expressed in co-dominant manner, like most blood group system genes. It means that if the gene is present, its gene product is an enzyme or a protein. No gene or allele is dominant over another gene or allele. Hence, one gene does not suppress the other gene in co-dominant system. The particular alleles at specific gene locus in person constitute genotype, and their outward expression is known as phenotype.
- In ABO blood group system, person lacking an antigen on red blood cells has corresponding anti-A or anti-B antibodies. Both A and B red blood group genes are dominant; while O blood group gene is recessive. ABO blood group system, genotype, quantity of H antigen on red blood cells, antigens on ABO, corresponding antibodies in plasma, reaction of antigen with antiserum and Indian frequency are given in Table 12.3.

AMORPHIC 'O' GENE

Amorphic gene describes that does not express a detectable functional gene product. The O gene is amorph, meaning no functional gene product (a protein or an enzyme) is produced from the sequence of DNA. As ABO genes are co-dominant, the phenotype of person with blood group A can result from one of two allelic combinations: AA or AO. Similarly, the phenotype of person with blood group B can result from one of two allelic combinations: BB or BO. Person with blood group AB has AB genotype.

ABO GENE PRODUCTS

Genes usually code only for proteins. ABO antigens are not proteins, rather carbohydrates. A gene codes for the production of an enzyme that makes the A antigen. Likewise, the B antigen codes for the synthesis of an enzyme that makes the B antigen. The flow of genetic information in the ABO blood group system is in the following sequence: DNA → RNA → synthesis of enzymes → A or B antigen (sugar) on the red blood cell membrane.

Hh GENES AND ABO PHENOTYPES

H antigen is the precursor substance for both A and B antigens. Hh system is different from the ABO system. ABO and Hh genes are inherited independently. ABO genes are located on chromosome 9. On the other hand, Hh genes are found on chromosome 19. 'H' gene encodes α1, 2-L-fucosyltransferase 1 (FUT-1) enzyme essential for A and B antigens. Like the O gene, the H gene is also amorph. Only one copy of the H gene is essential to make the H antigen. In person having two copies of the H gene (Hh), neither the enzyme nor the H antigen is produced.

Table 12.3 ABO blood group system, genotype, quantity of H antigen on red blood cells, antigens on ABO, corresponding antibodies in plasma, reaction of antigen with antiserum and Indian frequency

Blood Group Phenotype	Genotype	Quantity of H Antigen on RBCs	Antigens on RBCs	Corresponding Antibodies in Plasma	RBC Antigen Reacts with Antiserum Antibodies	Indian Frequency
A	▪ AA (homozygous) ▪ AO (heterozygous)	++	A	Anti-B antibody (IgM)	Anti-A antibodies	27%
B	▪ BB (homozygous) ▪ BO (heterozygous)	++	B	Anti-A antibody (IgM)	Anti-B antibodies	30%
AB	AB (heterozygous)	+	AB	None	Anti-A and anti-B antibodies	8%
O	OO	++++	None	Anti-A, anti-B (IgG), hence crosses placenta resulting in hemolytic disease of newborn	None	34%

Large amount of H antigen is present on O blood group red blood cells, while AB blood group has least amount of H antigen on their red blood cells. Immunohematology is study of blood group antigens and antibodies. Antigens are foreign molecules that bind specifically to an antibody or a T cell receptor. Antigens that exhibit great degree of foreignness from the host elicit the strongest immune response.

Immunodominant Sugars

Immunodominant sugar molecules are responsible for ABO blood group specificity, which are attached at the end of the carbohydrate precursor substance, which is H antigen. Immunodominant sugars of the ABO and Hh blood group systems are given in Table 12.4. Interaction of the Hh and ABO genes is shown in Fig. 12.1. Diagrammatic representation of the oligosaccharides representing H, A, B, Le^a and Le^b and the biosynthetic precursor of H and Le is shown in Fig. 12.2. Molecular basis of A and B antigens on the red blood cells are shown in Fig. 12.3.

- 'H' gene encodes the α1,2-L-fucosyltransferase enzyme, which adds L-fucose immunodominant sugar to precursor carbohydrate chain to form the H antigen.
- 'A' gene encodes the α1,3-N-acetyl-galactosaminyl-transferase enzyme, which adds N-acetyl-D-galactosamine immunodominant sugar to H antigen and not the precursor substance of H antigen.
- 'B' gene encodes the α-1,3-D-galactosyltransferase enzyme, which adds D-galactose immunodominant sugar to H antigen and not the precursor substance of H antigen.
- 'O' gene synthesizes an inactive transferase resulting in unchanged H antigen, individuals with blood group O has large amount of H antigen.

Type 1 and Type 2 Carbohydrate Chains

ABH precursor substance is of two types: type 1 (abundant) and type 2. Type 1 ABH precursor substance is present in the plasma, gut lining and secretions such as saliva, milk and urine. Type 2 ABH precursor substance is found on red blood cells and some in plasma. The difference between type 1 and type 2 ABH precursor substance is the attachment of the last galactose sugar molecule on the precursor substance. ABO and Hh blood group system carbohydrate chains are composed of hexoses which are sugar molecules with six carbon atoms.

ABO AND Hh BLOOD GROUP ANTIGENS

The red blood cell membrane is bilipid bilayer. ABO red blood antigens are carbohydrate structures expressed predominantly oligosaccharide molecules, which are attached to glycoproteins, glycolipids and glycosphingolipids in the red blood cell membrane.

ABO Antigens Expression

The red blood cell membranes contain over two million ABO antigens. ABO gene locus has been mapped on chromosome 9q34 and encodes the A and B glycosyltransferases. ABO gene contains seven exons, which encodes 354-amino acid transferase activity. Gene mutation may result in loss of glycosyltransferase activity, thus resulting in blood group '**O**' red blood cells.

Table 12.4 Immunodominant sugars of the ABO and Hh blood group systems

Red Blood Cell Antigen	Immunodominant Sugar	Enzymes that Add Sugar
H	L-fucose	α1, 2-L-fucosyltransferase
A	N-acetyl-D-galactosamine	α1, 3-N-acetylgalactosaminyltransferase
B	D-galactose	α1, 3-D-galactosyltransferase

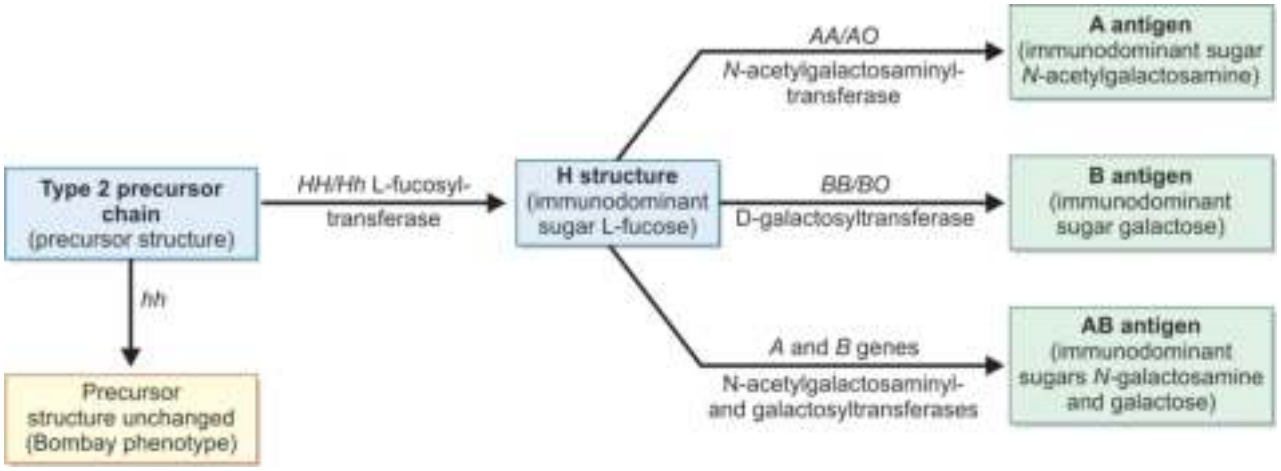


Fig. 12.1: Interaction of the Hh and ABO genes.

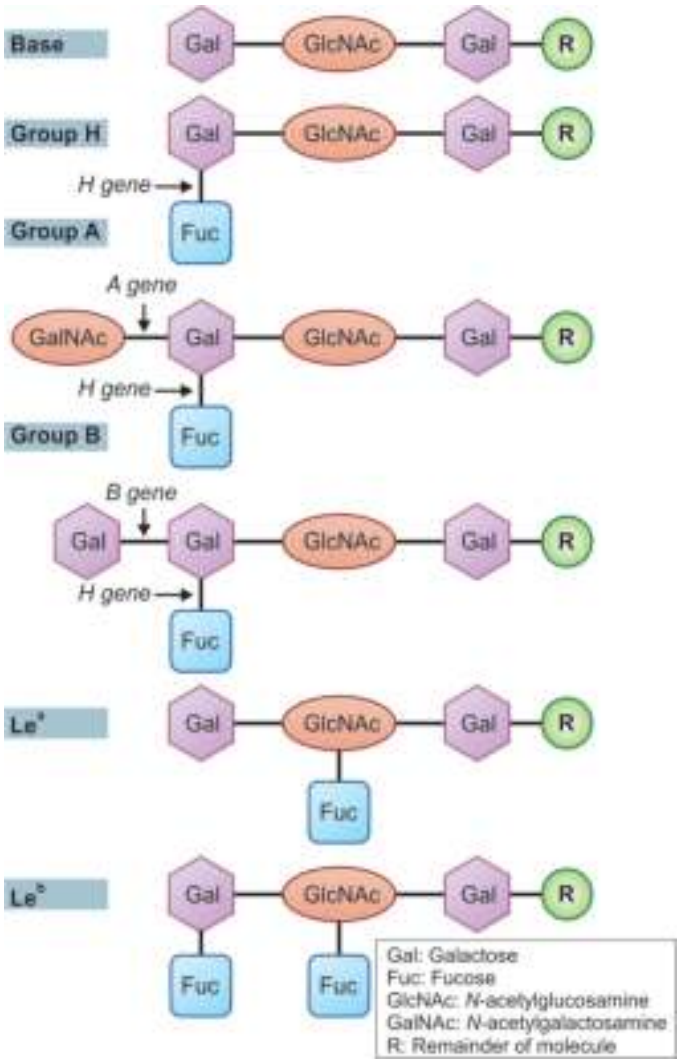


Fig. 12.2. Diagrammatic representation of the oligosaccharides representing H, A, B, Le^a and Le^b and the biosynthetic precursor of H and Le^a.

Many other mutations of these transferase genes result in decreased expression of antigens leading to weak/absent reactivity with standard anti-A and anti-B sera.

- ABO antigens are also expressed on vascular endothelial cells and epithelial cells of the lung, gastrointestinal tract and genitourinary tract in their secretions. Hence, these ABO antigens are very important in solid organs transplant. ABO matching is considered to be more important than human leukocyte antigen matching. ABO antibodies in the recipient are capable of binding antigens in the transplanted organ, resulting in activation of complement system and acute rejection of transplant.
- ABO antigens are also present in saliva, milk and urine in persons who carry the Se (FUT2) gene in 80% of population. Se (FUT2) gene encodes fucosyltransferase 1 (FUT1) enzyme similar to the H gene product and permits the formation of the H antigen on type 1 glycoproteins versus type 2 glycoproteins present in the secretions synthesized by the epithelial cells. Subsequently, persons who have the appropriate genes coding for A and B glycosyltransferases can synthesize ABO antigens and are called secretors.

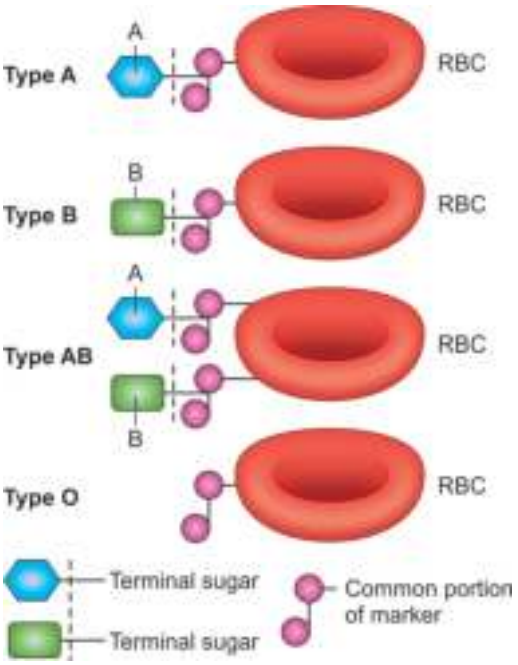


Fig. 12.3. Molecular basis of A and B antigens on the red blood cells. In general persons with blood group A, B and AB inherit a gene for the transferase enzyme that adds a certain terminal sugar to the basic red blood cell membrane. Blood group O persons do not have such an enzyme and lacks terminal sugar.

- Blood group O persons lack A and B glycosyltransferases and therefore only have H antigen present on the surface of the red blood cells. Rare persons lacking H antigen are designated as the 'Bombay phenotype'. These persons lacking 'H' antigen have potent anti-H antibody in addition to anti-A and anti-B antibodies, which must be transfused blood only from other persons with the 'Bombay phenotype'.
- ABO antigens are synthesized by glycosyltransferase enzymes, which participate in addition of terminal monosaccharide to sugars of the backbone carbohydrate chain, which is termed the H antigen. The A glycosyltransferase adds N-acetylgalactosamine in α_1, 3-linkage to the H antigen. The glycosylation may be (a) N-glycosylation, large, branched sugars attached to asparagine residues of the amino acid backbone or (b) O-glycosylation, smaller glycans (usually tetrasaccharides) attached to serine or threonine residues.

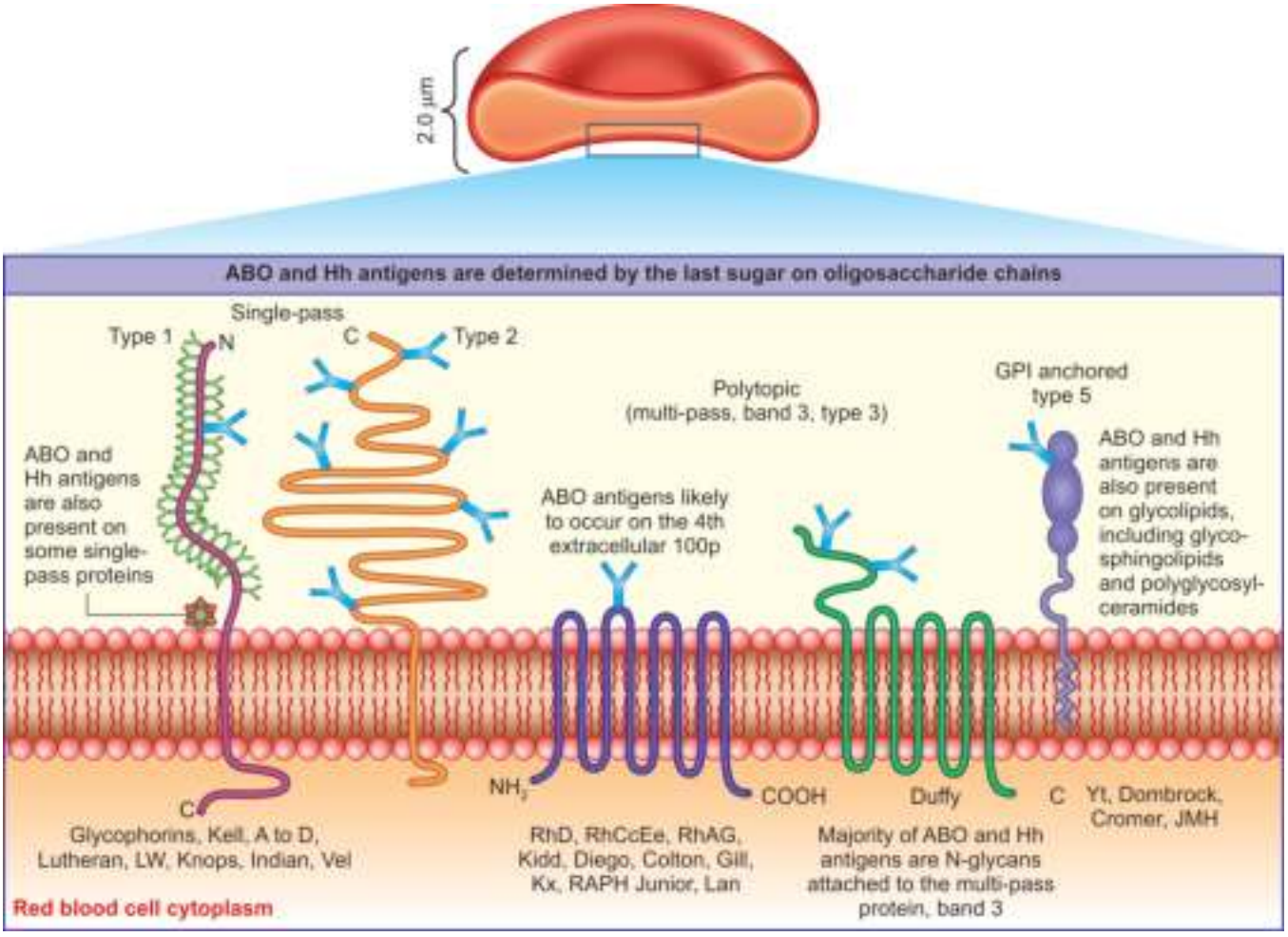


Fig. 12.4: Simplified schematic of different types of blood group active proteins and glycoproteins based on their integration into the red blood cell surface membrane. These are examples of blood group antigens for each type. Type 4 proteins are cytoplasmic and not present in the red blood cells. Single-pass type 1 and type 2 blood group antigens, multi-pass (polytopic) type 3 blood group antigens crossing the red blood cell membrane several times, and GPI anchored 5 blood group antigens are demonstrated.

- The majority of ABO and Hh antigens are N-glycans attached to the multi-pass proteins and band 3. Other multi-pass proteins that carry ABO and Hh antigens include RHAG and band 4.5. ABO and Hh antigens can be less frequently attached as O-glycans.
- ABO and Hh antigens are also present on some single-pass proteins. ABO and Hh antigens are also found on glycolipids, including glycosphingolipids and polyglycosylceramides. Red blood cell (RBC) antigens on RBC membrane include single-pass type 1 and type 2, multi-pass (polytopic) type 3 and GPI anchored type 5 blood group antigens are shown in Fig. 12.4.

Hematology Pearls: Red Blood Cell (RBC) Antigens on RBC Membrane

Red blood cell antigens on RBC membrane include single-pass type 1 and type 2, multi-pass (polytopic) type 3 and GPI anchored type 5 blood group antigens.

Single-pass Type 1 and Type 2 Blood Group Antigens

- Some blood group proteins pass through the RBC membrane, which have an external N-terminal domain and cytoplasmic C-terminal domain.
- Examples of single-pass type 1 and type 2 blood group antigens are Kell proteins A to D, Lutheran, LW, Knops, Indian, Vel.

Multi-pass (Polytopic) Type 3 Blood Group Antigens

- Multi-pass (polytopic) type 3 blood group antigens cross the red blood cell membrane several times.
- Usually both termini are cytoplasmic, but the Duffy glycoprotein has an odd number of membrane-spinning domains and an extracellular domain and an extracellular N-terminal domain.
- The multi-pass proteins that carry ABO and Hh antigens include RHAG and band 4.5.
- Examples of multi-pass (polytopic) type 3 proteins are RhAG, RhD, RhCcEe, Kidd, Diego, Colton, Gill, Kx, RAPH Junior and Lan.

GPI Anchored Type 5 Blood Group Antigens

- Finally, some red blood cell antigens lack membrane spinning domain, but are anchored to the RBC membrane by lipid tail (called glycosylphosphatidylinositol or GPI anchor), which is attached to the C-terminus of the protein through carbohydrate (type 5).
- There is deficient type 4 glycoprotein, which has no external domain in the red blood cell membrane.

Hematology Pearls: ABO Subgroups

- Most common ABO blood group is group O. Genetic variations in the ABO gene can produce subgroups of A, B and AB.
- Individuals have most often variations in A gene than B gene. A_1 and A_2 are the common subgroups of blood group A.
- Antigen frequency is approximately 45% for blood group O, 40% for blood group A, 10% for blood group B, and 5% for blood group AB. ABO subgroups are blood group A, blood group B, blood group AB and blood group O.
- Genes, antigens of the ABH and ABO type of donor RBCs suitable for patients are given in Table 12.5. Major blood group system, their genes and locus on chromosome are given in Table 12.6.

Blood Group A

- Genotype of individuals with blood group A is AA (homozygous) or AO (heterozygous). These individuals have both B and H antigens on red blood cells. Their plasma contains anti-B antibodies of IgM in nature.
- There are two subgroups of blood group A: A_1 (80%) and A_2 (20%). Both A_1 and A_2 antigens have same immunodominant sugar, i.e. N-acetyl-D-galactosamine. Both subgroups A_1 and A_2 antigens encode transferase, α-1,3-N-acetylgalactosaminyltransferase. However, the enzyme encoded by A_1 gene is better at converting H substance to A antigen. Both A_1 and A_2 phenotypes demonstrate 3+ to 4+ reactions with commercial anti-A reagents.
- Anti-A_1 lectin agglutinates only A_1 RBCs. This lectin is used to distinguish the A_1 and A_2 phenotypes in resolving ABO typing problems when A_2 phenotypes develop anti-A_1.
- It is important to identify donors as a weak subgroup of A. Donors with a very weak A antigen expression may be mistyped as blood group O.
- The presence of the antigen can be demonstrated by adsorption (a process by which unwanted antigen is removed from the RBCs) and elution.
- Other methods to resolve the discrepancy include molecular analysis, family studies and saliva analysis.

Blood Group B

Genotype of individuals with blood group B is BB (homozygous) or BO (heterozygous). These individuals have both B and H antigens on red blood cells. Their plasma contains anti-A antibodies of IgM in nature.

Blood Group AB

Genotype of individuals with blood group AB has both A and B antigens on RBCs. Their plasma lacks anti-A and anti-B antibodies. Subgroups of B are extremely rare: B3, Bx, Bel and Bm. Sometimes patients/donors with a very weak subgroup of B will produce a weak anti-B.

Blood Group O

- Genotype of individuals with blood group O contains large amount of H antigen on RBCs.
- 'O' gene synthesizes an inactive transferase resulting in unchanged H antigen. Individuals with blood group O have large amount of H antigen.
- Their plasma contains both anti-A and anti-B antibodies of IgG in nature. IgG can cross the placenta and induce hemolytic disease of fetus and newborn.

Hematology Pearls: Secretors and Nonsecretors

Nonsecretor is the individual who inherits the genotype sese and does not express soluble H substance in secretions. Comparison of secretors and nonsecretors is given in Table 12.7.

Secretors

- Secretor is the individual, who inherits Se allele (amorph Se gene also called FUT2 located on chromosome 19, close to H gene) and expresses soluble forms of H antigens in secretions.
- A, B, and/or H antigens can be present in various fluids in 80% of general population. ABO antigens are present in large quantity in their plasma (except those with Bombay phenotype), saliva, gastric juice, tears, semen, milk and urine.

Nonsecretors

- The Se gene encodes an α2-L-fucosyltransferase that is similar to the enzyme encoded by the H gene. Secretor studies may be useful in identifying a subgroup A or B antigens.
- The people who are 'sese' do not produce any A, B, or H antigen in their secretions are called nonsecretors. These individuals do not produce a functional fucosyltransferase.
- The type 1 precursor substance is not converted to H substance in their secretions, therefore cannot be converted to A or B antigens, regardless of their ABP genotype.
- ABH antigens found in secretions are primarily glycoproteins except ABH antigens found in milk and urine. ABH antigens found in milk and urine are oligosaccharides. ABH antigens found are either glycoproteins or glycosphingo lipids.
- ABH antigens in plasma can adsorb onto the cell membrane of platelets and lymphocytes, these ABH antigens are expressed on cell membrane of the platelets. But granulocytes and monocytes do not possess A, B or H antigens.

Table 12.5 Genes, antigens of the ABH and ABO type of donor red blood cells suitable for patients

Gene	Glycosyltransferase	Antigen Determining Sugar	ABH Antigen	ABO Type	Antibody Present	Preferable Donor	Other Acceptable Donors*
A	α1, 3-N-acetyl-galactosaminyltransferase	N-acetylgalactosamine	A	A	B	A	O
B	α1, 3-D-galactosyltransferase	D-galactose	B	B	A	B	O
H	α1, 2-L-fucosyltransferase	L-fucose	H	O	A and B	O	None
A and B	All of the above	All of the above	A and B	AB	None	AB	A or B or O**

**If blood is used as red blood cells. **Blood group A is preferable.*

Table 12.6 Major blood group system, their genes and locus on chromosome

Blood Group System	Chromosome of Gene Locus	RBC Membrane Associated with Antigen Expression	Antigen Composition
AB blood group system	Chromosome 9	Anionic transport protein (band 3)	Glycoprotein
H blood group system	Chromosome 19	CD173	Glycoprotein
Lewis blood group system	Chromosome 19	Not applicable	Carbohydrate
P blood group system	Chromosome 22	Globoside I	Carbohydrate
Rh blood group system	Chromosome 1	Polypeptides	Lipoprotein
LW blood group system	Chromosome 19	CD242, ICAM; 1qSF	Lipoprotein
MNSs blood group system	Chromosome 4	Glycophorin A, glycophorin B	Glycoprotein
Kell blood group system	Chromosome 7	CD239 endopeptidase	Glycoprotein
Kx blood group system	X chromosome	Unknown	Protein
Duffy blood group system	Chromosome 1	CD234 receptor	Glycoprotein
Lutheran blood group system	Chromosome 19	CD 239 IgSF	Glycoprotein
Gerbich blood group system	Chromosome 2	Glycophorin C, glycophorin D	Glycoprotein
Kidd blood group system	Chromosome 18	Urea transporter	Glycoprotein
Xg blood group system	X chromosome	CD99	Glycoprotein
Colton blood group system	Chromosome 7	Aquaporin	Glycoprotein
Chido/Rogers blood group system	Chromosome 6	C4A; C4B	Glycoprotein
John Multon Hagen blood group system	Chromosome 15	CDW108 semiphorin	Glycoprotein
Raph blood group system	Chromosome 11	Unknown	Glycoprotein
Ok blood group system	Chromosome 19	CD147	Glycoprotein
Diego blood group system	Chromosome 17	Band 3	Glycoprotein
Yt blood group system	Chromosome 7	Acetylcholinesterase	Glycoprotein
Cromer blood group system	Chromosome 1	Decay accelerating factor (DAF)	Glycoprotein
Dombròck blood group system	Chromosome 12	Ribosyltransferase	Glycoprotein
Scianna blood group system	Chromosome 1	Unknown	Glycoprotein
Knops blood group system	Chromosome 1	CD35; CR1	Glycoprotein
Indian blood group system	Chromosome 11	CD44	Glycoprotein
Ii blood group system	Unknown	Glucose transport protein (band 4.5), poly-N-acetyl-lactosaminyl glycolipids	Glycoprotein; glycolipid

Table 12.7 Comparison of secretors and nonsecretors

Features	Secretors (Fluids Containing A, B and H Antigens)	Nonsecretors (Fluids with Absence of A, B and H Antigens)
Genotype	Sese/Sese	Not applicable
Frequency	80%	20%
Plasma	Present in all individuals except Bombay phenotype	Traces/absent
Amniotic fluid, saliva, bile, digestive juices, milk, tears and urine	Present	Absent

ABO AND Hh BLOOD GROUP ANTIBODIES

Immunohematology is study of blood group antigens and antibodies. Most ABO antibodies are IgM. Red blood cell antibodies must be detected and identified to avoid serious blood transfusion reactions.

- Red blood cells of an individual contain antigens on their cell surfaces that correspond to their blood group. Antibodies in the serum that identify such antigens present on surface of red blood cells of another blood group.
- Blood group A has A antigen on their red blood cells and anti-B antibodies in the plasma. **Blood group B** has B antigen on their red blood cells and anti-A antibodies in plasma. **Blood group O** has neither A nor B antigens on red blood cells and anti-A, anti-B, and anti-A, B antibodies in plasma. Blood group AB has both A and B antigens on their red blood cells and neither anti-A nor anti-B in the plasma.
- Blood group antibodies are usually IgM or IgG. Naturally occurring antibodies are usually predominantly IgM, whereas immune antibodies are predominantly IgG.
- IgM antibodies cause directly agglutinate antigen present red blood cells in the saline medium. On the other hand, most IgG antibodies require potentiators or anti-human globulin to affect agglutination. Currently three methods are routinely used to detect antibodies: tube, gel and solid phase testing. Antigens and antibodies in common ABO blood groups are given in Table 12.8. ABO blood group reactions are given in Table 12.9.

Table 12.8 Antigens and antibodies in common ABO blood groups

ABO Blood Group	Antigens Present on Red Blood Cell	Antibodies Present in Plasma
A	A antigen	Anti-B antibody
B	B antigen	Anti-A antibody
AB	Neither A nor B antigens	Anti-A, anti-B, and anti-A, B antibodies
O	Both A and B antigens	Neither anti-A nor anti-B antibodies

Table 12.9 ABO blood group reactions

Phenotype	Reactions with Red Blood Cells with		Reactions with Plasma Tested with		Interpretation
	Anti-A Serum	Anti-B Serum	A1 Red Blood Cells	B Red Blood Cells	ABO Blood Group
Group A	+	0	0	+	0
Group B	0	+	+	0	A
Group O	0	0	+	+	B
Group AB	+	+	0	0	AB

+: Agglutination; O: No agglutination; Blood group O lacks both A and B antigens. Blood group AB lacks circulating ABO antibodies in their plasma.

Rh BLOOD GROUP SYSTEM

Landsteiner and Wiener in 1941, injected rhesus monkey red blood cells into the rabbits and guinea pigs and observed increase in antibodies titer. Red blood cells of rhesus monkey and human (white residents of New York city) have similar antigens. Antigen involved was called Rh factor. In 1939, Levin and Steven later discovered anti-D antibody that is responsible for hemolytic disease of newborn.

ANTIGENS OF Rh BLOOD GROUP SYSTEM

Rh blood group system has about 40 antigens. Most antigens are **C, D, E, c, d** and **e**. The genes encoding Rh proteins determine the phenotype of person. A related protein, the Rh glycoprotein is essential for assembly of Rh complex in the red blood cell membrane and expression of Rh antigens. CDE (Fisher-Race) and Dr Alexander Wiener nomenclature systems are employed for Rh blood group inheritance.

CDE (FISHER-RACE) NOMENCLATURE SYSTEM

According to Fisher-Race nomenclature of Rh blood group system, three closely linked loci located on chromosome 1 are inherited from each parent. The alleles occupying these loci are known as C, D, E, c, d and e. Out of these, **D gene is dominant over d gene**. On the other hand, loci such as C, c, E and e are codominant. Each gene (except d gene) encodes a specific antigen on RBCs. Each person inherits one haplotype from each parent making 8 possible genotypes (Fig. 12.5).

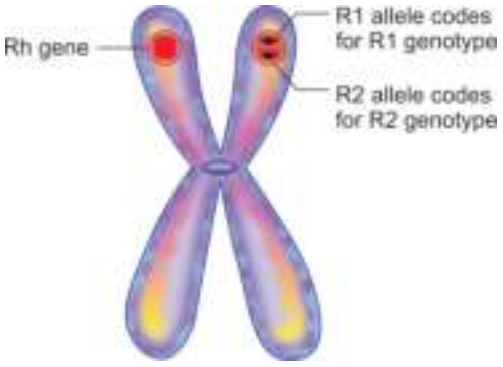


Fig. 12.6: Dr Alexander Wiener nomenclature of Rh blood group system.

Dr ALEXANDER WIENER NOMENCLATURE SYSTEM

According to Dr Alexander Wiener nomenclature of Rh blood group system, single Rh gene having multiple alleles is inherited from each parent. R_1 gene encodes Rh1 antigen comprising three factors, which correspond with C, D, and e of Fisher-Race nomenclature system. Similarly, R_2 gene encodes one Rh_2 antigen comprising factors, which correspond with Fisher-Race nomenclature system (Fig. 12.6).

CURRENT GENETIC ANALYSIS NOMENCLATURE SYSTEM

According to current genetic analysis nomenclature system, RHCE and RHD genes are located on chromosome 1. Presence of D gene makes the person Rh positive, while Rh negative lacks D antigen. RHCE gene can synthesize C or c, CE and ce (Fig. 12.7).

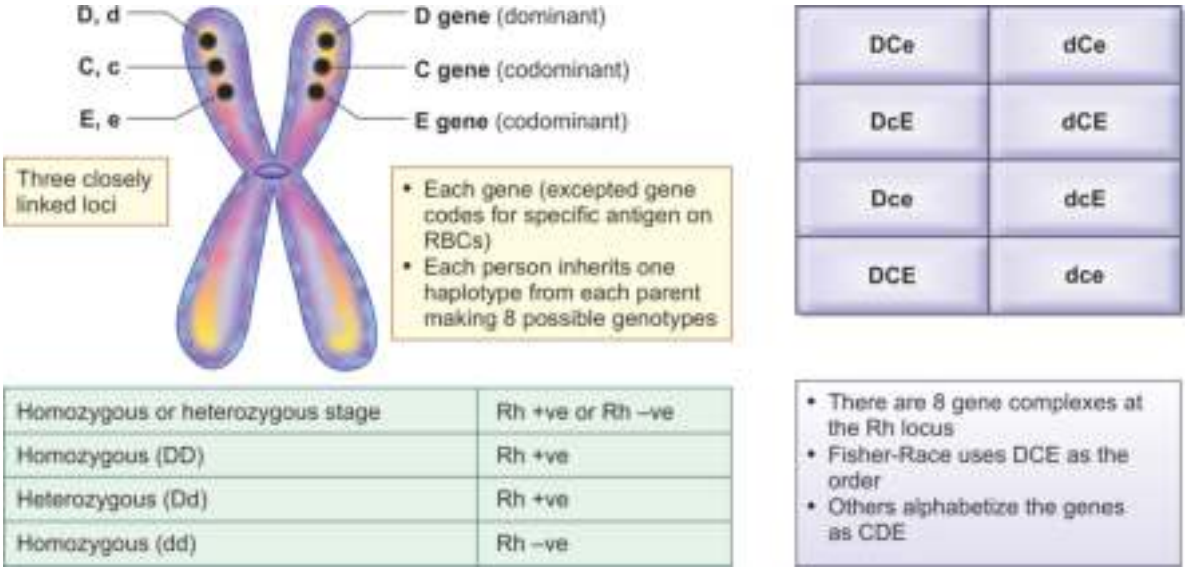


Fig. 12.5: Fisher-Race nomenclature system of Rh blood group inheritance.

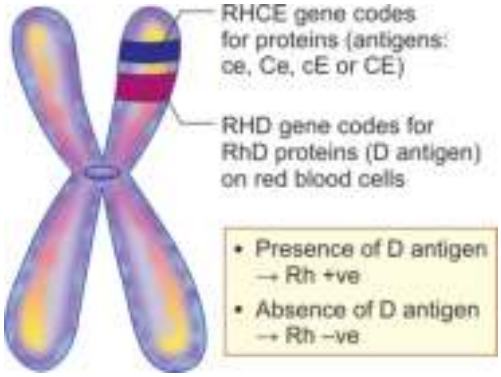


Fig. 12.7: Current genetic analysis nomenclature system of RHCE and RHD genes.

Rh FACTOR INCOMPATIBILITY DURING PREGNANCY

Rh factor incompatibility can cause lysis of red blood cells known as hemolytic disease of newborn. Naturally occurring red blood cell incompatibility results when Rh positive (+ve) fetus develops within Rh negative (–ve) mother. Initial sensitization of the immune system occurs when fetal red blood cells pass the placental barrier. In most cases, the fetus develops normally. However, subsequent pregnancy with Rh +ve fetus results in severe fetal red blood cell hemolysis and causes hemolytic disease of newborn. Pathogenesis of hemolytic disease of newborn and its prevention is shown in Fig. 12.8A to C.

ANTI-D ANTIBODY (RhoGAM) ADMINISTRATION

Routine antenatal prophylaxis is to infuse 500 IU anti-D antibody (RhoGAM) to Rh –ve mothers at 28 weeks and 34 weeks of pregnancy to inactivate and remove any Rh factor that may be transferred from the fetus in maternal circulation. Post-delivery anti-D immunoglobulin must be administered to Rh –ve mother, if an infant is Rh +ve.

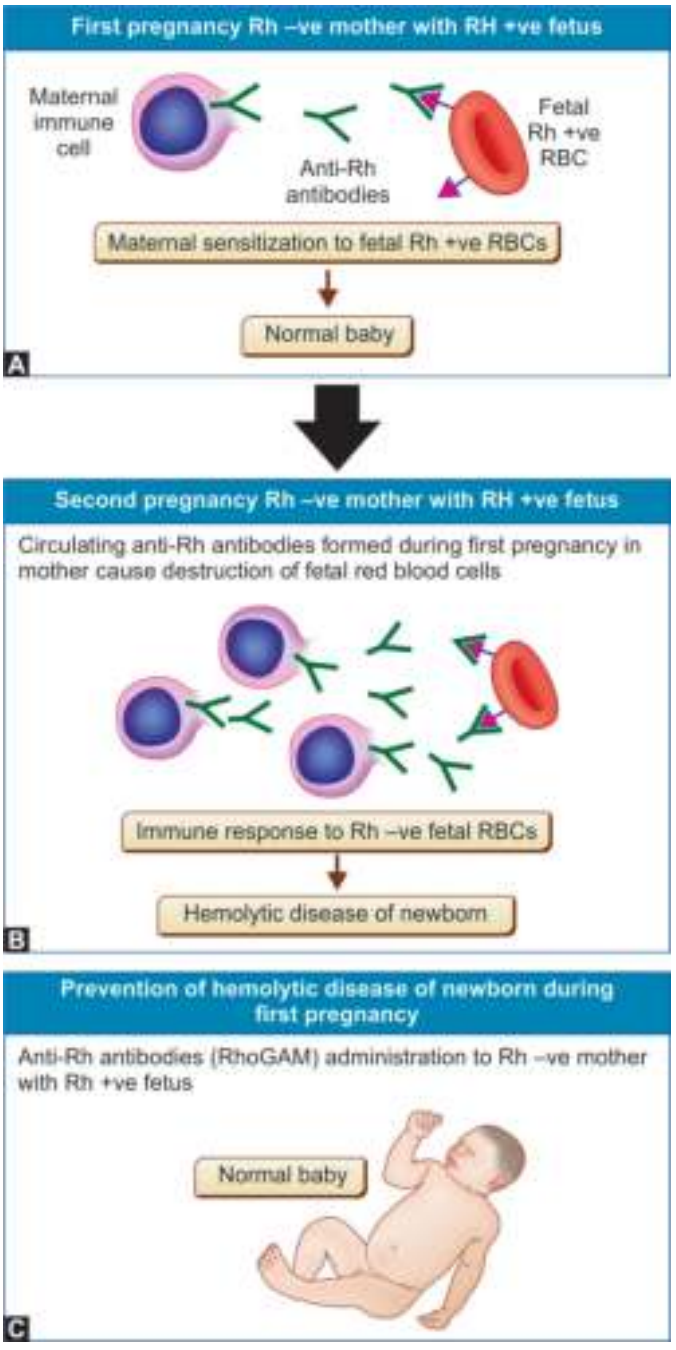


Fig. 12.8A to C: Pathogenesis of hemolytic disease of newborn and its prevention.

BOMBAY PHENOTYPE AND PARA-BOMBAY PHENOTYPE

Bombay phenotype persons lack H substance on the surface of RBCs and anti-H in their secretions. There are some individuals, who appear to be Bombay phenotype, but have very small amounts of A and/or B antigen on the surface of RBCs.

BOMBAY PHENOTYPE

Bombay blood group is a rare blood group reported by Dr YM Bhende in 1952 in India. **Bombay phenotype results from hh and sese genotypes**.

These individuals lack H substance on the surface of RBCs and anti-H in their secretions. Their RBCs and secretions also lack A and/or B antigens, because there is no H substance to be converted to A and/or B antigens.

- Red blood cells of Bombay phenotype appear to be group O in ABO blood grouping analysis.
- Bombay phenotype has autosomal recessive pattern of inheritance due to mutation of H gene on chromosome 19 that leads to synthesis of non-functional H-glycosyltransferase. Their serum contains anti-A and anti-H antibodies, which react with all with O blood group. These persons are nonsecretors and not demonstrated in body fluids.

PARA-BOMBAY PHENOTYPE

There are some individuals who appear to be Bombay phenotype, but have very small amounts of A and/or B antigens on their red blood cells. It was discovered that their genotype is hh but they possess at least one Se gene. The Se gene synthesizes a fucosyltransferase that acts on type 1 precursor substance in fluids and secretions.

- The H substance type 1 formed by the Se gene can be converted into A and/or B antigens depending on which ABO genes are inherited.
- Red blood cell membrane of para-Bombay individuals lacks intrinsic A, B, or H antigens, because the type 2 precursor substance on their RBCs remains unconverted due to lack of H gene.

OTHER BLOOD GROUP SYSTEMS

Blood group systems other than ABO Rh are Lewis, Kell, MNS, Duffy, P, Kidd, Lutheran, Yt, Dombròck, Diego, Colton, Indian, XG, Landsteiner-Wiener and John-Milton Hagen systems. Lewis, Kell, MNS, Duffy, P, Kidd blood group systems are given in Table 12.10.

Table 12.10 Lewis, Kell, MNS, Duffy, P, Kidd blood group systems

Blood Group System	Gene Locus	Antigens	Antibodies	Comments
Lewis blood group system	Lewis (Le) gene on chromosome 19 codes for fucosyltransferase	Present on glycosphingolipids (integral part of RBC)	IgM (not crossing placenta, hence does not cause hemolytic disease of newborn)	Lewis antigens present in plasma are passively adsorbed on RBCs
Kell blood group system	Kell locus on chromosome 7p23	Present on surface of RBC	IgG (crossing placenta, hence causing hemolytic disease of newborn)	▪ Acute hemolytic blood transfusion reaction (10%) ▪ Anemia but no hemolysis in hemolytic disease of newborn ▪ Some persons express weak Kell antigens resulting in McLeod syndrome characterized by acanthocytes, progressive cardiomyopathy, muscle wasting and neurological disorders
MNS blood group system	MNS genes created by swapping of DNA between GYPA and GYPB genes lying close together on chromosome 4	MN antigens present on glycophorin A, while S antigen on glycophorin B on surface of RBC	IgM	▪ Hemolytic blood transfusion reaction in rare case ▪ Hemolytic disease of newborn is rare case ▪ Anti-M and anti-N antibodies are rarely associated with transfusion reactions ▪ Anti-S antibody is formed following sensitization to transfusion and determined by indirect Coombs' test
Duffy blood (Fy) group system	Duffy antigens are resistant to *Plasmodium vivax*	Fya and Fyb present in glycoprotein of RBCs	IgG (crossing placenta, hence causing hemolytic disease of newborn)	▪ Anti-Fya more common ▪ Hemolytic blood transfusion reaction in occasional case

Contd...

Table 12.10 Lewis, Kell, MNS, Duffy, P, Kidd blood group systems (*Contd...*)

Blood Group System	Gene Locus	Antigens	Antibodies	Comments
				▪ Hemolytic disease of newborn in occasional case ▪ *Plasmodium vivax* is commonly seen in Duffy negative antigen
P blood group system	P blood group system represented by two chromosome loci	P1 and P2 antigens on surface of RBC most important (other antigens P+, P–, Pk)	IgG	▪ P negative persons are resistant to Parvovirus B19 infection ▪ Auto-anti-P persons are demonstrated in paroxysmal nocturnal hemoglobinuria
Kidd blood group system	Kidd/urea transporter gene	Jks on RBC	IgG	▪ Dangerous fatal ('killer Kidd') ▪ Hemolytic blood transfusion reaction in occasional case ▪ Hemolytic disease of newborn in occasional case ▪ IgG causes delayed hypersensitivity reaction and mild hemolytic disease of newborn

BLOOD COLLECTION, PROCESSING AND STORAGE

DONOR SELECTION CRITERIA

Donor selection criteria must meet safety criteria to protect both donor and recipient. Blood donor must have age 17–60 years, hemoglobin ≥13 g/dl for men and 12 g/dl for women, minimal donation interval of 12 weeks (16 weeks advised) and three donations per year maximum, afebrile, normal pulse and blood pressure. There should not be evidence of skin infections in antecubital fossa of both arms. Donor exclusion criteria include intravenous drug abuse, positivity for HBV, HCV, HIV, syphilis and malaria; pregnant and lactating woman due to increased iron requirement, insulin-dependent diabetes mellitus, chronic diseases related to urinary system, cardiovascular system, respiratory system and central nervous system. Donor selection and exclusion criteria are given in Table 12.11.

BLOOD DONATION PROCESS

Under aseptic conditions, large 16 gauze needle is used to puncture antecubital vein and ensure uninterrupted continuous blood flow in a span of 8 minutes. Approximately 30% of donors during donation process may develop local or systemic complications.

- Local adverse effects include pain, hematoma, accidental arterial puncture, thrombophlebitis, allergic rash and infection.

Table 12.11 Donor selection and exclusion criteria

Donor Selection Criteria
▪ Age 17–60 years ▪ Weight >50 kg ▪ Hemoglobin ≥13 g/dl for men and ≥12 g/dl for women ▪ Hematocrit ≥38% ▪ Temperature ≤37.50°C (afebrile) ▪ Minimal donation interval of 12 weeks (16 weeks advised) and three donations per year maximum ▪ Pulse rate 50–100 beats/minute without pathological abnormalities or <50 beats/minute in athletes ▪ Normal blood pressure
Donor Exclusion Criteria
▪ Donor showing positivity for HBV, HCV, HIV, syphilis and malaria ▪ Intravenous drug abuse ▪ Pregnant and lactating woman due to increased iron requirement ▪ Donation deferred for nine months post-pregnancy ▪ Insulin-dependent diabetes mellitus ▪ Cardiovascular diseases including hypertension ▪ Significant respiratory disorders ▪ Epilepsy and another CNS disorders ▪ Chronic renal disease ▪ Defer blood donation for six months after body piercing or tattoo, after acupuncture ▪ Defer blood donation for two months after vaccinations, e.g. measles, mumps ▪ Exclusion of any donor returning to driving bus, plane or heavy machine or crane operator, mining because delayed faint would be dangerous

- Systemic complications include vasovagal shock, syncope, nausea, vomiting, fatigue, cerebral stroke and myocardial infarction.
- Each blood donation removes 200–250 g of iron. Blood collected in >8 minutes is unsuitable for preparation of blood components such as platelet concentrate, fresh frozen plasma and cryoprecipitate. Blood bag should be stored at 20–25°C within 6–8 hours of collection for harvesting platelets.
- Double pack system is used to prepare red blood cells and plasma under sterile closed system conditions.
- Triple pack system is used to prepare red blood cells, platelet concentrate and fresh frozen plasma.
- In quad pack system, the blood collection center prepares a unit of red blood cells and divides it into four bags with equal volume under sterile closed system conditions used for pediatric and neonatal multiple blood transfusions in these patients.

ANTICOAGULANT PRESERVATIVES USED IN BLOOD BANK

In India, blood collection, processing, storage and preparations of blood components are regulated by Food and Drug Administration (FDA).

- Anticoagulants are used to prevent the activation of coagulation factors and to maintain blood in the liquid state for blood transfusion. Blood bags also contain preservatives that extend the shelf-life of the blood components.
- The anticoagulant preservative solutions approved in the blood bank include citrate-phosphate-dextrose (CPD), citrate-phosphate-dextrose-dextrose (CP2D), acid-citrate-dextrose (ACD), and citrate-phosphate-dextrose-adenine solution 1 (CPDA-1).
- Constituents of approved blood bank anticoagulant preservative solutions are given in Table 12.12. Contents of anticoagulant preservative solutions are given in Table 12.13.

ACID-CITRATE-DEXTROSE

Acid-citrate-dextrose (ACD) contains trisodium citrate (22 g), citric acid (8 g), and dextrose (24.5 g) dissolved in 1000 ml of distilled water with initial pH 5.6. Each blood bag of 450 ml contains 67.5 ml of ACD preservative. Initial pH of preservative is 5.6. Initial pH of blood bag on first day is 7. Storage time of blood bag is 21 days at 2–6°C.

CITRATE-PHOSPHATE-DEXTROSE

Citrate-phosphate-dextrose (CPD) contains trisodium citrate (26.30 g), citric acid (3.27 g), dextrose (25.5. g), and monobasic sodium phosphate (2.22 g) dissolved in 1000 ml of distilled water with initial pH 5.6. Each blood bag of 450 ml contains 63 ml of preservative. Initial pH of preservative is 5.6. Initial pH of blood bag on first day is 5.6. Storage time of blood bag is 21 days at 2–6°C.

CITRATE-PHOSPHATE-DEXTROSE-ADENINE 1

Citrate-phosphate-dextrose-adenine 1 (CPDA-1) contains trisodium citrate (26.35 g), citric acid (3.27 g),

Table 12.12 Constituents of approved blood bank anticoagulant preservative solutions

Constituents of Anticoagulant Preservative Solutions	Purpose	Blood Bank Preservative
Sodium citrate	Sodium citrate binds calcium and prevents coagulation cascade	▪ Citrate-phosphate-dextrose (CPD) ▪ Citrate-phosphate-dextrose-dextrose (CP2D) ▪ Acid-citrate-dextrose (ACD) ▪ Citrate-phosphate-dextrose-adenine 1 (CPDA-1)
Citric acid	Citric acid slows down glycolysis that occurs through cell metabolism	▪ Citrate-phosphate-dextrose (CPD) ▪ Citrate-phosphate-dextrose-dextrose (CP2D) ▪ Acid-citrate-dextrose (ACD) ▪ Citrate-phosphate-dextrose-adenine 1 (CPDA-1)
Dextrose	Dextrose supports generation of adenosine triphosphate (ATP) by glycolytic pathways for cell metabolism	▪ Citrate-phosphate-dextrose (CPD) ▪ Citrate-phosphate-dextrose-dextrose (CP2D) ▪ Acid-citrate-dextrose (ACD) ▪ Citrate-phosphate-dextrose-adenine 1 (CPDA-1)
Monobasic sodium phosphate	Monobasic sodium phosphate (buffer) maintains pH of blood bags	▪ Citrate-phosphate-dextrose (CPD) ▪ Citrate-phosphate-dextrose-dextrose (CP2D) ▪ Citrate-phosphate-dextrose-adenine 1 (CPDA-1)
Adenine	Adenine maintains ATP levels during cell metabolism, thus extending shell-life of red blood cells to 42 days	Citrate-phosphate-dextrose-adenine 1 (CPDA-1)

Table 12.13 Contents of anticoagulant preservative solutions

Contents of Preservative Solutions	Anticoagulant Preservatives used in Blood Bank			
	Anticoagulant-Citrate-Dextrose (ACD)	Anticoagulant-Citrate-Phosphate-Dextrose (CPD)	Anticoagulant-Citrate-Phosphate-Dextrose-Dextrose (CP2D)	Anticoagulant-Citrate-Phosphate-Dextrose-Adenine 1 (CPDA-1)
Trisodium citrate	22.0 g/L	26.3 g/L	26.3 g/L	26.3 g/L
Citric acid	8.0 g/L	3.7 g/L	3.7 g/L	3.7 g/L
Monobasic sodium phosphate	Nil	2.22 g/L	2.22 g/L	2.22 g/L
Dextrose	24.5 g/L	24.5 g/L	51.1 g/L	31.9 g/L
Adenine	Nil	Nil	Nil	0.275 g/L
Distilled water	1000 ml	1000 ml	1000 ml	1000 ml
Anticoagulant preservative used per 450 ml of blood	67.5 ml	63 ml	63 ml	63 ml
Initial pH of prepared anticoagulant preservative	5.6	5.6	5.6	5.6
Initial pH of anticoagulant preservative in blood bag on first day	7.0	7.2	7.2	7.3
Shelf life	21 days	21 days	21 days	35 days

dextrose (31.9 g) and monobasic sodium phosphate (2.22), and adenine (0.27 g) dissolved in 1000 ml of distilled water with initial pH 5.6. Initial pH of preservative is 5.6. Each blood bag of 450 ml contains 63 ml of CPDA-1 preservative. Initial pH of blood bag on first day is 7. Storage time of blood bag is 42 days at 6°C.

PREPARATION OF BLOOD COMPONENTS FROM WHOLE BLOOD

Whole blood is collected from a donor into a plastic bag containing anticoagulant preservative. The volume of the anticoagulant preservative in the collection bag varies based on the target amount for whole blood collection volume in order to maintain appropriate ratio of the anticoagulant preservative to the whole blood.

- Generally, 63 ml of anticoagulant preservative is used to collect a target volume of 450 ml of whole blood. The blood collection set also includes a number of satellite containers also known as satellite collection bags that are integrally attached to the main collection bag with hollow tubing.
  - Satellite collection bags become storage sites for the blood components prepared from whole blood.
  - The entire closed system prevents bacterial contamination of blood from outside environment. Blood component preparation and donor blood processing may occur simultaneously. Processing of whole blood units from collection to labeling is shown in Fig. 12.9.
- Separation of whole blood into blood components is also moderately expansive. Due to different specific gravity of blood components, these are separated by

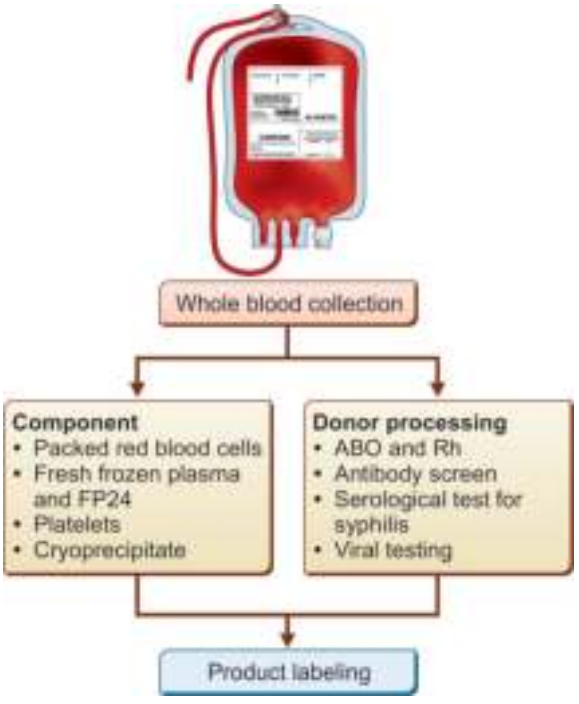


Fig. 12.9: Processing of whole blood units from collection to labeling.

centrifuging blood bag at different centrifugal force for different time. Packed red blood cells are obtained and stored at 1–6°C. Plasma containing platelets are obtained and centrifuged.

- Separated plasma is stored at <18°C. Separated platelet concentrate is stored at 20–40°C. Fresh frozen plasma is prepared within twenty-four hours and used to prepare cryoprecipitate, immunoglobulin, albumin and coagulation factor concentrate. Preparation of plasma products from whole blood is shown in Fig. 12.10.
- Each unit of blood (450 ml) contains 65 g of hemoglobin, 225 mg of iron, platelets and coagulation factors. Coagulation factors V and VIII begin to deteriorate within an hour of blood collection. Refrigerated red blood cells are viable up to six weeks.
- Production of red blood cell component, plasma, buffy coat pool and pooled platelet component is shown in Fig. 12.11.
- Preparing blood components from whole blood allow multiple patients to benefit from a single blood donation unit. Production of red blood cell component, platelet-rich plasma, fresh frozen plasma, plasma cryoprecipitate reduced and cryoprecipitate antihemophilic factor from whole blood is shown in Fig. 12.12.

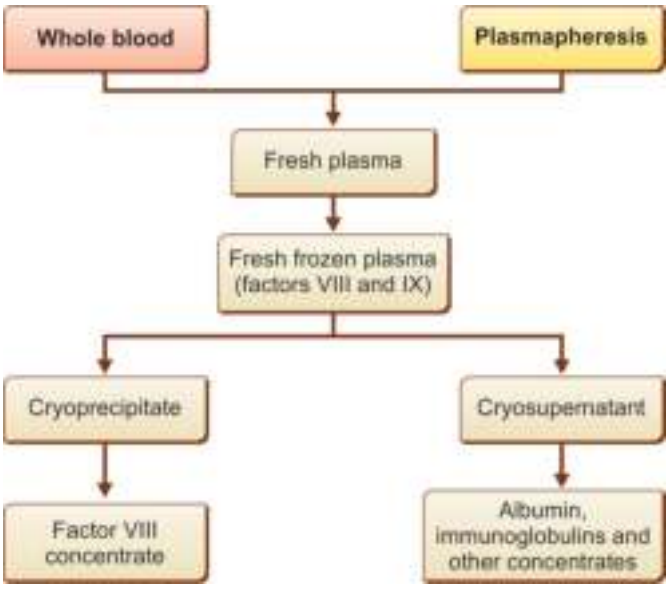


Fig. 12.10: Preparation of plasma products from whole blood.

WHOLE BLOOD COMPOSITION

Whole blood contains plasma and cellular component, which is not commonly used anymore because most patients do not require all the elements in the whole blood. Fresh whole blood is sometimes used by defense

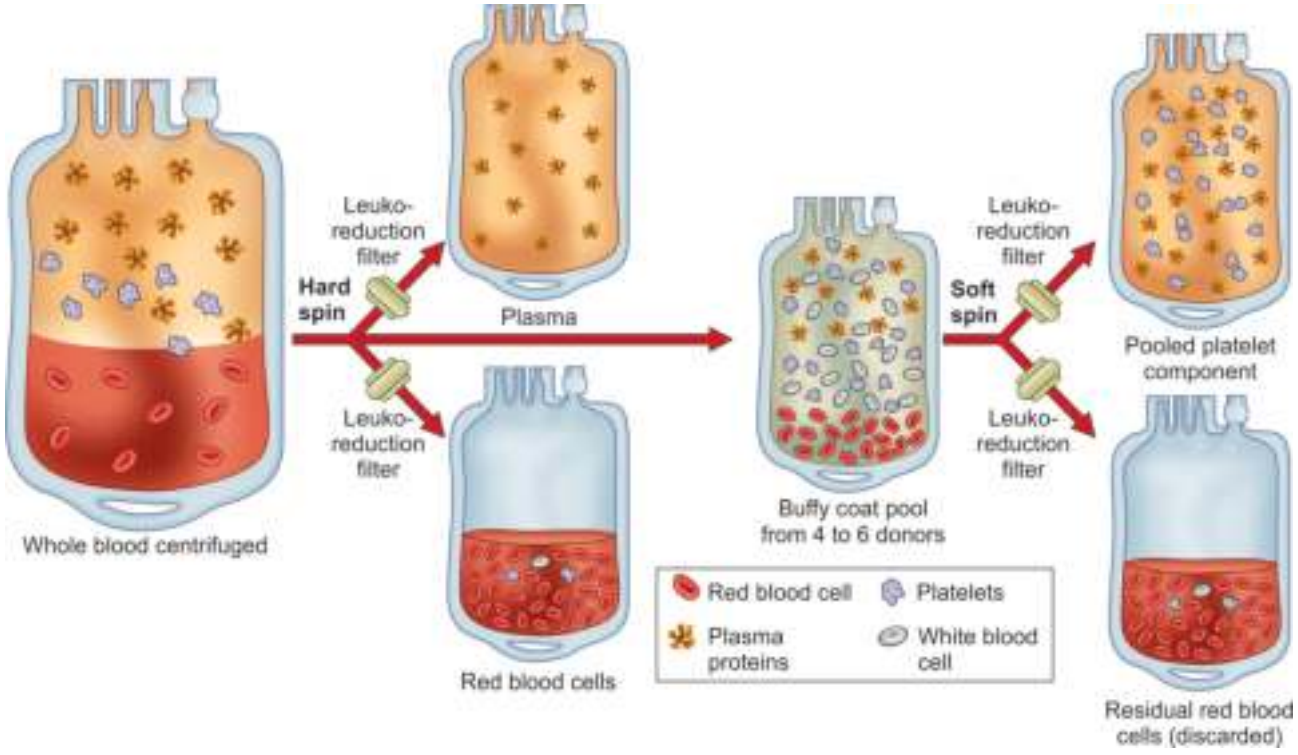


Fig. 12.11: Production of RBC component, plasma and buffy coat is done by hard spin and using leukoreduction filter. Buffy coat pool from 4 to 6 donors is centrifuged with soft spin by using leukoreduction filter to prepare pooled platelet component.

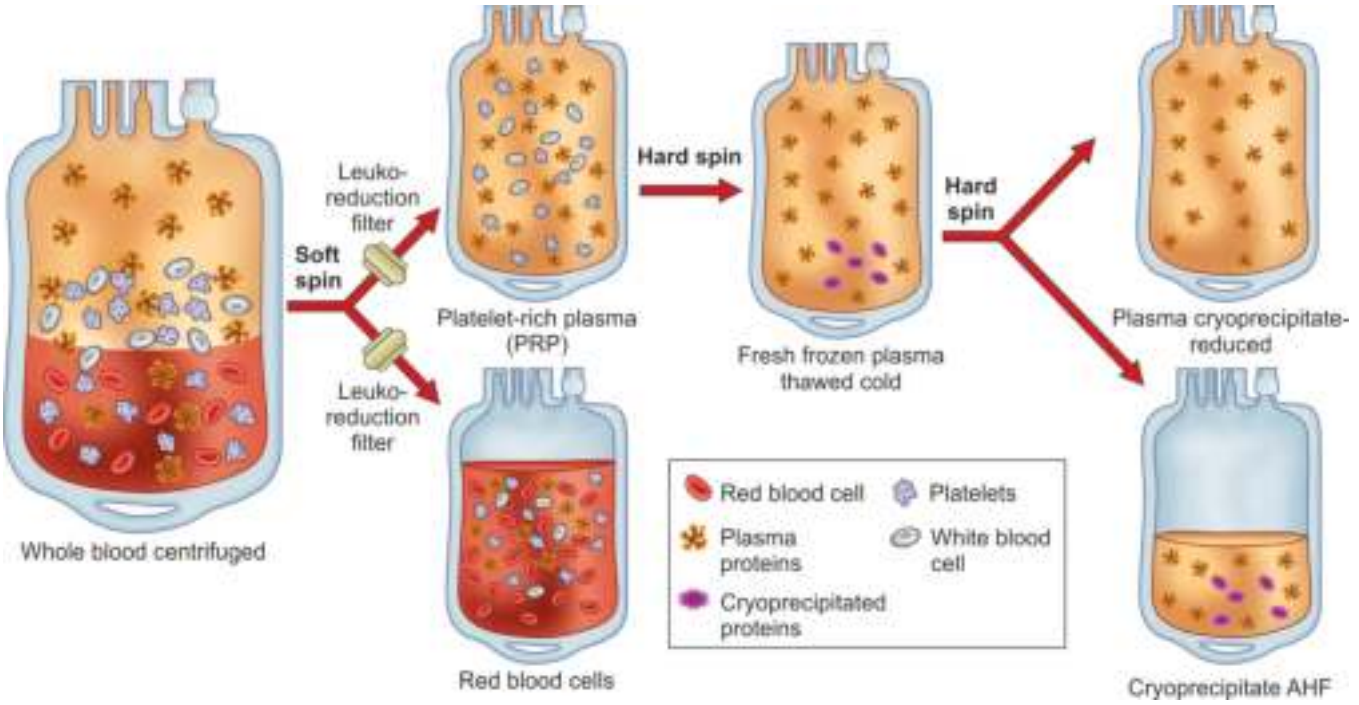


Fig. 12.12: Production of RBC component and platelet-rich plasma is prepared by centrifuging whole blood with soft spin and using leukoreduction filter. Platelet-rich plasma is centrifuged with hard spin to prepare fresh frozen plasma, plasma cryoprecipitate-reduced and cryoprecipitate antihemophilic factor.

forces in cases of requiring massive blood transfusion support.

RED BLOOD CELL COMPONENT

Red blood cell component is prepared by centrifuging whole blood in large centrifuge. Centrifugation results in settling of heaviest cellular components at the bottom of the collection bag and lighter components at the top. In general, RBCs tend to settle at the bottom of the container followed by white blood cells and platelets, which settle at top of the RBC layer.

- After centrifugation, the blood unit is placed into a device known as a 'plasma press' also known an expressor. Once the plasma has been pressed, the resultant RBCs and plasma bags are separated by using a tubing sealer. Tube sealers ensure the closure of the system for maintenance of sterility.
- RBC component in blood bag has volume of 225–350 ml and hematocrit of 65–80%. Transfusion of one unit of RBC component increases 1 g/dl in recipient, who is not actively bleeding.
- Typical RBC component unit prepared from whole blood contains 50–80 g of hemoglobin.

PLASMA COMPONENT

Plasma separated from whole blood can be used depending on the collection, processing and storage conditions.

- Plasma is frozen to preserve the activity of labile coagulation factors such as factor V and factor VIII. Stored frozen plasma must be thawed prior to transfusion.
- Fresh frozen plasma can be further processed to produce cryoprecipitate containing antihemophilic factor (factor VIII).
- On a large scale, plasma from 2500 donors are pooled and treated with solvent/detergent solution, which inactivates lipid-enveloped viruses such as hepatitis B virus (HBV), human immunodeficiency virus (HIV) and cytomegalovirus (CMV). Viruses without envelops are not inactivated by solvent/detergent and hence treated with second process to inactivate them.
- The pooled plasma is stored frozen into 200 ml bags. The process leads to slight reduction in all coagulation system proteins. But its use is similar to fresh frozen plasma. Plasma can be separated into individual proteins such as immunoglobulin, albumin and specific coagulation factors. Preparation of plasma cryoprecipitate-reduced and cryoprecipitate containing antihemophilic factor is shown in Fig. 12.13. Plasma fractionation process is used to prepare immunoglobulin, albumin and coagulation factor concentrate from plasma pool as shown in Fig. 12.14.

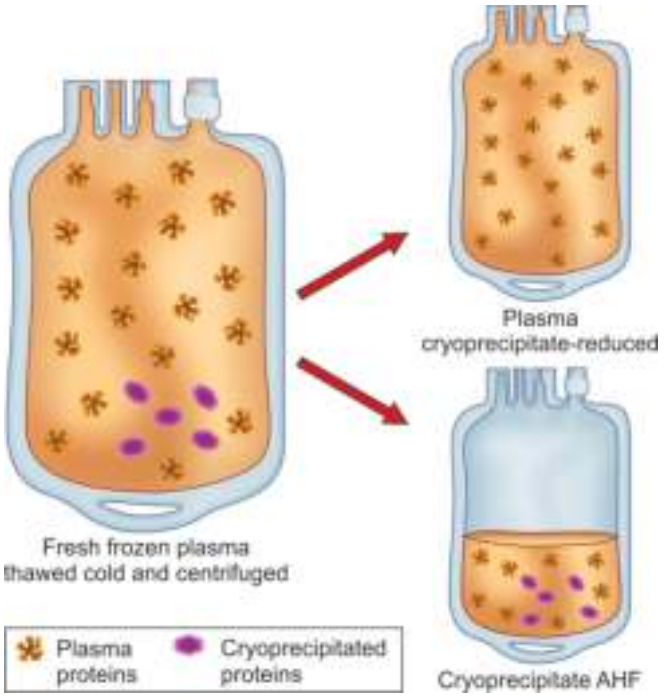


Fig. 12.13: Preparation of plasma cryoprecipitate-reduced and cryoprecipitate containing antihemophilic factor by thawing and centrifuging fresh frozen plasma.

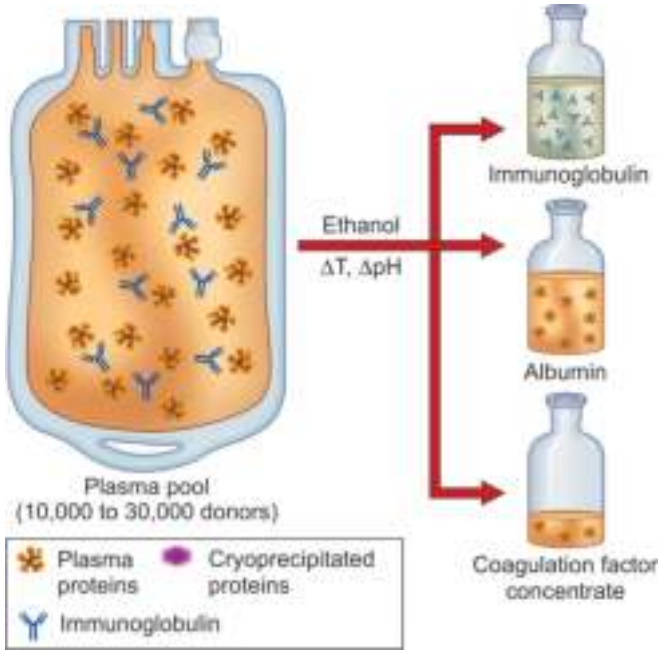


Fig. 12.14: Plasma fractionation process is used to prepare immunoglobulin, albumin and coagulation factor concentrate from plasma pool of 10,000 to 30,000 donors.

PLATELET COMPONENT

Platelet function is impaired following storage at colder temperature. Therefore, donated blood bags must be maintained at room temperature during all stages of collection, storage and production of platelet component. Platelet components are prepared by platelet-rich plasma (**PRP**) and buffy coat methods.

Platelet-rich Plasma Method to Prepare Platelet Component

Platelet-rich plasma method produces single platelet component from single whole blood donated bag. It is achieved by centrifuging the whole blood at low relative centrifugal force. Soft centrifugal spin produces platelet-rich plasma. Hard spin leads to platelets concentrate. Platelet-rich plasma is obtained by resuspension of aggregated platelets into supernatant plasma. Production of platelet-rich plasma component by centrifuging whole blood by soft centrifuge spin is shown in Fig. 12.15. Production of platelet component and plasma by centrifuging platelet-rich plasma with hard centrifuge spin is shown in Fig. 12.16.

- **Advantages of platelet-rich plasma method:** Platelet-rich plasma is used in pediatric age group. RBCs are not lost during the processing.
- **Disadvantages of platelet-rich plasma method**: Concentrated platelets into pellet causes platelet activation and platelet aggregation. Soft centrifugal force of whole blood traps a significant amount of plasma and platelets into the red blood cell layer.

Buffy Coat Method to Prepare Platelet Component

Buffy coat method produces a pool of platelets from four to six whole blood donated bags. Hard centrifugal spin

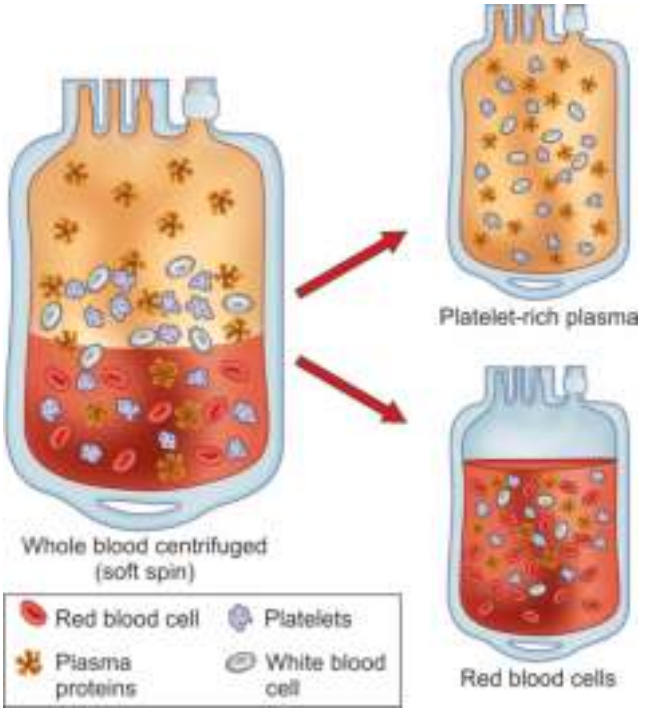


Fig. 12.15: Production of platelet-rich plasma component from whole blood by soft centrifuge spin.

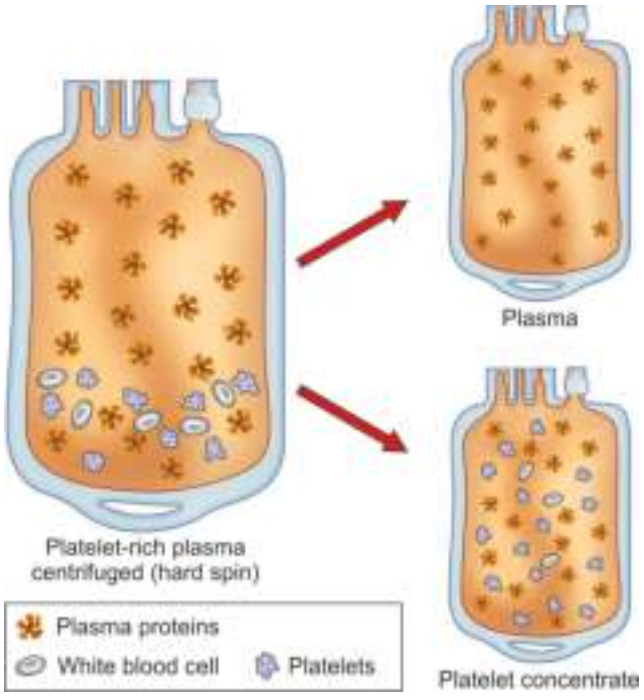


Fig. 12.16: Production of platelet component and plasma by centrifuging platelet-rich plasma with hard centrifuge spin.

is used to concentrate platelets in the buffy coat layer. Pooling and soft centrifugal spin is used to remove RBC contamination. Production of RBC component, plasma and buffy coat pool of platelets by centrifuging with hard spin of whole blood obtained from 4 to 6 donors is shown in Fig. 12.17. Production of pooled platelet component by centrifuging buffy coat pool with soft centrifuge spin of the buffy coat pool obtained from 5 to 6 donors is shown in Fig. 12.18.

- **Advantages of buffy coat method:** Concentrating platelets in the buffy coat layer reduce platelet activation resulting in better platelet quality. Hard centrifugal spin of whole blood minimizes the amount of plasma and platelets trapped in the red blood cell layer.
- **Disadvantages of buffy coat method:** Pooled product is not ideal for pediatric age group. Some RBCs are lost in the buffy coat. Buffy coat method requires the use of specialized equipment such as semi-automated plasma press and sterile docking device.

GRANULOCYTE COMPONENT

Granulocyte component is usually not prepared from whole blood. Apheresis technique is used to achieve higher number of granulocytes. Granulocytes component can be prepared from whole blood by isolating the buffy coat. Hydroxyethyl starch (**HES**), is a precipitating agent added to the whole blood and then the blood bag is left to settle by gravity. The plasma and buffy layer are then extracted into satellite container using manual or semiautomatic press. The satellite container is then centrifuged using hard spin to concentrate the granulocytes. Granulocyte component prepared from the whole blood contains 1.25×10^9 granulocytes.

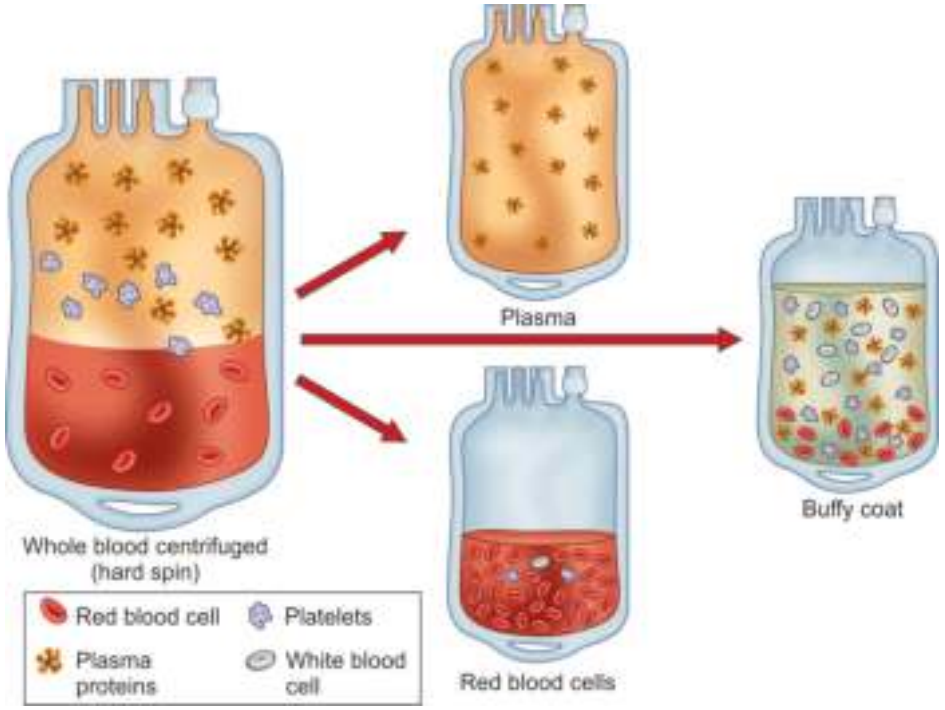


Fig. 12.17: Production of RBC component, plasma and buffy coat pool of platelets by hard centrifuge spin of whole blood obtained from 4 to 6 donors.

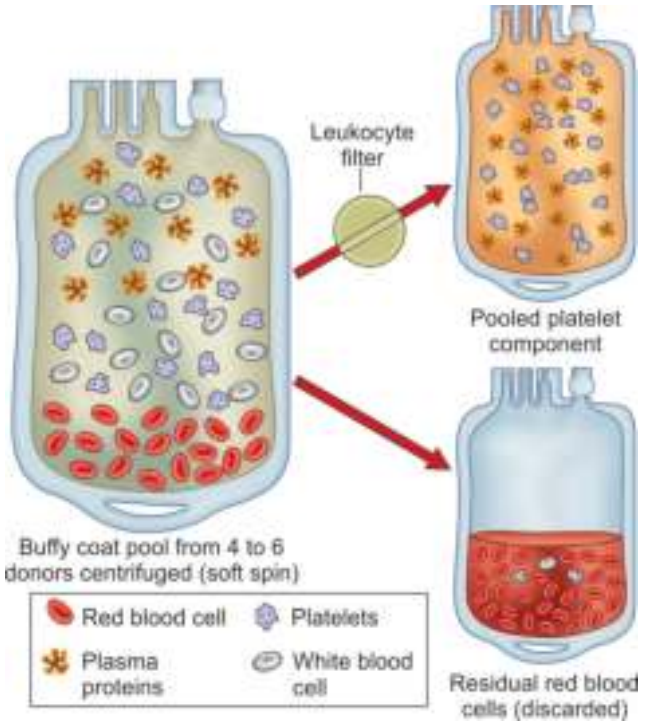


Fig. 12.18: Production of pooled platelet component by centrifuging buffy coat pool with soft centrifuge spin of the buffy coat pool obtained from 5 to 6 donors.

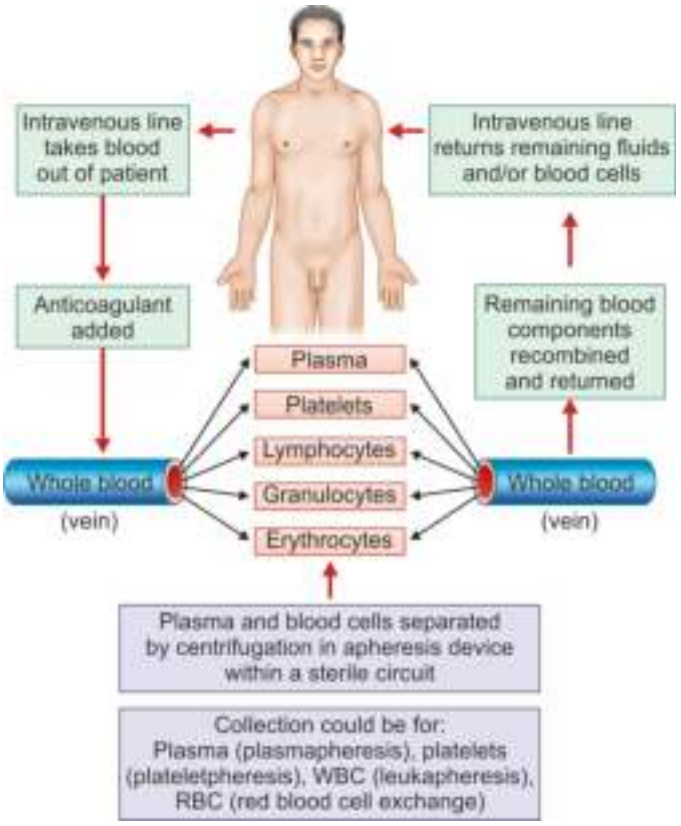


Fig. 12.19: Principle of apheresis: Apheresis is a technique by which a particular blood component is removed from the whole blood by using machine, and the main volume is returned back to the donor through the same collection line.

REMOVAL OF BLOOD COMPONENTS BY APHERESIS

Apheresis is a technique by which particular blood component is removed from the whole blood by using machine, and the main volume is returned back to the donor through the same collection line. The separation of the blood components is based on the specific gravity of each individual blood constituent. The entire process occurs in a closed system. Principle of apheresis is shown in Fig. 12.19. Intravenous line takes blood out of patient.

- Anticoagulant is added to prevent coagulation in the machine used for apheresis. The anticoagulated blood is centrifuged, distinct layers develop with the heavier red blood cells at the bottom and the lighter plasma portion at the top. Between these two layers, a small layer is known as buffy coat composed of white blood cells and platelets. Sedimented blood sample shows various blood constituents is shown in Fig. 12.20.
- Most apheresis machines use centrifugation to separate the various whole blood components. Collection set consisting of centrifuge bowl is loaded into a machine.
  - The donor is attached to the collection set through phlebotomy. As the machine rotates the centrifuge bowl, whole blood is drawn into the bowl and then mixed with acid-citrate-dextrose (ACD) anticoagulant preservative.
  - When the centrifuge bowl reaches capacity, the machine increases rate of rotation to optimal centrifugation speed resulting in separation of the whole blood into components.
  - The selected component is collected into container. The remainder of the donation is returned back to the donor through same collection tube.
- Membrane filtration technology can also be used to separate blood components. Membrane separators have specific size of pores, which separate specific blood component. Remaining blood constituents are returned back to the donor.
- Apheresis is used to collect plasma, platelet, RBCs and granulocytes. Fresh frozen plasma and cryoprecipitate are prepared whole blood. Procedures of apheresis are given in Table 12.14.

COLLECTION OF RED BLOOD CELLS BY APHERESIS

Collection of RBCs by apheresis is termed **erythropheresis**. RBCs are collected as a double unit.

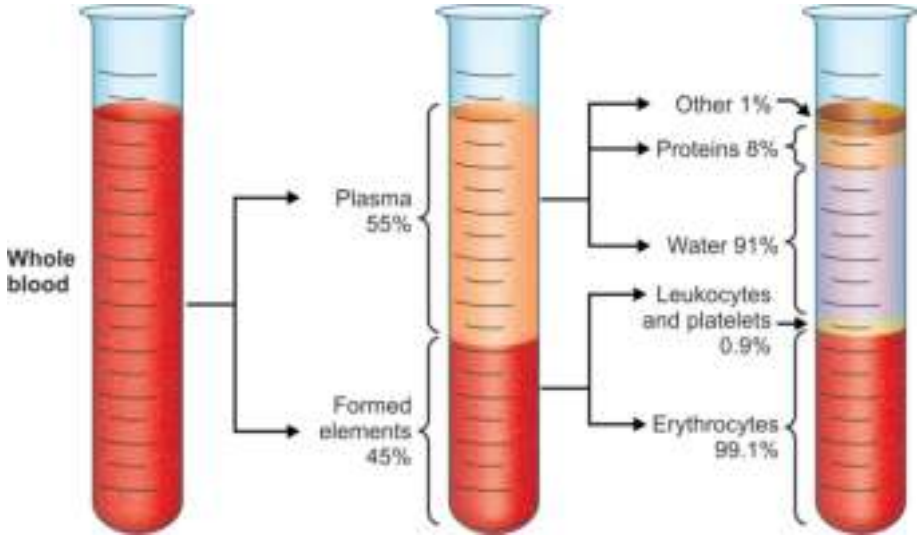


Fig. 12.20: Sedimented blood sample shows various blood constituents.

Table 12.14 Procedures of apheresis

Procedure	Blood Component Removed
Plasmapheresis (plasma exchange)	Removal of plasma
Plateletpheresis	Removal of platelets
Leukapheresis	Removal of white blood cells (e.g. granulocytes and lymphocytes)
Erythrocytapheresis	Removal of red blood cells
Hematopoietic progenitor cells (HPCs) apheresis	Hematopoietic progenitor cells (HPCs) also known as peripheral blood stem cells

- Double RBC apheresis components can be collected from one donor if the donor meets eligibility requirements and no other apheresis components are collected during the same donation. Depending on the instrument used, plasma and platelets are returned back to the donor. The period between two units of red blood cells obtained by erythropheresis from the donor is 16 weeks.
- The red blood cells are transfused back to the donor in near future for clinical management. It reduces exposure to other donor red blood cells. Each unit contains about 60 g of hemoglobin per component.

COLLECTION OF PLASMA BY APHERESIS

Collection of plasma by apheresis is termed **plasmapheresis**. Maximum volume of plasma obtained by plasmapheresis is 600 ml. In plasmapheresis, whole blood from the donor is centrifuged. The plasma is diverted into a collection bag. The cellular components (red blood cells, platelets, white blood cells) are returned back to the donor.

- The plasmapheresis process allows to collect large volume of plasma. The plasma can be used as fresh frozen plasma (FFP). Plasma can be collected from donors with high titers of antibodies against infections as hepatitis B virus, cytomegalovirus and herpes zoster to prepare immunoglobulin. These immunoglobulins are administered to provide prophylaxis against these infectious agents.
- Plasmapheresis is used to collect plasma for further manufacturing of various products such as intravenous immunoglobulin (**IVIg**), hepatitis immunoglobulin and Rh immunoglobulin. Donors undergoing frequent plasmapheresis must be evaluated periodically to analyze total proteins, serum electrophoresis and measurement of immunoglobulin levels.

COLLECTION OF PLATELETS BY APHERESIS

In plateletpheresis procedure, platelets along with a portion of plasma (100–500 ml) are removed and remaining red blood cells, white blood cells and major portion of plasma are returned back to the donor. Platelets are suspended in the donor plasma in the collection bag designed for platelet storage. A routine plateletpheresis takes 45–90 minutes.

- Prior to plateletpheresis, it is essential to analyze donor's platelet count. Donor should have at least 1,50,000/µl.

- Minimal acceptable platelet count in platelet component is 3,00,000/μl. Plateletpheresis should not be performed on donors taking medicines that interfere with the functioning of the platelets. Plateletpheresis should be performed after every seven days.

COLLECTION OF GRANULOCYTES BY APHERESIS

Granulocyte transfusion is beneficial in patients with **neutropenia** induced by chemotherapeutic agents. Patient with neutropenia unresponsive to antibiotics and antifungal drugs need granulocyte transfusion. Granulocyte transfusion has favorable results in the treatment of neutropenic neonates with sepsis.

- Collection of granulocytes by leukapheresis provides a high yield. A minimum of therapeutic dose of granulocyte component is 1×10^{10} per day. During centrifugation of whole blood, granulocytes are present in the buffy coat between red blood cells and plasma. Leukocyte depletion filter should not be used. A standard blood administration filter is sufficient for leukapheresis.
- Adding the red blood cell sedimenting agent, e.g. hydroxyethyl starch (**HES**) allows demarcation of layers resulting in improved granulocyte yield. During leukapheresis, some quantity of hydroxyethyl starch may enter donor blood circulation, which is cleared by reticuloendothelial system. Side effects of hydroxyethyl starch include expansion of circulatory volume, headache and peripheral edema.
- Granulocytes collected by leukapheresis contain large number of viable lymphocytes. Transfusion of lymphocytes in severely immunocompromised patient, there is significant risk for graft-versus-host disease (**GVHD**). Therefore, it is essential to irradiate leukocyte product prior to transfusion.
- Donor may be administered hematopoietic growth factors (**G-CSF**) or corticosteroids or hydroxyethyl starch (**HES**) to increase the number of granulocytes. The granulocytes can be collected. The minimum number of granulocytes is 1×10^{10} per granulocyte component. Drugs used in leukapheresis are given in Table 12.15.

Table 12.15 Drugs used in leukapheresis

Drugs	Actions
Hydroxyl starch	Hydroxyl starch that permits enhanced granulocyte harvest
Corticosteroids (i.e. prednisone, dexamethasone)	Corticosteroids mobilize granulocytes from the marginal pool, thus increasing harvest
Growth factors (i.e. colony stimulating factors)	Colony stimulating factor increases granulocyte yields

THERAPEUTIC APHERESIS

Therapeutic apheresis is based on existence of pathogenic substance in the blood that contributes to a disease process and its symptoms; and removal of the substance by apheresis.

- Pathogenetic substance is removed from the body by apheresis and remaining blood constituents are returned back.
- Therapeutic apheresis has become a standard therapy for many hematologic, renal, metabolic, autoimmune and other disorders.
- Therapeutic apheresis is accepted as a relatively safe procedure. But adverse effects may be observed in therapeutic procedures.
- Adverse effects of therapeutic apheresis include citrate toxicity, hematoma, sepsis, phlebitis, neuropathy, vasovagal reactions, hypovolemia, allergic reactions, hemolysis, depletion of coagulation factors, cardiopulmonary distress, transfusion-transmitted diseases, loss of lymphocytes and depletion of proteins and immunoglobulins.

THERAPEUTIC PLASMA EXCHANGE

Therapeutic plasma exchange (**TPE**) is the removal of undesirable harmful substances in blood circulation and retention of the plasma, with return of all the cellular components to the patient.

- Purpose of plasmapheresis is to remove the undesirable harmful agents present in the plasma such as immune complexes, alloantibodies, autoantibodies, immunoglobulins causing hyperviscosity, protein bound toxins or drugs, lipoproteins and phytanic acid. Indications of therapeutic apheresis are given in Table 12.16.

Hematology Pearls: Factors Removed by Therapeutic Plasmapheresis

- Immune complexes (e.g. systemic lupus erythematosus)
- Alloantibodies (e.g. antibody-mediated transplant rejection)
- Autoantibodies (e.g. Guillain-Barré syndrome, Goodpasture's syndrome)
- Immunoglobulins causing hyperviscosity (e.g. Waldenström's macroglobulinemia)
- Protein bound toxins and drugs (e.g. Amanita mushroom)
- Lipoproteins (e.g. familial hypercholesterolemia)
- Phytanic acid (e.g. Refsum disease involving peripheral neuropathy).

- Therapeutic plasmapheresis is performed in clinical practice in patients suffering from glomerular basement membrane disease, renal focal glomerulosclerosis, renal transplant antibody-mediated rejection, renal transplantation, HLA desensitization,

recurrent focal glomerulosclerosis, hemolytic uremic syndrome (HUS), familial hypercholesterolemia, cryoglobulinemia, thrombotic thrombocytopenic purpura, hyperviscosity associated with monoclonal gammopathy, acute inflammatory demyelinating polyneuropathy (Guillain-Barré syndrome), chronic inflammatory demyelinating polyneuropathy, Lambert-Eaton myasthenic syndrome, myasthenia gravis and paraproteinemic polyneuropathy due to IgG, IgA or IgM.

Table 12.16 Indications of therapeutic apheresis

Disorders	Procedure	Indication Category	Comments
Renal disorders			
Antiglomerular basement membrane disease	Plasma exchange	Category I	Standard and curative
Renal focal glomerulosclerosis	Plasma exchange	Category I	Standard and curative
Renal transplant antibody rejection	Plasma exchange	Category I	Standard and curative
Renal transplantation	Plasma exchange	Category I	Standard and curative
HLA desensitization	Plasma exchange	Category I	Standard and curative
Recurrent focal glomerulosclerosis	Plasma exchange	Category I	Standard and curative
Hemolytic uremic syndrome (HUS)	Plasma exchange	Category I	Standard and curative
Rapidly progressive glomerulonephritis (RPGN)	Plasma exchange	Category III	Conflicting results
Metabolic disorders			
Familial hypercholesterolemia	▪ Selection absorption ▪ Plasma exchange	▪ Category I ▪ Category II	▪ Standard and curative ▪ Supportive
Overdose or poisoning	Plasma exchange	Category II	Supportive
Acute hepatocellular failure	Plasma exchange	Category III	Conflicting results
Sepsis and multiorgan failure	Plasma exchange	Category III	Supportive
Thyrotoxicosis	Plasma exchange	Category III	Supportive
Autoimmune disorders			
Cryoglobulinemia	Plasma exchange	Category I	Standard and curative
Rheumatoid arthritis (refractory)	Immunoadsorption	Category II	Supportive
Systemic lupus erythematosus (SLE) severe	Plasma exchange	Category II	Supportive
Autoimmune hemolytic anemia (warm antibody mediated)	Plasma exchange	Category III	Conflicting results
Scleroderma (progressive systemic sclerosis)	▪ Plasma exchange ▪ Photopheresis	▪ Category III ▪ Category IV	▪ Conflicting results ▪ May be harmful
Hematologic disorders			
Thrombotic thrombocytopenic purpura	Plasma exchange	Category I	Standard and curative
Hyperviscosity associated with monoclonal gammopathy	Plasma exchange	Category I	Standard and curative
Sickle cell disease	Red blood cell apheresis	Category I	Standard and curative
Malaria or babesiosis	Red blood cell exchange	Category I	Standard and curative
Leukocytosis and thrombocytosis (symptomatic)	Cytapheresis	Category I	Standard and curative
ABO-incompatible hematopoietic stem cell transplant	Plasma exchange	Category II	Supportive
Cutaneous T cell lymphoma	Photopheresis	Category II	Supportive
Red blood cell alloimmunization in pregnancy (if intrauterine transfusion in not available)	Plasma exchange	Category II	Supportive
Aplastic anemia	Plasma exchange	Category III	Supportive
Post-transfusion purpura	▪ Plasma exchange ▪ Immunoadsorption ▪ Plasma exchange	▪ Category III ▪ Category III ▪ Category IV	▪ Supportive ▪ Supportive ▪ May be harmful

Contd...

Table 12.16 Indications of therapeutic apheresis (*Contd...*)

Disorders	Procedure	Indication Category	Comments
Neurological disorders			
Acute inflammatory demyelinating polyneuropathy (Guillain-Barré syndrome)	Plasma exchange	Category I	Standard and curative
Chronic inflammatory demyelinating polyneuropathy	Plasma exchange	Category I	Standard and curative
Lambert-Eaton myasthenic syndrome	Plasma exchange	Category I	Standard and curative
Myasthenia gravis	Plasma exchange	Category I	Standard and curative
Paraproteinemic polyneuropathy due to IgG, IgA or IgM	Plasma exchange	Category I	Standard and curative
Pediatric autoimmune neuropsychiatric disorder associated with streptococci (PANDAS)	Plasma exchange	Category I	Standard and curative
Paraproteinemic polyneuropathy due to multiple myeloma	Plasma exchange	Category II	Supportive
Rasmussen's encephalitis	Plasma exchange	Category III	Supportive

- IgM is distributed in the intravascular compartment, and IgG in intravascular and extravascular compartments. Therefore, therapeutic plasmapheresis performed to remove IgG antibodies is most effective when combined with immunosuppressive drugs. A large volume of plasma (maximum 600 ml) must be removed during therapeutic plasmapheresis and replaced with sufficient physiologic fluid to maintain the intravascular compartment.

THERAPEUTIC LEUKAPHERESIS

Therapeutic leukapheresis has been widely used to treat patients with very high leukocyte count of >100,000/µl. High leukocyte count causes organ dysfunction due to formation of microthrombi in cerebral and pulmonary circulation.

- Leukocytosis is more common in acute myelogenous leukemia (AML) than acute lymphoblastic leukemia (ALL). Single procedure of therapeutic leukapheresis should reduce the leukocyte count by 30–60%.
- However, more than one procedure is essential to mobilize leukocytes from the extracellular compartment. About one liter of fluid is lost in the therapeutic leukapheresis, hence these patients require fluid replacement.

THERAPEUTIC ERYTHROCYTAPHERESIS

Therapeutic erythrocytapheresis (red blood cell exchange) procedure removes a large number of red blood cells from the patients and returns to the patient's plasma and platelets along with compatible allogeneic donor red blood cells.

- Indication of red blood cell exchange (therapeutic erythrocytapheresis) includes sickle cell disease in order to decrease the number of hemoglobin S containing red blood cells, to prevent complications of sickle cell disease such as acute chest syndrome, painful crises and cerebral stroke. Basic aim of red blood cell exchange is to reduce the number of hemoglobin S containing red blood cells below 30%. Patient may require more than one red blood cell exchange procedure.
- The donor red blood cells selected for transfusion should be ABO and Rh compatible with the patient. Fresh red blood cells donated in last days should be preferred to transfuse the patient with sickle cell disease. Some of these patients need regular red blood cell exchange to reduce the complications of sickle cell disease. Finally, red blood cell exchange can be done to remove incompatible red blood cells from patient's blood circulation, e.g. ABO and Rh incompatibility.

THERAPEUTIC FLUID REPLACEMENT

In therapeutic fluid replacement procedures, the extracorporeal circuit (tubing, collection chamber, blood warmer) is normally primed with normal saline with an initial bonus of crystalloid. It is important to maintain intravascular volume and oncotic pressure. Human serum albumin may be administered as 5% solution. The use of fresh frozen plasma may be reserved for treatment of thrombotic thrombocytopenic purpura (TTP) and preexistent coagulopathy.

HEMATOPOIETIC PROGENITOR CELLS COLLECTION

Hematopoietic progenitor cells (**HPCs**) are also known as peripheral blood stem cells (**PBSCs**), which can be collected by apheresis from an autologous or allogeneic donor. After centrifuging blood, HPCs are found in the upper portion of the **buffy coat**. Large volumes of blood are processed to obtain the required yield.

- Donor may be administered hematopoietic growth factors (G-CSF) or corticosteroids or hydroxyethyl starch (HES) to increase the number of PBSCs.
- Hematopoietic progenitor cells express the cell surface glycoprotein CD34+, and measurement of CD34+ in the peripheral blood prior to collection is performed to ensure adequate mobilization of HPCs has taken place.
- Hematopoietic growth factors (G-CSF) should be administered four to five times to mobilize sufficient HPCs. Collection of PBSCs is done in outdoor department without the help of anesthetist.
- Peripheral blood stem cells (PBSCs) are also used for bone marrow transplantation.

IMMUNOADSORPTION/SELECTIVE ADSORPTION

Immunoadsorption/selective adsorption is alternative blood purification technique used to eliminate pathogenic antibodies.

- Plasma is removed by apheresis and then passed through an adsorption medium to remove harmful antibodies by column immunoadsorption.
- Indications for immunoadsorption include removal of high-titer ABO antibodies before ABO-incompatible renal transplantation.
- Immunoadsorption technique is also used to treat patients with either refractory idiopathic thrombocytopenic purpura, rheumatoid arthritis and familial hypercholesterolemia.
- Other uses of immunoadsorption include removal of endotoxin, bile acids, granulocytes and monocytes.

PHOTOPHERESIS

Photopheresis utilizes leukapheresis to obtain the buffy coat from the whole blood. The remaining cells are treated with 8-methoxypsoralen (8-MOP), exposed to ultraviolet A light and then reinfused into the patient. The combination of 8-methoxypsoralen and ultraviolet irradiation leads to cross-linking of leukocyte DNA resulting in apoptosis. Photopheresis is used to treat cutaneous T cell lymphoma, acute and chronic graft-versus-host disease, solid organ transplant rejection and selected immunomediated disorders.

BLOOD COMPONENT LABELING

Blood component labeling is the stage where all donation, production, and testing records available in blood banks. The label on a blood component gives all the information essential to handle the blood component appropriately, which also provides a link through the blood donation identification number, back to the original donor of blood product.

- According to International Society for Blood Transfusion (ISBT), a barcode containing the same information may be available. **Barcode scanners** are used to electronically transfer the data to the information systems. At minimum, the following information may be present. Unique identification number assigned at the time of blood donation provides back to the original donor of blood product. The blood group must consist of details of ABO group and Rh type of blood component.
- The product code provides information about type and volume of blood component, anticoagulant used; and/or the target whole blood collection volume. It is mandatory to mention expiry date of the blood component. Special tests are performed on donor cells. Tests performed on donor cells are given in Table 12.17. Sequence of testing of donated blood and confirmatory test are given in Table 12.18.

Table 12.17 Tests performed on donor cells

Tests on Donor Cells	Comments
ABO blood grouping	Resolution of ABO blood group discrepancies
Rh blood grouping	Resolution of Rh blood grouping
Antibody screening	Fresh frozen plasma cannot be prepared from donor plasma containing antibody
Serologic tests for transmission disease	Rapid plasma regain (RPR) for syphilis and antibody to *Trypanosoma cruzi*
Screening of viral diseases for transmission disease	▪ HbsAg (hepatitis B surface antigen) ▪ Hepatitis B nucleic acids ▪ Hepatitis B core antigen (anti-hepatitis B core antigen) ▪ Hepatitis C antibody (anti-HCV antibody) ▪ Hepatitis C nucleic acids ▪ HTLV-I/II antibody (human T cell lymphotrophic virus (HTLV) causes leukemia and lymphoma) ▪ Cytomegalovirus in immunodeficient patients ▪ NAT (nucleic acid testing) for HIV-RNA, HCV-RNA, West Nile RNA and HBV-DNA

Table 12.18 Sequence of testing of donated blood and confirmatory test

Pathogen	Sequence of Testing	Confirmatory Test
HIV 1, HIV 2	▪ HIV 1/2 EIA* ▪ HIV-NAT (HIV—nucleic acid testing)	▪ HIV 1 Western blot or immunofluorescence assay ▪ HIV 2 Western blot or immunofluorescence assay ▪ HIV 2 EIA
HCV	▪ EIA/ChLIA ▪ HCV-NAT (HCV—nucleic acid amplification testing)	▪ RIBA or RNA test ▪ Confirmatory NAT, demonstration of seroconversion on follow-up
HBV	▪ HBsAg EIA/ChLIA ▪ Anti-HBc EIA/ChLIA	▪ Neutralization test ▪ No license test

**Enzyme-linked immunosorbent assay also called ELISA.*

CHANGES IN STORED BLOOD COMPONENTS

Storage of blood components is essential for safe blood transfusion. As soon as blood is obtained from the donor, it starts to undergo changes that influence how its component function, e.g. red blood cells, platelets, white blood cells, plasma proteins and bacterial contamination. Some changes in stored blood components are reversible. Shelf-life of blood components to 42 days may be achieved by additive solutions containing AS-1 (Adsol), AS-3 (Nutricel) and A-5 (Optisol) admixed with dextrose, adenine, monobasic sodium phosphate, mannitol, sodium chloride, sodium citrate and citric acid in different proportions. Recently, it has been discovered that addition of potassium, magnesium and L-carnitine significantly improve platelet viability during storage. Storage conditions for blood components are given in Table 12.19.

Table 12.19 Storage conditions for blood components

Blood Component	Storage Temperature	Expiry Time from Collection
Whole blood and red blood cells, granulocytes and platelets storage		
Whole blood (ACD/CPD/CP2D anticoagulant preservative)	1–6°C	21 days
Whole blood (CPDA-1 anticoagulant preservative)	1–6°C	35 days
Red blood cells (ACD/CPD/CP2D anticoagulant preservative)	1–6°C	21 days
Red blood cells (CPDA-1 anticoagulant preservative)	1–6°C	35 days
Red blood cells (additive solution)	1–6°C	42 days
Platelets	20–24°C (continuous gentle agitation)	24 hours to 5 days
Granulocytes	20–24°C (continuous gentle agitation)	24 hours
Plasma products storage		
Fresh frozen plasma or plasma frozen within 24 hours of phlebotomy	≤–18°C	12 hours
Fresh frozen plasma or plasma frozen within 24 hours of phlebotomy after thawing	1–6°C	24 hours
Thawed plasma	1–6°C	5 days after thawing
Liquid plasma	1–6°C	5 days after expiration of whole blood
Cryoprecipitate containing antihemophilic factor	≤–18°C	12 months
Cryoprecipitate containing antihemophilic factor thawed	20–24°C	6 hours
Plasma cryoprecipitate reduced	≤–18°C	12 months
Plasma cryoprecipitate reduced and thawed	1–6°C	5 days after thawing

RED BLOOD CELL CHANGES IN STORED BLOOD

The aim of blood preservative is to provide functional and viable blood components for patients requiring blood transfusion.

- Blood stored at 2–6°C maintains optimal viability of RBCs. Normally, RBCs contain abundant 2,3-diphosphoglycerate (2,3-DPG) level, which increases delivery of oxygen to the tissues.
- Various biochemical changes affecting viability of RBCs include decrease in pH, production of lactic acid, decrease in consumption of glucose and low levels of 2,3-diphosphoglycerate (2,3-DPG).
- Many of biochemical changes in red blood cells are reversible after blood transfusion, with intracellular potassium levels returning to normal within a few hours RBC component of transfusion. Levels of ATP and 2,3-DPG are slower to return to normal; 2,3-DPG levels usually recover within 12–24 hours.
- Red blood cell membrane changes due to reduced levels of ATP may be irreversible depending on blood storage conditions. RBC storage changes are given in Table 12.20.

Hemolysis of Red Blood Cells in Stored Blood

When whole blood is collected from donor, it contains red blood cells (RBCs) of various life span. Over the time, older RBCs undergo apoptosis. When RBCs undergo apoptosis, their cell membrane ruptures resulting in release of free hemoglobin into the stored blood. The stored RBCs maintain their metabolic activity. Basic approach to improve the quality and efficacy of stored RBCs has been focussed on reducing oxidative damage to RBCs by removing oxygen at the beginning of storage and maintaining the anaerobic state throughout the storage period.

Table 12.20 Red blood cell storage changes

Features	Changes in Red Blood Cells Observed
Percentage of viable red blood cells	Decreased
Glucose	Decreased
Adenosine triphosphate (ATP)	Decreased
Lactic acid	Decreased
pH	Decreased
2,3-diphosphoglycerate (2,3-DPG)	Decreased
Oxygen dissociation curve	Shift to the left (increase in hemoglobin and oxygen affinity; less oxygen delivered to the tissues)
Plasma K^+	Increased
Plasma hemoglobin	Increased

Reduction of ATP and 2,3 Diphosphoglycerate (2,3-DPG) in Stored Blood

Normally, adenine participates in synthesis of adenosine triphosphate (ATP) resulting in increase in ATP level and shelf life of red blood cells to 42 days. Sufficient ATP is associated with the viability of red blood cells. Metabolic activity and release of oxygen cause reduction of adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) in stored blood. Reduction in ATP levels leads to changes in red blood cell membrane such as reduction in RBC flexibility and their removal from circulation by reticuloendothelial system when transfused. Reduction in 2,3-DPG leads to impaired oxygen release by the red blood cells following blood trans fusion. Red blood cell membrane changes also cause intracellular potassium to leak into extracellular storage fluid. Blood stored in citrate-phosphate-dextrose with adenine 1 (CPDA-1) maintains high levels of 2,3-DPG for 10–14 days in stored blood.

Decreased pH in Stored Blood

There is increased glycolysis resulting in production of lactic acid and decline in pH in stored blood. Storage of blood at low temperature keeps the rate of glycolysis at lower limit so pH is maintained. Preservative solutions used in blood bag provide a buffering capability to minimize changes in pH and optimize the storage period. Fall in pH in stored blood leads to decrease in level of 2, 3-DPG in the RBCs resulting in impaired capacity to deliver oxygen to the tissues.

Maintenance of Stored Blood at Lower Temperature

Lower temperature at 2–6°C minimizes the growth of bacteria that might have entered the blood bag during venepuncture. Labile coagulation factors in plasma are best maintained at low temperature. Low temperature storage of blood bags slows glycolytic activity and allows the shelf life of the red blood cells component to be extended. At higher temperature, red blood cell membrane changes cause intracellular potassium to leak into extracellular storage fluid.

PLATELET CHANGES IN STORED BLOOD

Platelets are very fragile cells and adversely affected by temperature and forces applied during centrifugation resulting in changes in platelet cytoskeleton and impaired ability to aggregate in stored blood. These platelet changes are reversible in the early stage of storage of platelet component. But storage of platelet

Table 12.21 Platelet storage changes

Features	Changes in Platelets Observed
Lactate	Increased
pH	Decreased
Adenosine triphosphate (ATP)	Decreased
Morphology scores change from discoid to spherical (loss of swirling effect)	Decreased
Degranulation (β-thromboglobulin, platelet factor 4)	Increased
Platelet activating markers, e.g. P-selectin (CD62P) or CD63	Increased
Platelet aggregation	Drop in responses to some agonists

component over several days results in platelet degranulation and irreversible platelet membrane changes. Platelet storage changes are given in Table 12.21.

- Platelets have increased metabolic activity during storage. Lactic acid is produced through glucose metabolism and carbon dioxide through fatty acid metabolism. Oxygen is consumed during these activities, while pH falls and carbon dioxide levels rise. Prolonged storage of platelet component in this environment eventually leads to apoptosis.
- Preservation of viable and functional platelets depend on temperature, pH and use of plastic bag. Platelets should be stored at 20–24°C. Temperature of the platelet component plastic bag should be controlled with continuous gentle agitation in platelet incubator and agitator.
- Maintenance of pH above 6 and function of platelets are dependent on the permeability of the storage bag. New plastic blood bags made of polyolefin without plasticizer maintain pH and functions of platelets up to 7 days. It is recommended that platelets must be stored in blood bag for 5 days from the date of blood collection. The pooled platelets may be stored for 4 hours at 22–24°C before used for platelet transfusion.

WHITE BLOOD CELL CHANGES IN STORED BLOOD

Granulocytes and other white blood cells (WBCs) are fragile, which deteriorate very quickly after obtaining through blood donation.

- Rapid deterioration of WBCs can have serious consequences for preparation of other blood components from whole blood.
- When WBCs disintegrate, these release cytokines and intracellular enzymes resulting in acceleration of apoptosis of RBCs and platelets.
- The cytokines and enzymes released by white blood cells may cause serious reactions in patients when the blood components are transfused.
- Shelf life of granulocytes is 24 hours at 22–24°C, which do not require agitation. WBCs should be transfused within 3 hours of blood storage to reach to the site of inflammation.

PLASMA PROTEIN COMPONENT CHANGES IN STORED BLOOD

Most of the plasma proteins of interest in transfusion medicine are very stable in a wide range of storage conditions. However, coagulation factors V and VIII undergo degradation rapidly when plasma components are stored at temperatures above freezing.

Fresh Frozen Plasma Preparation

Shelf life of fresh frozen plasma (FFP) storage below –18°C is 12 months. After thawing, FFP may be stored at 2–6°C for 12 hours before use for transfusion. Fresh frozen plasma (FFP) contains factors VIII and IX, which is administered in patients with hemophilia A (factor VIII deficiency) and Christmas disease (factor IX deficiency). Fresh frozen plasma (FFP) contains coagulation factors I, II, VII, VIII, IX, X, XI; and vWF in the same concentration present in plasma.

Cryoprecipitate Preparation

Cryoprecipitate is a cold insoluble fraction of plasma proteins obtained from FFP, which contains coagulation factors such as fibrinogen, factor VIII, factor XIII, von Willebrand factor (vWF) and fibronectin. Cryoprecipitate can be stored at –18°C or lower temperature for 12 months. Thawed cryoprecipitate can be stored for 6 hours at 2–6°C. One unit of cryoprecipitate is 15–20 ml in volume.

BACTERIAL CONTAMINATION IN STORED BLOOD

At every step of blood collection and production of blood components are done in closed system to reduce bacterial contamination. But there is a little chance for bacterial contamination of the blood. Bacteria may

flourish in blood components under ideal conditions. Bacterial contamination accelerates the destruction of RBCs, WBCs and platelets. Transfusion of contaminated blood components with bacteria can also cause severe blood components transfusion reactions in the patients.

TRANSPORTATION OF BLOOD COMPONENTS

Transportation of blood is a critical step in preparation of blood components. Blood collected from the donors is transported to laboratory for preparation of blood components to be used in hospitals.

- It is essential to maintain appropriate temperature range for the blood components being transported. In general, blood products should be transported at temperature of 1–10°C.
- Granulocyte and platelet components transportation should be carried out at temperature of 20–24°C. Frozen blood components should be transported in dry ice.

MODIFICATION OF BLOOD COMPONENTS

Blood components may be modified to increase the safety of the blood product. Some modifications affect blood storage conditions by increasing expiry time.

LEUKOREDUCTION BEFORE BLOOD STORAGE

Pre-storage leukoreduction is preformed while preparing blood components. Leukoreduction is a process that removes WBCs from the whole blood or blood components.

- Leukoreduction is performed to reduce the undesirable effects that WBCs have on stored blood components and to reduce the risk of adverse febrile reactions; and organ transplant rejection due to alloimmunization to HLA antigens in patients receiving blood components.
- Whole blood is centrifuged by using hard spin to remove granulocytes from buffy coat layer. Leukoreduction may also be achieved through the use of a specialized filter that trap WBCs in a matrix. Both these methods remove majority of granulocytes and monocytes sparing lymphocytes in final leukoreduction process.
- The acceptable number of WBCs in the final leukoreduction process has 5×10^9 leukocytes per blood component.
- After leukoreduction, there is decreased risk for graft-versus-host disease, immunosuppression, transmission of cytomegalovirus, Epstein-Barr virus, T cell lymphotropic virus and bacteria.

INACTIVATION OF PATHOGENS IN STORED BLOOD

Inactivation of bacteria and viruses is essential in prepared blood components. Few manufactures of blood components have developed processes and equipment to achieve pathogen inactivation.

- Plasma is treated by adding methylene blue, psoralen, or riboflavin to the component in a closed system, then exposing the blood component to light at specific wavelengths. The methods cause some reduction in plasma coagulation factors after treatment.
- A similar process has been developed for platelets using psoralen or riboflavin, but both methods have adverse effects on functioning of platelets. No manufactures have successfully developed commercial methods for treating RBCs.

ALIQUOTING OF BLOOD COMPONENTS

Aliquoting is most often performed to prepare a product for low-volume transfusion to neonates or pediatric patients. It is performed in an open system or closed system. Blood components may be aliquoted into syringes or transfer satellite containers. Blood components prepared in an open system must be transfused within short time after modification. There is always risk for bacterial contamination in open system. To maintain a closed system, aliquot containers must be attached to the original blood component bag with sterile docking device.

POOLING OF BLOOD COMPONENTS

Pooling of blood components may be performed to reduce the number of blood product bags that have to be accessed at the time of transfusion, which can be done in an open. Pooling of blood components is usually performed with platelet-rich plasma or cryoprecipitate containing antihemophilic factor (factor VIII) to create a single treatment dose. RBCs can also be pooled with thawed plasma to recreate most of the constituents of whole blood for transfusion.

VOLUME REDUCTION FROM BLOOD COMPONENTS

Volume reduction is performed to remove plasma or additive solution from blood components. It is done by centrifuging cellular components and removing supernatant fluid usually with plasma press either by open or closed system. Both methods affect survival of the RBCs or platelets. Volume reduction reduces risk for circulatory overload. Removal of excess plasma reduces the risk for adverse reactions related to transfusion of certain plasma proteins.

IRRADIATION OF BLOOD COMPONENTS

Cellular blood components containing active T cells can cause transfusion-associated graft-versus-host disease (GVHD) in susceptible persons.

- T cells are inactivated by exposure to gamma rays or X-rays radiation without affecting function of RBCs and platelets.
- Radiation dose may be minimum of 25 Gy or maximum 50 Gy. Radiation does not adversely affect function of platelets.
- But radiation has some impact on the cell membrane of red blood cells with increased potassium level in the storage fluid.

WASHING OF BLOOD COMPONENTS

Washing of RBC or platelet components is performed to remove plasma proteins that can cause severe reactions in certain persons. Washing of these blood components is done with normal saline either manually or in automated cell washer. Washing of blood components is performed in an open system or closed system resulting in shortened expiry date.

FREEZING, THAWING AND DEGLYCEROLIZING RED BLOOD CELLS

Red blood cells may be stored for a long time by way of freezing. Red blood cells normally undergo cell lysis when frozen and then thawed. It is essential to reduce red blood cell damage during frozen storage by using cryoprotective agent such as glycerol.

- Glycerol limits the formation of ice crystals within Red blood cells (RBCs) resulting in reduction of damage to the RBC membrane. Frozen RBCs are thawed at 37°C. It is essential to remove glycerol prior to transfusion.
- Deglycerolization is performed by washing the thawed red blood cell component with successive concentration changes of sterile saline solution, starting with hypertonic saline, which can also be done in an automated closed system, if red blood cell component has been originally frozen in closed system.

REJUVENATION OF RED BLOOD CELLS

Rejuvenation is a method used to reverse adverse effects of stored RBCs. Rejuvenating solutions contain adenine, pyruvate, inosine and phosphate, which are capable to return ATP and 2,3-DPG levels to normal levels in stored RBC component. It is essential to wash rejuvenating RBC component prior to transfusion. Rejuvenation may also be done to improve the condition of red blood cells prior to freezing in glycerol.

BLOOD PLASMA FRACTIONATION

Blood plasma fractionation refers to the general processes of separating the various components of blood, which in turn is a component of blood obtained through blood fractionation.

- Separation of blood plasma components is achieved by pooling plasma from up to 10,000 donors. Ethanol is added to the blood plasma and then manipulated the pH and temperature of the solution. Blood plasma fractional process causes individual plasma proteins to precipitate of the solution at specific pH and temperature.
- Three major categories of plasma proteins that are fractioned from pooled plasma include albumin, immunoglobulins and coagulation proteins (e.g. factor VIII, factor IX, fibrinogen, anti-thrombin III), and protein C. Albumin is usually packaged and distributed as a liquid in 5% or 25% concentration.
- Specific immunoglobulin contains IgG antibody to specific antigen, which include Rh(D) immunoglobulin and hepatitis B immunoglobulin.
- Nonspecific immunoglobulin is available as an intravenous solution and referred as intravenous immunoglobulin (**IVIg**). Fractionation of 1000 kg of plasma yields about 4 kg of IVIg.
- Commercially available coagulation proteins include von Willebrand factor combined with factor VIII and prothrombin complexes that contain factor II, factor VII, factor IX and factor X, with or without protein C. Many plasma products, plasma pool undergoes solvent/detergent treatment is to inactivate lipid-enveloped viruses. Plasma may also be filtered through small pore nanofilter to trap bacteria and viruses to prepare safe plasma products.

Plasma Fractionation Products

- Albumin
- Immunoglobulins
- Coagulation factor concentrates factor VIII, factor IX, fibrinogen, anti-thrombin III and protein C
- Rh(D) immunoglobulin
- Hepatitis B immunoglobulin
- Intravenous immunoglobulin (IVIg)

RECOMBINANT PLASMA PROTEIN PRODUCTION

Genetic engineering has developed recombinant plasma protein products. Recombinant plasma protein products are prepared by inserting the genetic information that codes for a specific plasma protein into the DNA of plant or animal cells. Currently, recombinant factor VIII and factor IX are available for clinical use.

BLOOD TRANSFUSION AND BLOOD COMPONENT THERAPY

BLOOD TRANSFUSION: GOALS

Blood transfusion is indicated in the abnormalities in the hematologic or coagulation systems, whether congenital or acquired. Basic aim of blood transfusion is to increase tissue oxygenation and/or restore hemostasis. Common investigations performed prior to transfusion include complete blood count, hemoglobin, hematocrit values, platelet count, prothrombin time and international normalized ratio (PT–INR), activated partial thromboplastin time (APTT).

BLOOD TRANSFUSION: INDICATIONS

Patient with hemoglobin levels or hematocrit <20%, 10 g/dl or hematocrit <30%, while those with hemoglobin level <7 g/dl frequently require blood transfusion.

- Fresh frozen plasma (**FFP**) is transfused in the treatment of the bleeding patient with disseminated intravascular coagulation (**DIC**) and massive blood transfusion cases.
- Cryoprecipitate is a rich source of fibrinogen and factor XIII. Blood components are classified in four categories given in Table 12.22. Indications of blood component transfusion are given in Table 12.23. Plasma-derived products obtained by blood plasma fractionation and their indications are given in Table 12.24.

Table 12.22 Categories of blood components

Categories	Blood Components
Cellular components	▪ Whole blood ▪ Red blood cell component ▪ Granulocytes
Plasma components	▪ Platelet products ▪ Fresh frozen plasma ▪ Cryoprecipitate ▪ Other transfusion products
Hematopoietic stem cell (HSC) products	▪ Bone marrow stem cells ▪ Peripheral blood stem cells ▪ Cord blood preparations
Plasma fractionation products	▪ Albumin ▪ Immunoglobulins ▪ Coagulation factor concentrates (factor VIII, factor IX, fibrinogen, anti-thrombin III) and protein C ▪ Rh(D) immunoglobulin ▪ Hepatitis B immunoglobulin ▪ Intravenous immunoglobulin (IVIg) ▪ Commercially available coagulation proteins (von Willebrand factor combined with factor VIII and prothrombin complexes that contain factor II, factor VII, factor IX and factor X, with or without protein C)

Table 12.23 Indications of blood component transfusion

Blood Component	Indications	Comments
Red blood cell component	▪ Hemoglobin 7 g/dl or hematocrit <21% in a patient with uncompromised cardiovascular function ▪ Hemoglobin 10 g/dl or hematocrit <30% with cardiovascular disease, sepsis or hemo globinopathy	▪ Red blood cell concentrate obtained by additive solution saline-adenine-glucose mannitol (OAS), e.g. SAGM; and stored at 4°C ▪ Common adult dose one unit
Platelet component	▪ Prophylactic platelet transfusion in patient with $<10 \times 10^9$/L (adults) or 50×10^9/L (neonates) ▪ Platelet count $<30 \times 10^9$/L in patient with bleeding or minor bedside procedure ▪ Platelet count $<50 \times 10^9$/L in a patient with intraoperative or postoperative bleeding ▪ Platelet count $<100 \times 10^9$/L in a patient with bleeding post-cardiopulmonary bypass	▪ Buffy coat pooled plasma is obtained from four donors. Buffy coat residue is discarded ▪ Platelets are stored at room temperature ▪ Do not transfuse platelets in the setting of thrombotic thrombocytopenic purpura, heparin-induced thrombocytopenia. Platelet transfusions are unlikely to be useful in idiopathic thrombocytopenic purpura or post-transfusion thrombocytopenic purpura
Fresh frozen plasma	▪ Activated partial thromboplastin time (APTT) and/or prothrombin time (PT) increased ▪ Bleeding in patient with international normalized ratio (INR) ≥2 ▪ Bedside procedure and international normalized ratio (INR) ≥2	Fresh frozen plasma (FFP) replaces coagulation factors in those patients with an elevated prothrombin time (PT), activated partial thromboplastin time (APTT) or INR and bleeding. It is used as replacement with plasmapheresis. It is not the choice for patients with hemophilia A, hemophilia B or von Willebrand disease

Contd...

Table 12.23 Indications of blood component transfusion *(Contd...)*

Blood Component	Indications	Comments
	▪ Prophylactic (bleeding) with international normalized ratio (INR) ≥10 ▪ Fresh frozen plasma (FFP) is not indicated for patients with international normalized ratio (INR) <1.5 and thrombotic thrombocytopenic purpura ▪ Fresh frozen plasma (FFP) is indicated in the treatment of bleeding in a patient with disseminated intravascular coagulation (DIC)	▪ Fresh frozen plasma (FFP) for fractionation (e.g. albumin, γ-globulin, specific antiviral immunoglobulin, anti-D and coagulation factors)
Cryoprecipitate	Cryoprecipitate transfusion is done in the setting of dysfibrinogenemia, fibrinogen concentration <100 mg/dl and von Willebrand disease	Cryoprecipitate is rich in fibrinogen, factor VIII, factor XIII, von Willebrand factor and fibronectin
Granulocytes	Documented sepsis unresponsive to antimicrobial therapy	Normally, granulocytes phagocytose microbes

Table 12.24 Plasma-derived products obtained by blood plasma fractionation and their indications

Plasma Products	Indications in Clinical Practice
Hemophilia A (factor VIII deficiency)	
Factor VIII (antihemophilic factor)	Treatment or prevention of bleeding in patients with hemophilia A
Factor VIII (high purity)	Hemophilia A with HIV infection
Recombinant factor VIII	Hemophilia A
Porcine factor VIII	Treatment of bleeding episodes in hemophilia A in the presence of complex factor VIII inhibitor
Activated prothrombin complex concentrates	Treatment of bleeding episodes in hemophilia A in the presence of complex factor VIII inhibitor
Hemophilia B (Christmas disease) (factor IX deficiency)	
Factor IX complex	Prophylaxis and treatment of hemophilia B bleeding episodes and other bleeding disorders
Factor IX (high purity)	Hemophilia B
Recombinant factor IX	Hemophilia B
von Willebrand disease	
von Willebrand factor/factor VIII concentrate	Treatment or prevention of bleeding in von Willebrand disease
Hemophilia C (factor XI deficiency)	
Fresh frozen plasma (FFP)	Treatment or prevention of bleeding in hemophilia C associated with cardiovascular disorders
Factor I (fibrinogen) deficiency	
Fibrinogen	Treatment of hemorrhagic diathesis in hypofibrinogenemia, dysfibrinogenemia and afibrinogenemia
Factor VII (stable factor) deficiency	
Factor VII	Treatment of factor VII deficiency, anticoagulant overdose, severe liver disease
Recombinant factor VII	Treatment of factor VII deficiency with factor VII inhibitor
Fibrin stabilizing factor (factor XIII deficiency)	
Factor XIII	Treatment of bleeding disorders of wound healing due to factor XIII deficiency
Multiple factors deficiency and other diseases	
Prothrombin complex concentrate	Treatment of factors I and X deficiency; factor VIII inhibitors, oral coagulants overdose and severe liver disease

Contd...

Table 12.24 Plasma-derived products obtained by blood plasma fractionation and their indications (*Contd...*)

Plasma Products	Indications in Clinical Practice
Antithrombin III plasma product	
Antithrombin III	Treatment of bleeding associated with liver disease, antithrombin III deficiency and thromboembolism
Hemolytic disease of fetus and newborn	
RhO(D) immunoglobulin	Treatment and prevention of hemolytic disease of fetus and newborn resulting from Rh incompatibility and incompatible blood transfusions
Restoration of plasma volume	
Albumin	Restoration of plasma volume subsequent to shock, trauma, surgery and burns
Plasma protein fraction	Restoration of plasma volume subsequent to shock, trauma, surgery and burns
Immunoglobulin deficiency	
Immunoglobulin (intravenous and intramuscular)	Treatment and agammaglobulinemia, chronic lymphocytic leukemia, immune-mediated thrombocytopenic purpura; and passive immunization for hepatitis A and measles
Dissolution of intravascular clots	
Fibrinolysin	Dissolution of intravascular clots
Alpha-1 proteinase inhibitor for emphysema and cirrhosis	
Alpha-1 proteinase inhibitor	Treatment of emphysema and cirrhosis due to genetic alpha-1 antitrypsin deficiency
Hereditary angioedema	
C1 esterase inhibitor	Treatment of angioedema
Apnea after administration of succinylcholine chloride	
Serum cholinesterase	Treatment of prolonged apnea after administration of succinylcholine chloride
Bacterial diseases	
IgM-enriched immunoglobulin	Treatment and prevention of septicemia and septic shock due to toxin liberation in the course of antibiotic treatment
Tetanus immunoglobulin	Passive immunization subsequent to exposure to tetanus
Viral diseases	
Haptoglobin	Supportive therapy in viral hepatitis and pernicious anemia
Hepatitis B immunoglobulin	Passive immunization to hepatitis B
Immunoglobulin (intravenous and intramuscular)	Passive immunization to hepatitis A
Rabies immunoglobulin	Passive immunization subsequent exposure to rabies
Rubella immunoglobulin	Passive immunization subsequent to exposure to German measles
Varicella-zoster immunoglobulin	Passive immunization subsequent to exposure to smallpox
Varicella-zoster immune	Passive immunization subsequent to exposure to globulin chickenpox
Cytomegalovirus immune	Passive immunization subsequent to exposure to globulin cytomegalovirus

BLOOD COMPONENTS TRANSFUSION

Transfusion of blood components except fractionation products in <4 hours ensures the product would deliver the most therapeutic response with minimal bacterial contamination. Refrigerated storage of blood products inhibits bacterial growth. Basic transfusion set has flexible plastic tubing and standard inline blood filter with very small pore.

- Microaggregate filters may be used for transfusion of RBC component. These microaggregate filters also reduce the degenerated platelets, wWBC fragments and small strands of fibrin. While transfusion of RBC component or platelet component, leukocyte

reduction filters may be used to reduce the number of leukocytes. Leukocyte reduction may be performed in prestorage blood, while the blood components are prepared; or just before transfusion at bedside.

- Using too small needle for transfusion may cause RBC hemolysis especially rapid administration of the RBC component. Typically, 18 gauge or large needles are used for blood transfusion especially RBCs.
- Massive blood transfusion may cause hypothermia resulting in cardiac arrhythmia with fatal out. Therefore, body warmer should be used to treat hypothermia. Normal saline 0.9% sodium chloride may be added to most blood components. Ringer's lactate solution should not be added to the blood components, because high level of calcium content inactivates the anticoagulant resulting in formation of blood clots. When transfusing fractionation blood plasma products, instructions of manufacture should be checked.

WHOLE BLOOD TRANSFUSION

Whole blood consists of red blood cells, white blood cells, platelets and plasma. The platelets in stored whole blood in refrigerator become nonfunctional. Whole blood is transfused in medical and surgical emergencies in patients with excessive bleeding. Whole blood replacement is performed to treat hemolytic disease of newborn. Each unit of whole blood increases hemoglobin concentration by 1 g/dl and hematocrit by three points in adults.

- Autologous blood transfusion refers to collection of blood from the patient prior to surgery 1–3 weeks before surgery for whom the blood transfusion is used later. Benefits of autologous transfusion are to avoid transfusion transmitted infectious diseases and immunological adverse reactions.
- Autologous blood transfusion is safe and useful in patients with rare blood group and with multiple alloantibodies. Another form of autologous blood collection is intraoperative blood salvage. Blood is collected from the surgical site during surgery or postoperative period. Blood lost during surgery is collected by suction mixed with anticoagulant, and washed in a machine designed for this purpose. The salvage blood cells are reinfused. Contraindications for intraoperative salvage include the presence of infection or cancer cells at the site of surgery.

RED BLOOD CELL COMPONENT TRANSFUSION

Red blood cell component is most often called packed RBCs and obtained by removal of plasma, which are most often used to treat anemia. Transfusion of RBCs permits restoration of oxygen carrying capacity with less risk of volume overload. The hematocrit of packed RBCs is about 70–80%. Removal of plasma from whole blood, hematocrit is double of the normal range. Each unit of packed RBCs should raise the hematocrit by about 3 points per unit or 1 g/dl of hemoglobin.

- Patients with anemia fall into two categories: (a) patient developing anemia over time is hemodynamically stable and treated for anemia and (b) patient develops anemia as a result of an acute blood loss due to trauma, surgery or severe bleeding due to underlying medical illnesses. These patients most often require treatment for both anemia and volume depletion.
- The clinician should assess overall clinical picture of patient especially symptoms related to decreased oxygen carrying or not. Donation from first degree family member should be irradiated prior to infusion in order to prevent transfusion associated graft-versus-host disease (TA-GVHD). These blood units are screened for infectious diseases.

WHITE BLOOD CELL COMPONENT TRANSFUSION

Granulocyte units are collected by apheresis. Granulocytes are removed from whole blood, and remainder of the blood is transfused back to the donor.

- Granulocytes may be used to treat neutropenic patients suffering from bacterial and/or fungal sepsis, who have been resistant to antimicrobial therapy. Granulocyte transfusion may benefit patients with abnormality of neutrophil function.
- Continued neutropenia is treated with granulocyte colony-stimulating factor (G-CSF) to stimulate granulopoiesis. If the patient does not respond to G-CSF, donor granulocytes should be transfused. Recombinant growth stimulating hormones, usually **G-CSF** may be administered to mobilize the donor's bone marrow to release larger numbers of granulocytes to produce high yield of granulocytes.
- Granulocyte units should be administered within 24 hours of collection from donor, which are always irradiated prior to transfusion to prevent transfusion associated graft-versus-host disease (**TA-GVHD**).
- Granulocytes should not be infused through leukoreduction filters. The donor and recipient should be both human leukocyte antigen (HLA) and Rh compatible.

HEMATOPOIETIC STEM CELLS USED FOR TRANSPLANTATION

Hematopoietic stem cells (HSCs) are primitive stem cells that have the capacity for self-renewal and maturation into different lineages such as RBCs, WBCs and

platelets. Transplantation of HSCs is combined with high-dose chemotherapy or radiation to treat leukemia, lymphoma, multiple myeloma, severe aplastic anemia, myelodysplastic syndrome, myelodysplastic neoplasms, severe combined immunodeficiency (SCID) syndrome, some hemoglobinopathies, some solid tumors and metabolic disorders.

- Hematopoietic stem cells (HSCs) are obtained from bone marrow, peripheral blood or umbilical cord. By using recombinant colony-stimulating factor, HSCs are mobilized from bone marrow and usually collected from peripheral blood. Peripheral blood HSCs collection requires a catheter, that may carry pathogens. HSCs take short time to engraft, but there is increased risk for development of graft-versus-host disease (GVHD).
- Conversely, hematopoietic stem cells (HSCs) collection directly from bone marrow carries higher risk for the donor from surgical procedure and anesthesia. HSCs take longer time to engraft, but development of graft-versus-host disease is less severe.
- An alternative source of HSCs is umbilical cord blood. HSCs are collected from the umbilical cord and placenta immediately after birth of newborn. Umbilical cord has high number of hematopoietic stem cells, which are cryopreserved using 10% diethyl sulfoxide and stored frozen in public cord banks for further use. Hematopoietic stem cell transplants can be either autologous or allogeneic depending on the disease being treated. Autologous HSCs refer to cells or tissue obtained from self. Allogeneic HSCs refer to cells or tissue obtained from a genetically different individual.

Hematology Pearls: Autologous and Allogeneic Hematopoietic Stem Cells

Autologous Hematopoietic Stem Cells used for Transplant

- Hematopoietic stem cells (HSCs) are collected from patient's peripheral blood or bone marrow after lower doses of chemotherapy and cryopreserved using 10% diethyl sulfoxide in frozen state.
- Hematopoietic stem cells (HSCs) are further treated by high doses of chemotherapy and radiation to eradicate remaining cancer cells. These cryopreserved HSCs are now thawed and infused to restore hematopoietic function.
- Hematopoietic stem cells (HSCs) are currently used for disorders such as Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, neuroblastoma and testicular cancer, which are more often resistant to first-line chemotherapy and irradiation.

Allogeneic Hematopoietic Stem Cells used for Transplant

- Allogeneic hematopoietic stem cell transplant is used in certain patients after failure of an autologous transplant.
- Hematopoietic stem cells (HSCs) are obtained from HLA-matched related healthy donor.
- Allogeneic HSCs are used in patients suffering from acute myelogenous leukemia, high risk cytogenetic acute lymphoblastic leukemia, aplastic anemia, high-risk cytogenetic myelodysplastic syndrome and late stage imatinib-resistant chronic myelogenous leukemia. Prior to infusion of HSCs, the patient is administered immunosuppressive therapy to prevent graft-versus-host disease.
- Allogeneic HSCs may be incompatible for ABO/Rh antigens for both donor and recipient.
- After an HLA-match allogeneic HSC transplant, some patients experience a relapse of their original disease. Donor lymphocytic infusion (CD3 positive) is done to induce destruction of malignant cells in the recipient.

MODIFICATION OF CELLULAR COMPONENTS

Modification of cellular components is performed by leukoreduction, irradiation of cells, washing and freezing and deglycerolization of RBCs in blood bags.

Pre-Transfusion Modifications of Blood Cellular Components

Leukoreduction

- Febrile reactions are common in sensitized patients, who receive components >5×10^9 leukocytes per blood component and alloimmunization to the HLA antigens of residual lymphocytes.
- Leukoreduction helps to prevent the formation of antibodies to HLA antigens expressed on lymphocytes. Leukocytes are removed by use of leukocyte reduction filters because of their large size compared with RBCs.
- Leukoreduction reduces risk of febrile nonhemolytic blood transfusion reactions, graft-versus-host disease (GVHD), transplant rejection, reactivation of cytomegalovirus, human T cell lymphotropic virus 1 (HTLV-1) and Epstein-Barr virus.

Irradiation of RBCs and Granulocytes

- Irradiation of granulocytes is performed to prevent transfusion associated graft-versus-host disease (TA-GVHD). Gamma-radiation disrupts the DNA in WBCs.
- Blood bags are given γ-irradiation dose of 25 Gy. Irradiated RBCs result in increased potassium leakage.
- Fresh frozen does not need to be irradiated because it seldom contains viable WBCs.
- In immunocompromised persons, cytomegalovirus can cause significant morbidity and mortality, which include premature and low birth neonates. Testing of blood component of donor for cytomegalovirus is essential prior to transfusion.
- cytomegalovirus seronegative blood component is transfused to the recipients.

Washing of RBCs

Washing of RBC units eliminates 85% of WBCs, 15% of RBCs and 99% of plasma; that can be transfused to IgA deficient patients having antibodies to IgA. Washing is also indicated in patients, who have severe allergic reactions to blood products.

Freezing and Deglycerolization of RBCs

- Blood from donors with rare RBC phenotypes may be frozen for a period of 10 years and used for autologous of allogeneic transfusion.
- Each blood unit is frozen with cryoprotective agent such as 20–40% of glycerol. Prior to blood transfusion, blood unit should be washed, a process known as deglycerolization.
- The blood product should be used within 24 hours for open system or two weeks for closed systems. Frozen platelet products can be stored for two years.

TRANSFUSABLE PLASMA COMPONENTS

Several blood components are used to treat deficiencies and abnormalities of coagulation proteins. Administration of fresh frozen plasma (**FFP**) provides many coagulation factors and improves hemostasis in patients with multiple deficient coagulation factors and massive transfusions. Cryoprecipitate provides factors VIII, XIII, von Willebrand factor, fibrinogen and fibronectin. Cryoprecipitate can be used prior to surgical procedures in patient with dysfibrinogenemia.

Fresh Frozen Plasma

Fresh frozen plasma (FFP) is obtained by separation of plasma and frozen at –18°C within 8 hours of whole blood collection. Plasma is frozen rapidly either in a dry ice-ethanol or in a dry ice-antifreeze bath or in a mechanical freezer kept at –65°C or lower. It can be stored at –65°C for 7 years.

- **Indications:** Fresh frozen plasma is administered by intravenous route in a dose 7–15 ml/kg body weight in patients suffering from hemophilia A and Christmas disease. It is also valuable in treatment of thrombotic thrombocytopenic purpura. It can also be used in the reversal of adverse effects of oral coagulant therapy associated with bleeding occurring due to depletion of coagulation factors.
- **Adverse effects:** Approximately 6–12% cases of hemophilia A patients develop antibodies against factor VIII after transfusion of fresh frozen plasma (FFP). Therefore, recombinant factor VIII is being transfused in hemophilia A patients.

Cryoprecipitate

Cryoprecipitate is a cold insoluble fraction of plasma proteins obtained from fresh frozen plasma (FFP) and prepared by process of centrifugation of fresh frozen plasma after thawing at 4°C. Cryoprecipitate is stored at –18°C within one hour of separation from fresh frozen plasma, which can be used within one year from the date of blood collection.

- **Constituents:** Important constituents of cryoprecipitate are fibrinogen, factor VIII, factor XIII, von Willebrand factor (vWF) and fibronectin. Cryoprecipitate cannot serve as a therapeutic source of the coagulation factors II, V, VII, IX, X, XI and XII. The coagulation factors should be obtained from fresh frozen plasma (FFP) in specific concentration.
- **Indications:** Currently, the major use of cryoprecipitate is a source of fibrinogen. Deficiency of fibrinogen is seen in massive blood transfusion and disorders involving activation of the coagulation system. Cryoprecipitate is administered by intravenous route to promote hemostasis in various coagulation disorders, e.g. fibrinogen deficiency, hemophilia A, factor XIII deficiency and von Willebrand disease. Advantage of administration of cryoprecipitate is exposure of the recipients to a few donors. Blood products are costly and limited worldwide. Excessive administration of cryoprecipitate is not beneficial to the patients suffering from hemophilia A, von Willebrand disease, fibrinogen deficiency and factor XIII deficiency.

Cryosupernatant Plasma

Cryosupernatant refers to plasma from which the cryoprecipitate has been removed. Thawing of fresh frozen plasma (FFP) between 1°C and 6°C, cold insoluble cryoprecipitate is separated. The remaining thawed plasma is known as cryosupernatant, which is stored at –18°C. It should be administered to the patients within one year from the date of whole blood collection.

- **Constituents:** Since cryoprecipitate contains fibrinogen, factor VIII, von Willebrand factor (vWF) and fibronectin, and these components are reduced in cryosupernatant. Levels of factor VIII and fibrinogen are greatly reduced.
- **Indications:** Cryosupernatant plasma can be used when replacement of factor VIII is not required. It may be used in refractory thrombotic thrombocytopenic purpura (**TTP**) and hemolytic uremic syndrome (**HUS**).

Factor VIII Concentrate

Factor VIII concentrate is the product of choice for patients with hemophilia A, which has a short half-life about 12 hours *in vivo*. High purity factor VIII concentrate is administered continuously during surgery.

Factor IX Concentrate

High purity factor IX concentrate is used for patients with hemophilia B also known as Christmas disease.

Prothrombin Complex Concentrate

Prothrombin complex concentrate contains coagulation factors IX, X, II and sometimes factor VII, which are more effective than fresh frozen plasma (FFP). Prothrombin complex concentrate is used to treat hemophilia A patients, who have circulating antibody (inhibitor) to factor VIII.

Specific Immunoglobulins

Specific immunoglobulins are obtained from donors, who have high concentration of IgG and administered in patients for passive prophylaxis against infectious agents. Anti-D is administered to the Rh –ve mother having Rh +ve fetus in the prevention of hemolytic disease of newborn.

PLATELET COMPONENT TRANSFUSION

Platelet component transfusion usually controls bleeding in thrombocytopenic patients or dysfunctional platelet disorders, either due to suppressed platelets production in leukemias, chemotherapy, radiotherapy; or dilutional thrombocytopenia after massive transfusions. Platelets circulate for a week in stable non-immunized thrombocytopenic patients.

- Prophylactic platelet component transfusion in a patient with $<10\times10^9$/L is administered to prevent intracranial hemorrhage.
- Platelet-rich plasma (PRP) is an autologous concentrate of platelets in a small volume of plasma used to stimulate acceleration of healing of bone fracture and soft tissue injury.
- Random donor platelet components are prepared from donor's whole blood within 4–5 hours of collection by process of centrifugation in platelet apheresis machine.
- Platelet components obtained from single donor are administered to treat acute hemorrhage secondary to thrombocytopenia.
- One unit of plateletpheresis can be divided into four equal packs for creating pediatric sized platelets component to minimize platelet wastage. An adult dose of pooled platelets is obtained from 4 donors.

FIBRIN GLUE AND PLATELET GEL

Commercial preparations of fibrin glue are used as sealant during surgery in order to produce tight closure of the wound. Platelet gel is composed of autologous platelet and WBC-rich plasma, calcium chloride and thrombin, that are used to stimulate coagulation system and healing of surgical wound.

PLASMA FRACTIONATION PRODUCTS

Plasma fractionation products include albumin, Rh immunoglobulin, intravenous immunoglobulin (IVIg), hyperimmunoglobulin and coagulation factor concentrates.

Hematology Pearls: Plasma Fractionation Products

Albumin

Albumin is most commonly used as a volume replacement in trauma, shock, burns and therapeutic plasma exchange.

Rh Immunoglobulin

Rh immunoglobulin is administered to prevent immunization to the D antigen. Rh immunoglobulin is administered to Rh negative mother with Rh positive fetus in the 28th week of pregnancy.

Intravenous Immunoglobulin

Intravenous immunoglobulin (IVIg) is administered in hypogammaglobulinemia/agammaglobulinemia, severe combined immunodeficiency syndrome, Wiskott-Aldrich syndrome, pediatric HIV with recurrent bacterial infections, post-allogeneic transplant, chronic lymphocytic leukemia, immune thrombocytopenic purpura, pure red cell aplasia, neonatal alloimmune thrombocytopenic purpura, hemolytic disease of the fetus and newborn, post-transfusion purpura, myasthenia gravis, Guillain-Barré syndrome, dermatomyositis/polymyositis and staphylococcal toxic shock syndrome.

Hyperimmunoglobulin

Hyperimmunoglobulin is administered to provide passive immunity in patients suffering from rabies, rubella, cytomegalovirus, tetanus, varicella zoster and hepatitis. Coagulation factor concentrates are administered to prevent or treat bleeding episodes in patients with coagulation factors deficiency. Hemophilia patients most often develop inhibitors to antihemophilic factor.

PHARMACEUTICAL DRUGS USED TO REDUCE/ PREVENT BLEEDING IN HEMATOLOGIC DISORDERS

There are several drugs administered to reduce or prevent bleeding in hematologic disorders. Recombinant products are available in modern age.

Hematology Pearls: Pharmaceutical Drugs used to Reduce/Prevent Bleeding in Hematologic Disorders

Desmopressin Acetate

Desmopressin acetate is a synthetic peptide, 1-deamino-8-D-arginine vasopressin (DDAVP). It is prophylactically administered prior to surgery to reduce bleeding or prevent bleeding in patients with mild hemophilia A or type 1 von Willebrand disease. DDAVP releases factor VIII and von Willebrand factor from the vascular endothelial cells.

Vitamin K Preparations

Vitamin K can be administered to reverse the effect of oral anticoagulants and prepare patients for surgery. Vitamin K-dependent coagulation proteins include: factors II, VII, IX and X.

Erythropoietin

Erythropoietin is synthesized by kidney to stimulate erythropoiesis. Recombinant erythropoietin is administered to stimulate erythropoiesis in patient with chronic renal failure.

Thrombopoietin

- Thrombopoietin is synthesized by liver and kidneys and to a lesser extent by bone marrow, spleen, testes, brain and muscle that stimulate megakaryopoiesis.
- Recombinant thrombopoietin can be administered to stimulate megakaryopoiesis in patients with suffering from bone marrow suppression that has caused thrombocytopenia.
- Biological agents such as romiplostim and eltrombopag are thrombopoietin receptor agonists stimulate platelet production in patient suffering from idiopathic thrombocytopenic purpura (ITP).

Colony Stimulating Factors

- Recombinant preparations of colony stimulating factors such as G-CSF and GM-CSF are administered in patients with neutropenia after chemotherapy in cancer patients.
- Plerixafor preparation is administered to patients, who are unable to produce sufficient amounts of autologous hematopoietic stem cells.

Antifibrinolytic Preparations

Antifibrinolytic preparations inhibit fibrinolysis. Fibrinolysis normally increases during cardiopulmonary bypass. These agents are administered during cardiac surgery to reduce bleeding due to increased fibrinolysis.

Recombinant Factor VIIa Product

Recombinant factor VIIa is administered to patients suffering from hemophilia A and hemophilia B having inhibitors, which initiates deposition of fibrin on platelets and induces platelet aggregation at the site of injury.

MASSIVE BLOOD TRANSFUSION

Massive blood transfusion is defined as replacement of recipient patient's total blood volume with donor components within 24 hours in cases of trauma, obstetrics related conditions, surgery, gastrointestinal tract bleeding, aneurysm and plasma exchange in thrombotic thrombocytopenic purpura or hemolytic uremic syndrome (HUS). Patients receiving multiple blood transfusions are at increased risk for febrile reactions, allergic reactions, volume overload, sepsis, viral infections, transfusion-related acute lung injury (TRALI), hemolytic reactions, alloimmunization, anticoagulant toxicity, hyperkalemia and hypothermia.

ADVERSE BLOOD TRANSFUSION REACTIONS

Blood transfusion reactions are defined as adverse events associated with the transfusion of whole blood or its components. Acute adverse blood transfusion reactions may occur within 24 hours of transfusion. Delayed blood transfusion reactions occur after one day to many weeks. Acute and delayed adverse blood transfusion reactions are given in Table 12.25. One important aspect of blood transfusion safety is the reporting and surveillance of adverse blood transfusion events. Hemolytic blood transfusion reactions and cytokines are given in Table 12.26. Clinical pearls of blood transfusion-related manifestations are given as follows.

Table 12.25 Acute and delayed adverse blood transfusion reactions

Adverse Blood Transfusion Reactions	Comments
Acute adverse blood transfusion reactions	
Acute hemolytic transfusion reactions	Donor red blood cells are destroyed by recipient's blood containing IgM. Intravascular hemolysis results in hemoglobinuria, acute renal failure and oliguria
Air embolism	Air embolism occurs due to entry of excess air >100 cc at infusion site and obstructing blood flow
Allergic transfusion reactions	Skin rashes, urticaria (hives), pruritus (itching), generalized flushing, edema of lips, tongue and/or uvula, erythema (redness of skin), edema in the periorbital area and conjunctival edema
Anaphylactic transfusion reactions	Bronchospasm and rarely anaphylactic shock
Citrate toxicity	Citrate binds with calcium and magnesium resulting in hypocalcemia and hypomagnesemia
Febrile nonhemolytic transfusion reactions	Fever, rigors and chills due to sensitization of donor WBCs within an hour of transfusion

Contd...

Table 12.25 Acute and delayed adverse blood transfusion reactions *(Contd...)*

Adverse Blood Transfusion Reactions	Comments
Hypotensive transfusion reactions	Hypotension occurs due to platelet component transfusion related to bradykinin production
Hypothermia	Rapid transfusion of cold-temperature blood products
Transfusion-associated circulatory overload (TACO)	Acute pulmonary edema with impaired cardiac functions
Transfusion-associated dyspnea (TAD)	Transfusion-associated dyspnea term is used when the patient develops respiratory distress; and exclusion of transfusion-associated acute lung injury and transfusion-associated circulatory overload
Transfusion-related acute lung injury (TRALI)	Acute respiratory distress syndrome occurs within six hours of transfusion due to formation of antibodies against RBC antigens and phagocytes
Delayed adverse blood transfusion reactions	
Delayed hemolytic transfusion reactions	Delayed hemolytic reactions occur four days to two weeks after blood transfusion due to extravascular destruction of RBCs
Transfusion related hemosiderosis	Occurs due to multiple blood transfusions. Chelating agents should be administered to prevent hemosiderosis
Post-transfusion purpura (PTP)	Pre-existing alloantibodies in recipient's plasma against donor's platelet antigens result in platelet destruction by reticuloendothelial cells
Transfusion associated graft-versus-host disease (TA-GVHD)	Occurs when immunocompetent T cells from the donor are transfused to immunodeficient recipients recognize HLA as foreign

Table 12.26 Hemolytic blood transfusion reactions associated with cytokines

Cytokines Terminology	Biological Activity	
Pro-inflammatory cytokines		
IL-1, TNF-α	▪ Hypotensive shock, death ▪ Mobilization of leukocytes from bone marrow ▪ Activation of T and B cells	▪ Induction of IL-1, TNF-α, IL-6, chemokines (CXCL-8 and CCL-2) ▪ Induction of adhesion of molecules
IL-6	▪ Fever ▪ Synthesis of acute phase reactants	▪ Activation of B and T cells
Chemokines		
CXCL-8 (C-X-C)	▪ Chemotaxis of neutrophils and their activation ▪ Chemotaxis of lymphocytes ▪ Induction of histamine release by basophils	
CCL-2 (C-C)	▪ Induction of IL-1, adhesion molecules and respiratory burst ▪ Chemotaxis of monocytes	
Anti-inflammatory cytokines		
IL-1Ra	Competitive inhibition of receptors of IL-1	

Clinical Pearls: Blood Transfusion-related Clinical Manifestations

Blood Transfusion-related ABO Incompatibility

- During blood transfusion, patient presents with hypotensive shock, tachycardia, chest pain, backache and passage of dark urine. Serum bilirubin and lactic acid dehydrogenase levels are elevated. Serum haptoglobin level is low.
- ABO incompatibility presents with acute symptoms of hemolysis while the blood transfusion is continuing.

Blood Transfusion-related Acute Hemolytic Reaction

Acute hemolytic reaction is a complication of transfusion-related blood to intravascular hemolysis, characterized by rapid onset with symptoms of fever, chills, hemoglobinemia and hypotension, major complications such as irreversible shock, renal failure and disseminated intravascular coagulation.

Blood Transfusion-related Acute Lung Injury (TRALI)

- Thirty minutes after a patient receives blood transfusion, patient presents with shortness of breath.

- Transfusion-related acute lung injury (TRALI) presents with acute shortness of breath from antibodies in the donor blood against recipient WBCs.
- Chest radiograph shows transient infiltrates in lungs. Symptom resolves spontaneously with any treatment.

Blood Transfusion-related IgA Deficiency

- As soon as patient receives blood transfusion, patient presents with hypotensive shock, shortness of breath and tachycardia. Serum bilirubin and lactic acid dehydrogenase levels are within normal range.
- IgA deficiency presents with anaphylaxis. Clinician should use blood donations from IgA deficient donor or washed red blood cells.

Minor Blood Group Incompatibility

- A few days after blood transfusion, patient develops jaundice. The hematocrit does not rise with blood transfusion.
- Minor blood group incompatibility to Kell, Duffy, Lewis or Kidd antigens or Rh incompatibility presents with delayed jaundice. There is no specific therapy for these patients.

Febrile Nonhemolytic Reactions

- A few hours after blood transfusion, patient presents with fever above 1°C. There is no evidence of hemolysis.
- Febrile nonhemolytic reactions result in a small rise in temperature. These reactions are against donor WBC antigens. These reactions are prevented by using filtered blood transfusions in the future to remove the WBC antigens.

BLOOD TRANSFUSION-RELATED IMMUNE HEMOLYTIC REACTIONS

Immune-mediated hemolytic blood transfusion reactions are most commonly associated with patient antibodies that are directed against antigens present on the transfused RBCs, which reactions can be divided into two categories.

- Blood transfusion-related acute hemolytic reactions occur immediately after exposure to the transfusion component, that is associated with complement-mediated intravascular hemolysis.
- Blood transfusion-related delayed hemolytic reactions may occur days to week after transfusion, that is associated with extravascular hemolysis by antibody and/or complement-coated RBCs are cleared through phagocytosis by macrophages in spleen, liver and bone marrow.
  - Proper pretransfusion testing can prevent immune-mediated hemolytic reactions.
  - Delayed hemolytic reactions usually occur due to the presence of patient antibodies to non-ABO red blood cell (RBC) antigens.
- Immediate and delayed blood transfusion-associated hemolytic reactions are given in Table 12.27.

BLOOD TRANSFUSION-RELATED ACUTE HEMOLYTIC REACTIONS

Blood transfusion-related acute hemolytic reactions occur when antibodies (IgM) react with antigens on RBCs. Antigen–antibody reactions activate complement system resulting in intravascular hemolysis.

Pathophysiology

Activated potent anaphylatoxins such as C3a and C5a stimulate the release of histamine and serotonin from mast cells resulting in vasodilation and smooth muscle contraction.

- Antigen–antibody complexes can activate factor XIII and trigger the intrinsic pathway of coagulation system.
- While activated complement system increases expression of tissue factor expressed on endothelial cells and leukocytes resulting in triggering extrinsic pathway of coagulation system.
- Both intrinsic and extrinsic pathways of coagulation lead to development of disseminated intravascular coagulation (**DIC**) resulting in formation of microthrombi in the capillaries associated with uncontrolled bleeding due to consumption of coagulation factors and platelets.

Table 12.27 Immediate and delayed blood transfusion associated hemolytic reactions

Parameters	Immediate Blood Transfusion Associated Hemolytic Reactions	Delayed Blood Transfusion Associated Hemolytic Reactions
Time of onset	Within one hour of blood transfusion	Within 7–10 days of blood transfusion
Antibody responsible for hemolysis	IgM (e.g. anti-A, anti-B) natural occurring antibody	IgG (e.g. anti-RhD) formed due to immune response
Site of hemolysis	Intravascular hemolysis	Extravascular hemolysis
Clinical features	Headache, backache, hypotension, renal failure	Asymptomatic or mild fever or jaundice
Laboratory findings	▪ Hemoglobinemia ▪ Methemalbuminemia	▪ Serum bilirubin raised ▪ Anemia positive/negative
Mortality	5–10%	0–1%

- Renal damage is most important complication of blood transfusion-related acute hemolytic process, which may be partially due to free hemoglobin in the tubules and reduced renal blood flow.

Clinical Features

Patient most often presents with fever with or without chills and hemoglobinuria. Other signs and symptoms are variable, which include hemoglobinemia (free hemoglobin in the plasma), positive direct antiglobulin test (DAT), pain at the intravenous site, pain in abdomen, chest, flank or back, nausea and vomiting, hypotension progressing to shock, dyspnea, disseminated intravascular coagulation (DIC) and renal failure. In anesthetized patients, hypotension and bleeding are the only findings.

BLOOD TRANSFUSION-RELATED DELAYED HEMOLYTIC REACTIONS

Blood transfusion-related delayed hemolytic reactions may occur days to weeks after blood transfusion, that is associated with extravascular hemolysis by antibody and/or complement system protein coated RBCs are cleared through phagocytosis by macrophages in spleen, liver and bone marrow. Delayed hemolytic reactions are less severe than acute hemolytic reactions.

Pathophysiology

Antibodies directed against the RBC antigens bind to the RBC membrane. There is absence or minimal activation of complement system. Hence, antigen–antibody-coated RBCs remain in the circulation, where these RBCs are cleared by reticuloendothelial cells in the spleen, liver and bone marrow resulting in extravascular hemolysis.

Clinical Features

Patient presents with fever, anemia, jaundice, hemoglobinuria and leukocytosis days to weeks after blood transfusion. Some patients progress to hyperhemolysis due to formation of autoantibodies in patients receiving multiple blood transfusions in cases of sickle cell anemia and thalassemia. Hyperhemolysis involves both intravascular and extravascular hemolysis resulting in fatal outcome.

BLOOD TRANSFUSION-RELATED FEBRILE NONHEMOLYTIC REACTIONS

Blood transfusion-related febrile nonhemolytic reactions involve increase in body temperature of at least 10°C either during blood transfusion or shortly after the blood transfusion especially patients receiving multiple blood transfusions or previously pregnant women.

PATHOPHYSIOLOGY

Blood transfusion-related febrile nonhemolytic reaction is a leukocyte-mediated reaction occurring by two mechanisms: (a) the recipient has been alloimmunized to human leukocyte antigens (HLA) as a result of exposure to foreign WBCs during a prior blood transfusion, organ transplant or pregnancy. The recipient's HLA antibody attacks the transfused WBCs that possess the concurrent antigen; (b) during blood storage, cytokines released by WBCs, which are passively transfused to the recipient resulting in initiation of inflammatory response.

- Both these mechanisms result in the release of pyrogens responsible for fever. The cytokine-mediated reactions are most often associated with the transfusion of platelet products.
- Leukoreduction of blood products reduces risk of febrile nonhemolytic reaction. Febrile blood transfusion reactions are also observed in acute hemolytic blood transfusion reaction, bacterial contamination of donor unit, blood transfusion-related lung injury and delayed blood transfusion reactions.

CLINICAL FEATURES

Patient presents with fever 39°C, rigors and chills, wheezing, coughing, dyspnea, hypertension or hypotension, headache or vomiting.

BLOOD TRANSFUSION-RELATED ALLERGIC REACTIONS

Blood transfusion-related allergic reactions occur due to protein-based allergens that react with preformed plasma IgG or IgE antibodies. The antibody binds to the allergen resulting in activation and degranulation of mast cells and releasing cell products, i.e. histamine and heparin, which stimulate vasodilation and influx of fluids to the tissues with subsequent edema.

CLINICAL FEATURES

Patient presents with at least two of the following signs and symptoms occur during or within two hours of blood transfusion, which include skin rash, urticaria (hives), pruritus (itching), generalized flushing, edema of lips, tongue and/or uvula, erythema (redness of skin), edema in the periorbital area and conjunctival edema.

BLOOD TRANSFUSION-RELATED ANAPHYLACTIC/ANAPHYLACTOID REACTIONS

Blood transfusion-related anaphylactic/anaphylactoid reactions are rare but severe life-threatening nonhemolytic type 1 hypersensitivity reactions.

PATHOPHYSIOLOGY

Blood transfusion-related anaphylactic reactions occur due to allergic responses to plasma-containing blood products. There is a reaction between a protein allergen in the transfused blood product and its corresponding antibody, which is either in the donated blood component or in the recipient's plasma.

- Antigen–antibody complexes activate mast cells, which release histamine and other chemical mediators resulting in anaphylactic reactions.
- Anaphylactoid blood transfusion reactions occur in IgA-deficient persons, they have made antibody to IgA after exposure to IgA through pregnancy or prior transfusion. When these persons are subsequently transfused with plasma-containing blood products, IgA in the blood-containing plasma blood product initiates the anaphylactoid response. Clinical presentation of anaphylactic and anaphylactoid blood transfusion reactions is indistinguishable.

CLINICAL FEATURES

Patient presents with immediate severe respiratory symptoms such as laryngeal edema, tightness in throat, dysphagia, dysphonia, hoarseness of voice, stridor, dyspnea, coughing, wheezing and bronchospasm. Anaphylactic and anaphylactoid transfusion reactions can be prevented by using washed RBCs or platelet units, autologous donation of blood and/or plasma and deglycerolized rejuvenated RBC units.

BLOOD TRANSFUSION-RELATED ACUTE LUNG INJURY

Blood transfusion-related acute lung injury is an acute adverse reaction, that occurs within 6 hours of blood transfusion.

- Patient develops inadequate oxygenation of blood (hypoxemia) in the absence of cardiac involvement. Hypoxemia is defined by oxygen saturation <90% on room air or PaO_2.
- Chest radiograph shows bilateral pulmonary infiltrates. It is presumed that neutrophils play an important role in causing damage to pulmonary capillaries resulting in pulmonary edema.
- Acute lung injury occurs by both immune and nonimmune mechanisms. Immune response is antibody-mediated reaction, in which antibody is directed against HLA class I, HLA class II or human neutrophil antigens. Antibody-coated neutrophils accumulate in the pulmonary microvasculature and activate complement system resulting in destruction of pulmonary capillaries.

CLINICAL FEATURES

Patient presents with chills, cough, fever, cyanosis, tachycardia and hypotension, pulmonary edema without cardiac involvement. Most cases recover within hours to days with supportive therapy. Blood transfusion-related acute lung injury has been identified as a leading cause of blood transfusion-related death. Diagnostic criteria for blood transfusion-related acute lung injury are given in Table 12.28.

BLOOD TRANSFUSION-RELATED CIRCULATORY OVERLOAD

Rapid rate of transfusion of blood products is the most important cause of transfusion-related circulatory overload. Excess fluid volume in the circulation can result in pulmonary edema and/or congestive heart failure. Blood transfusion-related circulatory overload is diagnosed by presence of at least three symptoms, e.g. acute respiratory distress (dyspnea, orthopnea, cough), evidence positive fluid balance, elevated brain natriuretic peptide, radiograph evidence of pulmonary edema, evidence of left heart failure and elevated central venous pressure. Infusion of red blood cell concentrate is helpful in the management of these cases.

BLOOD TRANSFUSION-RELATED DYSPNEA

Blood transfusion-related dyspnea term is used when the patient develops respiratory distress; and exclusion

Table 12.28 Diagnostic criteria for blood transfusion-related acute lung injury

Features	Comments
Onset of acute lung injury	Within 6 hours of transfusion
Oxygenation	▪ Partial pressure of arterial oxygen (PaO_2)/fraction of inspired oxygen in mm Hg (FiO_2) ▪ Oxygenation saturation of ≤90 and on room temperature
Chest radiograph	Bilateral pulmonary infiltrates
Blood pressure	▪ Pulmonary artery occlusion pressure ≤18 mm Hg when measures ▪ No evidence of left atrial hypertension

of transfusion-related acute lung injury and transfusion-related circulatory overload.

BLOOD TRANSFUSION-RELATED HYPOTENSION

Blood transfusion-related hypotension occurs during or within 1 hour of transfusion, which is characterized by sudden drop of blood pressure (systolic or diastolic) to >30 mm Hg.

PATHOPHYSIOLOGY

Platelet component infusion is usually implicated in hypotensive blood transfusion reactions. Whole blood and packed RBC component administration may cause blood transfusion reactions but to a lesser extent. Hypotensive blood transfusion reactions are related to **bradykinin** produced by kinin-kallikrein system of blood proteins. Activated factor XIII releases kallikrein from high molecular kininogen resulting in generation of bradykinin. Normally, angiotensin-converting enzyme (ACE) degrades bradykinin.

CLINICAL FEATURES

Patient receiving inhibitors of angiotensin-converting enzyme (ACE) is more prone to develop hypotensive transfusion reactions.

- Angiotensin-converting enzyme (ACE) inhibitor-related hypotensive reaction has been observed in liver transplantation in recipients. Patient receiving ACE inhibitors should not be transfused blood component. Leukoreduction of blood products should be preferred.
- Hypotensive blood transfusion reactions should be treated with vasopressor agents resulting in quick increase in blood pressure.
- Immunodeficient recipients fail to eliminate donor T cells resulting in blood transfusion-related graft-versus-host disease.

BLOOD TRANSFUSION-RELATED GRAFT-VERSUS-HOST DISEASE

Blood transfusion-related graft-versus-host disease is a rare complication, but fatal in 90% of cases within 1–3 weeks of onset of graft-versus-host disease.

PATHOPHYSIOLOGY

The blood transfusion-related graft-versus-host disease is caused by donor T cells of transfused blood products. Donor T cells carrying different human leukocyte antigen (HLA) attack the recipient host tissues.

- Immunocompetent recipients may be at risk of developing transfusion-related graft-versus-host disease.
- Recipient patient with fully functional immune system, patient's lymphocytes may not recognize donor's lymphocytes because both share the HLA A_1 antigen. However, donor's lymphocytes may recognize recipient's HLA A_2 as non-self and can attack the recipient's tissues.
- Patient develops blood transfusion-related graft-versus-host disease within a few days to six weeks after transfusion of blood products.

CLINICAL FEATURES

Patient presents with fever, skin rashes, hepatomegaly, diarrhea, anorexia, nausea and vomiting. Pancytopenia develops within 3–4 weeks after blood transfusion resulting in recurrent infections. Definite diagnosis of graft-versus-host disease includes evidence of liver dysfunction (elevated serum enzymes and serum bilirubin), pancytopenia, bone marrow suppression (bone marrow aspirate and bone marrow trephine biopsy show hypoplasia or aplasia) and characteristic histology of skin (blister and bullae formation) and liver pathology (bile duct damage with lymphocytic infiltration of the portal tracts).

BLOOD TRANSFUSION-RELATED SEPSIS

Blood transfusion-related sepsis is nonimmune transfusion reaction resulting in immediate fever (rise of temperature above 2°C of normal), rigor and hypotension shortly after blood transfusion.

PATHOPHYSIOLOGY

Blood transfusion-related sepsis occurs when bacteria-contaminated blood is transfused to the recipient. Bacterial endotoxins generated during blood storage may lead to transfusion associated sepsis. Sources of bacterial contamination of blood components may occur during blood collection and storage of blood products.

PREVENTION

Storage of red blood cell component in refrigerator reduces risk for blood transfusion-related sepsis.

- The blood component should be inspected for lysis of RBCs or platelet clumping, analysis of biochemical markers, light microscopy of gram stained smears and immunoassays prior to blood transfusion.
- Blood transfusion-related sepsis can be prevented by donor clinical history, proper phlebotomy technique,

Table 12.29 Blood transfusion-related sepsis

Gram Stain	Bacterial Contamination	Blood Transfusion-related Sepsis	Source
Gram-positive bacteria	▪ *Staphylococcus* species ▪ *Streptococcus* species ▪ *Bacillus* species	▪ Red blood cell component ▪ Platelet component	▪ Skin flora ▪ Natural environment
Gram-negative bacteria	▪ *Pseudomonas* species ▪ *Escherichia* species ▪ *Yersinia* species ▪ *Serratia* species	▪ Red blood cell component ▪ Platelets component	▪ Blood stream ▪ Natural environment

single arm collection technique, using diversion pouch, prestorage leukoreduction, pathogen inactivation, performing automated culture of bacteria, visual inspection of blood component, biochemical markers, microscopy of gram stained smear and immunoassays. Blood transfusion-related sepsis is given in Table 12.29.

BLOOD TRANSFUSION-RELATED HEMOSIDEROSIS

Blood transfusion-related hemosiderosis is an adverse response to multiple (usually >100) RBC component transfusion in patients suffering from thalassemia major, sickle cell disease and other hereditary hemolytic anemias. One RBC component unit contains approximately 250 mg of iron. Following multiple blood transfusions, iron begins to accumulate and deposit in tissues of the body. Iron deposits interfere with normal functions of major organs.

CLINICAL FEATURES

Patient presents with anemia, weakness and fatigue. Serum ferritin and total iron binding capacity should be assessed to confirm the diagnosis. Perls Prussian blue stain is applied to tissue section or bone marrow aspirate smear to demonstrate iron. Prevention of hemosiderosis is achieved by limiting blood transfusion of RBC products, administration of young RBCs and chelating agents.

BLOOD TRANSFUSION-RELATED PURPURA

Blood transfusion-related purpura presents as severe life-threatening thrombocytopenia within 5–12 days especially in females over the age of 60 years. Platelet level is <20% of pretransfusion level. Cerebral hemorrhage is the main cause of death.

PATHOPHYSIOLOGY

Transfusion of leukocyte-reduced blood products has decreased the incidence of blood transfusion-related thrombocytopenia over years.

- Pre-existing alloantibodies in recipient's plasma against donor's platelet antigens result in platelet destruction by reticuloendothelial cells in spleen.
- Majority of cases develop alloantibodies after exposure to platelet antigens during pregnancy and prior blood transfusion. Blood transfusion-related purpura occurs as a result of transfusion of whole blood, RBC component and any cellular blood product. Leukoreduction decreases the incidence of blood transfusion-related thrombocytopenia.
- Anti-HPA-1a is the most commonly identified antibody in blood transfusion thrombocytopenia accounting for more than 75% of cases. Patient presents with fever, chills and thrombocytopenia.

RAPID MASSIVE BLOOD TRANSFUSION-RELATED REACTIONS

Rapid massive blood transfusion-related reactions occur due to citrate toxicity (hypocalcemia and/or metabolic acidosis and/or hypomagnesemia), hypothermia and dilution coagulopathy.

CITRATE TOXICITY

Following rapid massive blood transfusion, there is transient increase in the plasma citrate level, because normal liver has capacity to metabolize the citrate.

- Citrate toxicity occurs in patients undergoing exchange blood transfusion in newborns and massive blood transfusion in adults, who have liver dysfunction.
- Elevated plasma citrate binds to calcium and magnesium resulting in hypocalcemia and hypomagnesemia.

- Hypocalcemia results in tingling sensations, muscle spasm, skeletal muscle tremors and electrocardiogram changes. Citrate toxicity can cause metabolic acidosis.

HYPOTHERMIA

Rapid massive blood transfusion of cold-temperature blood products especially requiring thawing prior to transfusion results in hypothermia. Patient presents with body temperature less than 35°C, lactic acidosis, ventricular arrhythmia and impaired immune system. Hypothermia can be prevented by the use of blood warming devices, when large amounts of blood products are being transfused.

DILUTIONAL COAGULOPATHY

Rapid massive transfusion of red blood cell component, fluids, colloids and crystalloids can cause dilution of platelets and coagulation factors resulting in coagulopathy.

BLOOD TRANSFUSION-RELATED NONIMMUNE HEMOLYSIS

Several conditions, both physical and chemical in origin such as improper storage of blood at higher or freezing temperature, use of small-bore needles during rapid transfusion, osmotic hemolysis using hypotonic/hypertonic solutions and drugs causing nonimmune hemolysis. Nonimmune hemolysis can be prevented through adherence to written protocols and standards. Equipment function must be regularly analyzed.

BLOOD TRANSFUSION-RELATED HYPERKALEMIA

Blood transfusion-related hyperkalemia in the blood is serious adverse blood transfusion reaction. If not immediately treated, patient can develop respiratory failure and death. Red blood cells release potassium during storage of old blood products resulting in hyperkalemia. Irradiation of red blood cell component also leads to hyperkalemia. Plasma exchange in neonates is performed if serum potassium level reaches 8 mEq/L.

BLOOD TRANSFUSION-RELATED HYPOKALEMIA

Hypokalemia in the blood occurs due to blood transfusion of washed RBCs and deglycerolized RBCs. Hypokalemia causes cardiac disturbances. Premature infants and patients suffering from renal disease are at high risk for developing hypokalemia.

BLOOD TRANSFUSION-RELATED AIR EMBOLISM

Air embolism is a rare adverse reaction of blood transfusion. Excess of air (>100 cc) is introduced into the infusion site as a result of leak or crack in the infusion set. Air embolism may also occur due to improper monitoring of intravenous blood transfusion.

- The consequence of an air bubble in the blood vessels leads to obstruction to circulatory blood flow.
- Patient presents with cough, chest pain and dyspnea. Use of plastic infusion sets has reduced incidence of air embolism.
- Air embolism can be prevented by expelling air from blood infusion set and changing of cracked infusion set before starting blood transfusion.

BLOOD TRANSFUSION-RELATED THROMBOPHLEBITIS

Blood transfusion-related thrombophlebitis occurs in patients with indwelling catheters on infusion site of blood transfusion, which is painful but not a serious complication. Cellulitis may extend from wrist to the elbow.

BLOOD GROUPING

ABO BLOOD GROUPING

ABO blood grouping is performed by two methods in blood bank: cell grouping (forward grouping) and serum grouping (reverse grouping). Both cell and serum groupings should be performed since each test acts as a check on the other.

- Cell grouping (forward grouping) identifies the ABO antigen on the recipient/donor red blood cells employing known (commercial) anti-A and anti-B sera (sometimes anti-A/anti-B sera).
- Serum grouping (reverse grouping) identifies antibodies in the recipient/donor plasma using (commercial) A_1 and B RBCs. ABO-incompatible blood transfusion results in immediate hemolytic blood transfusion reactions, which may have fatal outcome.
- ABO blood group system compatibility is critical in many transplant procedures. ABO discrepancy occurs

Table 12.30 Pretransfusion common blood bank tests

Blood Bank Tests	Blood Bank Test Reactants
ABO forward blood grouping	Recipient RBCs (unknown blood sample) are tested with known commercial antisera anti-A and anti-B
ABO reverse blood grouping	Recipient serum (plasma) is tested with known antigenic make up A1 and B RBCs
Rh testing	Recipient RBCs are tested with anti-D and Rh control, if needed
Antibody screening	Recipient serum (plasma) is tested with donor RBCs
Cross-matching	Recipient serum is tested with donor RBCs

when the cell grouping (forward grouping) and serum grouping (reverse grouping) results do not correlate. Further investigations must be performed before ABO blood group is interpreted.

- ABO discrepancies fall into four categories: (a) weak or missing reaction is observed in the forward grouping, (b) weak or missing reaction is observed in the reverse grouping, (c) unexpected positive reaction is demonstrated in the forward grouping, and (d) unexpected positive reaction is demonstrated in the reverse grouping. Pretransfusion common blood bank tests are given in Table 12.30.

METHODS OF ABO BLOOD GROUPING

There are two methods of ABO blood grouping: cell grouping (forward grouping) and serum grouping (reverse grouping). ABO blood grouping performed by two methods in blood bank: Cell grouping (forward grouping) and serum grouping (reverse grouping) are shown in Fig. 12.21.

Cell Grouping (Forward Grouping)

Cell grouping (forward grouping) identifies the ABO antigen on the recipient/donor RBCs employing known (commercial) anti-A (blue-colored vial), anti-B (yellow-colored vial) sera and anti-A/anti-B (colorless vial) sera. Now add antibodies to A, B and Rh antigens in three test tubes (1 for A, 1 for B, 1 for Rh) containing patient's RBCs.

- Clumping of the RBCs indicates presence of antigen on the patient's RBCs.
- Failure to clump RBCs indicates absence of antigen on the patient's RBCs. Sodium azide is added to antiserum and stored at 4–6°C to prevent bacterial growth.

Serum Grouping (Reverse Grouping)

Serum grouping (reverse grouping) identifies antibodies in the recipient/donor plasma using (commercial) A_1 and B RBCs. These antibodies bind to the RBC antigens.

- Now add patient's serum, which can bind to red blood cells in two test tubes (A cells in 1 test tube and B cells in 2 test tubes).
- Clumping of the RBCs indicates absence of antibody to RBC antigens.
- ABO antisera used for blood group typing include anti-A (blue-colored vial), anti-B (yellow-colored vial) and anti-A/anti-B (colorless vial).

METHODOLOGY

ABO blood grouping is performed by three methods: (a) slide test, (b) test tube, and (c) microplate. Tube and microplate tests are better than slide test; and employed in blood banking.

Blood Grouping by Slide Test

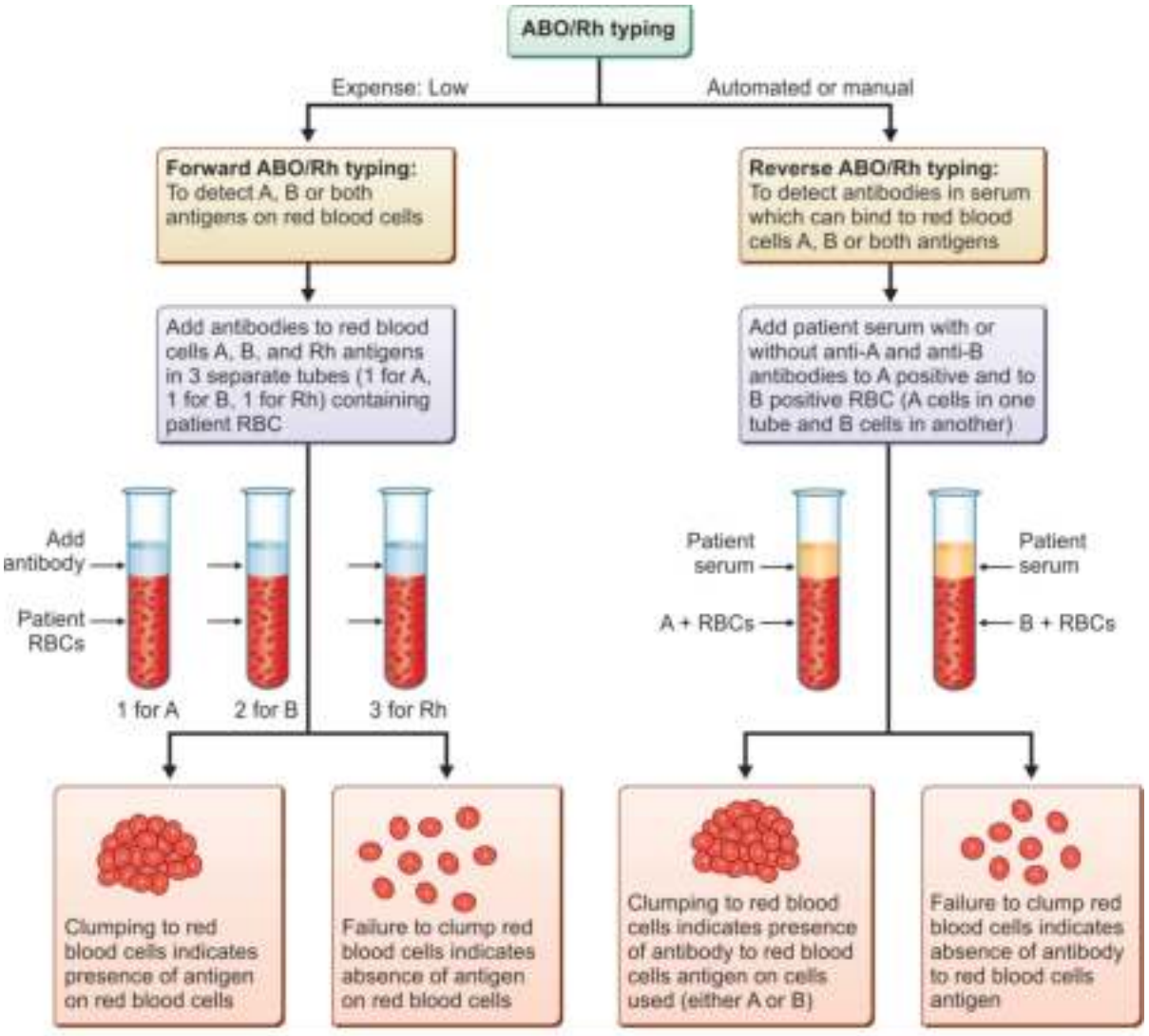
Red blood cells (RBCs) from blood sample are reacted with anti-A and anti-B sera. Demonstration of agglutination of RBCs indicates presence of corresponding antigen on the RBCs (Fig. 12.22).

- One drop of anti-A and one drop of anti-B are placed on different areas on the slide ensuring that these are not mixed. Now add one drop of blood sample containing RBCs to be tested to the anti-A and anti-B sera and then mixed by using a stick.
- Presence of RBCs agglutination is observed in both the areas of the slide. Results of the slide test are always confirmed by cell grouping (forward grouping) and serum grouping (reverse grouping) by test tube method.

Blood Grouping by Test Tube Method

Blood grouping by test tube method is more reliable than slide test. Red blood cells (RBCs) of blood sample are washed with saline and then mixed with known antiserum in the test tube.

- Mixture is incubated at room temperature and centrifuged. Following centrifugation, cell button formed is dislodged gently by tapping the base of the test tube and examined for agglutination of RBCs.
- Agglutination of RBCs is graded from 1+ to 4+ with naked eye. Blood grouping using test tube is shown in Fig. 12.23.



	Forward grouping reaction with serum		Reverse grouping reaction with RBCs	
Blood group	**Anti-A**	**Anti-B**	**A cells**	**B cells**
A	+	0	0	+
B	0	+	+	0
O	0	0	+	+
AB	+	+	0	0

Fig. 12.21: ABO blood grouping is performed by two methods in blood bank: Cell grouping (forward grouping) and serum grouping (reverse grouping).

Blood Grouping by Microplate Method

Microplate technique uses automated platforms in blood banking laboratory for testing blood specimens to detect weaker serum antigen–antibody reactions, i.e. RBC surface antigens and serum antibodies. The reactants are centrifuged and incubated in microplates, and the ABO/Rh(D) blood group is read through an automated system. Microplate composed of polystyrene consists of automated 96 microwells of either U or V shape. The U-shaped microplate can detect multiple blood specimens simultaneously.

Rh BLOOD GROUPING

Both ABO and Rh antigens are immunogenic. Therefore, RBCs for D antigen is routinely performed in blood banking by mixing RBCs with anti-RhD antibodies. Depending on the presence or absence of D antigen, persons are called Rh positive or Rh negative. Importance of Rh blood grouping in clinical practice is that Rh negative mother with Rh positive fetus develops anti-RhD antibodies against fetus Rh positive cells. Antibodies from mother's cross placenta result in hemolytic disease of newborn.

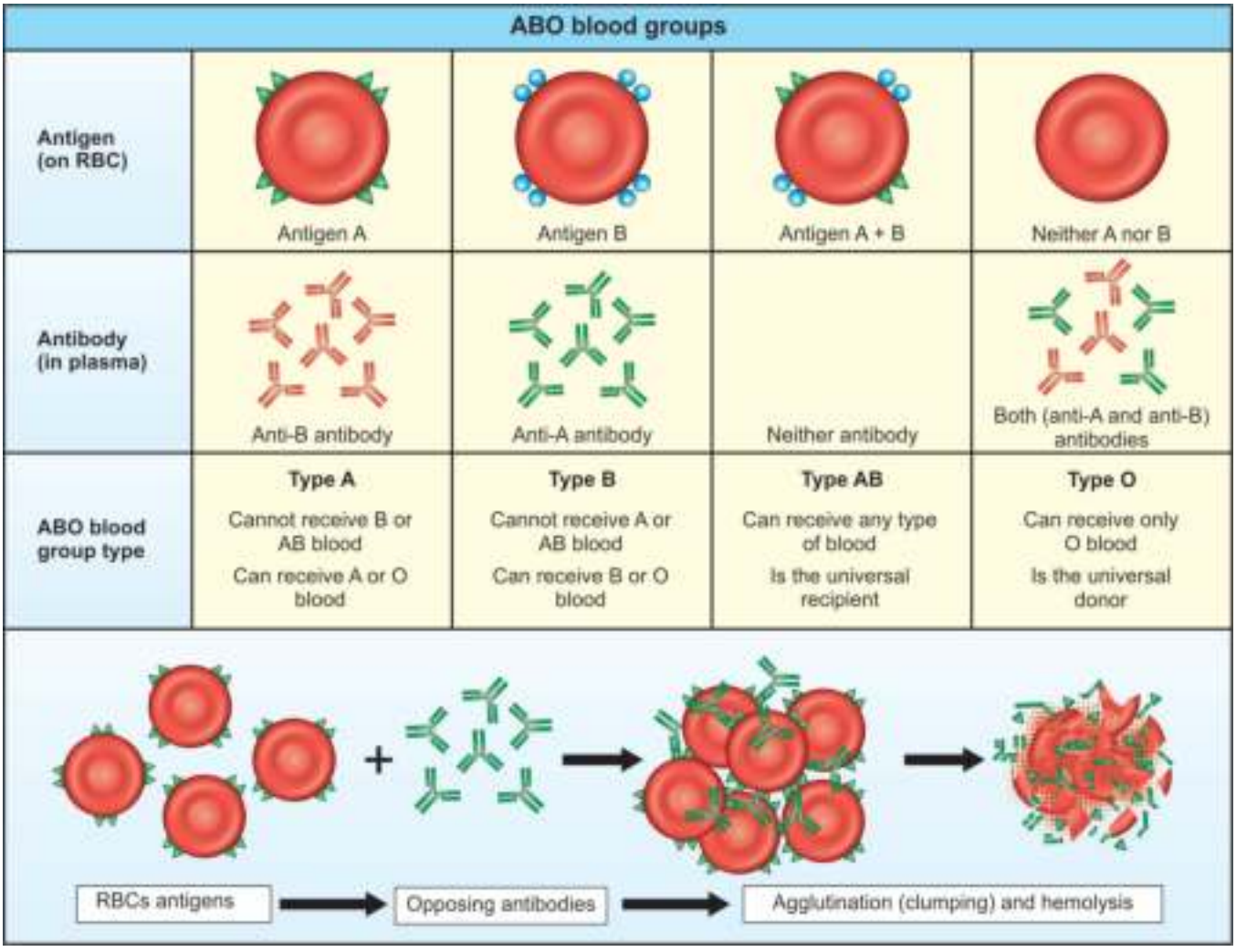


Fig. 12.22: Blood grouping performed by using slide test.

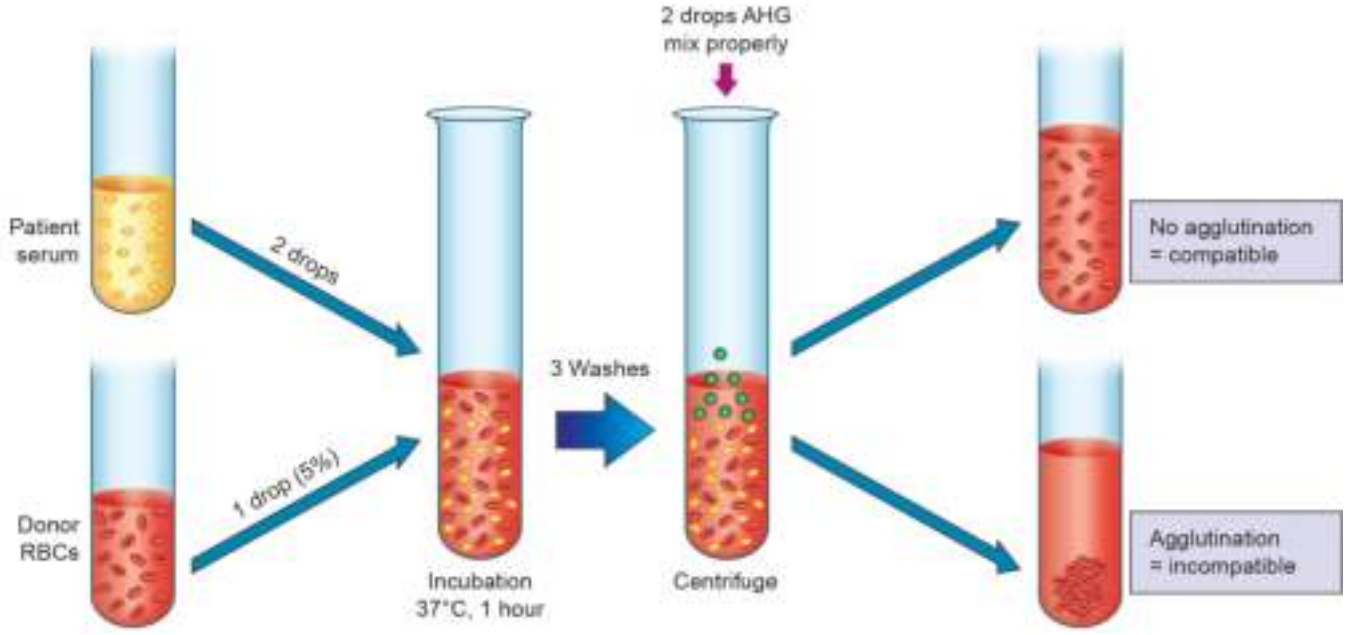


Fig. 12.23: Blood grouping performed by test tube method.

BLOOD CROSS-MATCHING (COMPATIBILITY) TEST

In blood transfusion medicine, cross-matching (part of series of steps in compatibility tests) is done before blood transfusion to determine if the donor's blood is compatible with the blood of an intended recipient. Blood cross-matching is also performed to determine compatibility between a donor and recipient in organ transplantation. Compatibility is determined through matching of different blood group systems. The most important is the ABO and Rh blood group system and/or by directly testing for the presence of antibodies against the RBC antigens in a sample of donor blood or the tissue. Blood cross-matching can be performed by electronic method with a database, if the recipient has previously been tested. Recipient blood sample should be tested to screen antibodies by indirect Coombs' test. Red blood cell antigens and antibodies and their significance are given in Table 12.31. Blood cross-matching is shown in Fig. 12.24.

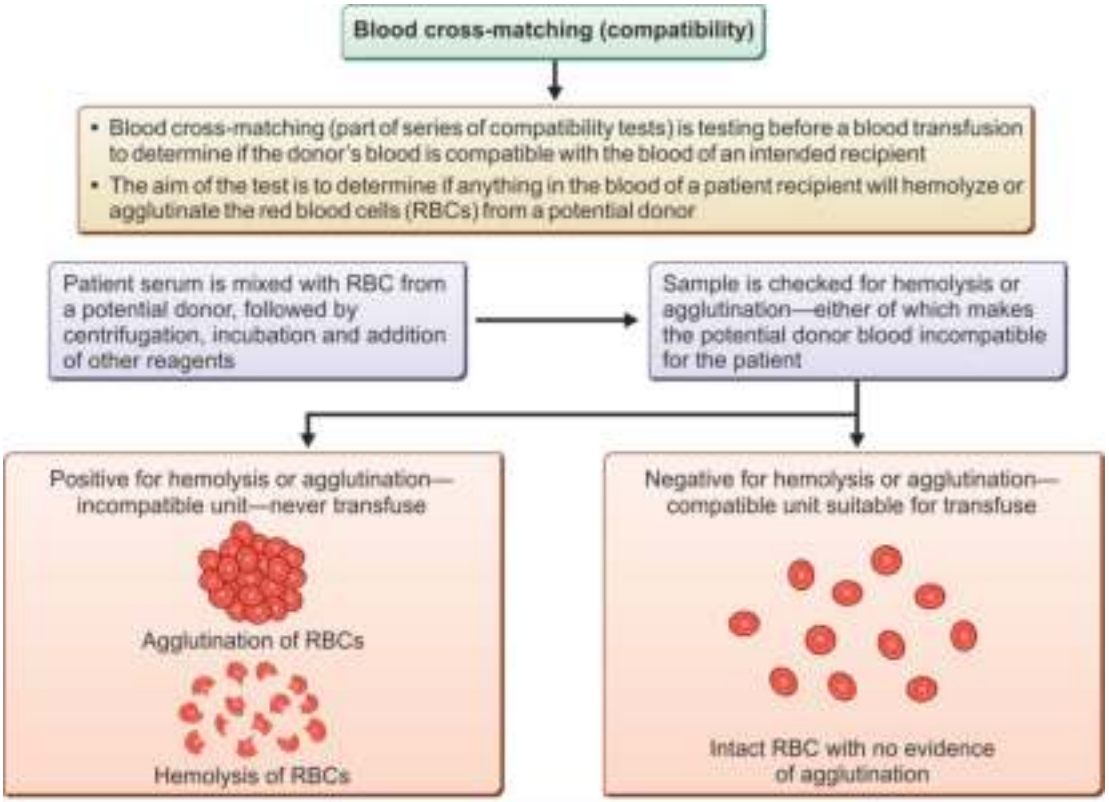
Table 12.31 Red blood cell (RBC) antigens and antibodies and their significance

Red Blood Cell Antigens	Natural Occurring Antibodies (Usually IgG)	Antibodies Only Occurring After Sensitization or Immune (Usually IgG)	Significance
ABO blood group			
A antigen	Anti-B antibody	Nil	Hemolytic blood transfusion reaction common
B antigen	Anti-A antibody	Nil	Hemolytic blood transfusion reaction common
AB antigen	Nil	Nil	Hemolytic blood transfusion reaction common
O antigen	Anti-A and anti-B antibody	Nil	Hemolytic blood transfusion reaction common
Rh blood group			
D antigen	Nil	Anti-D antibody	▪ Extremely common (90%) ▪ Hemolytic disease of newborn (most common)
Kell (KEL) blood group			
Kell antigen	Nil	Anti-Kell antibody	Acute hemolytic blood transfusion reaction (10%)
Duffy (Fy) blood group			
Duffy antigen	Nil	▪ Anti-Fya antibody ▪ Anti-Fyb antibody	▪ Anti-Fya more common ▪ Hemolytic blood transfusion reaction in occasional case ▪ Hemolytic disease of newborn in occasional case
Kidd (Jk) blood group			
Kidd antigen	Nil	▪ Anti-Jka antibody ▪ Anti-Jkb antibody	▪ Dangerous fatal ('killer Kidd') ▪ Hemolytic blood transfusion reaction in occasional case ▪ Hemolytic disease of newborn in occasional case
Lutheran (LU) blood group			
Lutheran antigen	Nil	Anti-Lutheran antibody	▪ Hemolytic blood transfusion reaction in rare case ▪ Hemolytic disease of newborn absent

Contd...

Table 12.31 Red blood cell (RBC) antigens and antibodies and their significance *(Contd...)*

Red Blood Cell Antigens	Natural Occurring Antibodies (Usually IgG)	Antibodies Only Occurring After Sensitization or Immune (Usually IgG)	Significance
Lewis (LE) blood group			
Lewis antigen	Nil	Anti-Lewis antibody	▪ Hemolytic blood transfusion reaction in rare case ▪ Hemolytic disease of newborn absent
P (PI) blood group			
P antigen	Nil	Anti-P antibody	▪ Hemolytic blood transfusion reaction in rare case ▪ Hemolytic disease of newborn in rare case
MNS (MNS) blood group			
MNS antigen	Nil	Anti-MNS antibody	▪ Hemolytic blood transfusion reaction in rare case ▪ Hemolytic disease of newborn in rare case



	Compatible with		
Patient's ABO group	**Whole blood**	**Red blood cells (RBCs)**	**Plasma**
O	O	O	O, A, B, AB
A	A	A, O	A, AB
B	B	B, O	B, AB
AB	AB	AB, A, B, O	AB

Fig. 12.24: Blood cross-matching.

IMMEDIATE SPIN BLOOD CROSS-MATCHING

Immediate spin blood cross-matching is faster, less expensive but less sensitive. It is an immediate test that combines recipient's serum and the donor's red blood cells (RBCs) at room temperature. No agglutination of RBCs indicates a negative test reaction, and thus compatible blood cross-matching.

ELECTRONIC BASED BLOOD CROSS-MATCHING

Electronic based blood cross-matching is a computer-assisted analysis using data from the donor unit, where a donor's blood is tested prior to donation. Recipient blood sample is also tested, which includes ABO/Rh typing of the blood unit and the recipient for the presence of antibodies. Electronic based blood cross-matching can only be performed if the recipient has a negative antibody screen below the detectable level of current test methods. Blood cross-matching falls into two groups: major cross-match and minor cross-match.

ELECTRONIC BASED MAJOR BLOOD CROSS-MATCH

In electronic based major blood cross-match, recipient serum is tested against donor packed red blood cells to determine if the recipient has preformed antibodies against any antigens on the donor's red blood cells. This is essential cross-match prior to release of a blood unit of packed cells.

ELECTRONIC BASED MINOR BLOOD CROSS-MATCH

In electronic based minor blood cross-match, recipient RBCs are tested against donor serum to detect donor antibodies directed against recipient's RBC antigens. This technique is no longer required. It is assumed that the small amount of donor serum antibodies left in the blood unit of packed RBCs would have been diluted in the recipient.

CHAPTER

13

Lymph Node, Spleen and Thymus Gland Disorders

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LEARNING OBJECTIVES

DISORDERS OF LYMPH NODE

- Lymph node organization
  - Lymph node compartments
  - Arterial supply and venous drainage of lymph node
- Developmental stages of hematopoietic stem cells
  - T cell development
  - B cell development
- Lymph node non-neoplastic disorders
  - Suppurative lymphadenitis
  - Granulomatous lymphadenitis
  - Reactive lymphoid hyperplasia
  - Sinus histiocytosis
  - Infectious mononucleosis
  - Kikuchi disease
  - Kumura's disease
  - Castleman disease
- Cervical lymphadenopathy
- Lymphoid neoplasms
  - 2024 WHO classification of lymphoid neoplasms
  - Predisposing factors
  - Molecular diagnostics
  - Staging of lymphoid neoplasms

HODGKIN'S DISEASE

- Hodgkin diseases: overview
  - Diagnostic criteria of Hodgkin's disease
    - Reed-Sternberg cells (growth and survival of Reed-Sternberg cells and role of EB virus in pathogenesis of Hodgkin's disease)
  - Clinical features
  - Chest radiographs
  - Mode of spread
  - Clinical staging
    - Ann Arbor staging system
  - Laboratory diagnosis
  - Differential diagnosis
- Hodgkin's disease: morphologic subtypes
  - Lymphocyte-rich classic variant of Hodgkin's disease
  - Lymphocyte-depletion classic variant of Hodgkin's disease
  - Nodular sclerosis classic variant of Hodgkin's disease
  - Mixed cellularity classic variant of Hodgkin's disease
  - Nodular lymphocytic–histiocytic predominant variant of Hodgkin's disease

NON-HODGKIN'S LYMPHOMAS

- Non-Hodgkin's lymphomas: overview
  - Non-Hodgkin's lymphomas: grades
  - Approach to NHL work up
  - Immunophenotyping
- Precursor B cell and T cell NHLs
  - Precursor B cell lymphoblastic leukemia/lymphoma
  - Precursor T cell lymphoblastic leukemia/lymphoma
  - Myeloid and lymphoid neoplasms with FGFR1 abnormalities
- Mature B cell non-Hodgkin's lymphoma
  - Burkitt's lymphoma
  - Diffuse large B cell lymphoma
  - Chronic lymphocytic leukemia/small lymphocytic lymphoma
  - Follicular lymphoma
  - Mantle cell lymphoma
  - Marginal zone lymphoma (nodal marginal zone lymphoma, extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue, splenic marginal zone lymphoma)
  - Plasmablastic lymphoma
  - Hairy cell leukemia
  - B cell prolymphocytic leukemia
  - Multiple myeloma
- Mature T cell and NK cell lymphomas
  - Angioimmunoblastic T cell lymphoma
  - Adult T cell leukemia/lymphoma
  - Anaplastic lymphoma kinase (ALK) positive anaplastic large T cell lymphoma
  - Extranodal NK cell/T cell lymphoma
  - Mycosis fungoides
  - Sézary syndrome

DISORDERS OF SPLEEN

- Spleen: structure
  - White pulp of spleen
  - Red pulp of spleen
- Functional disorders of spleen
  - Therapeutic splenectomy
  - Splenomegaly
  - Fibrocongestive splenomegaly
  - Hypersplenism
  - Autosplenectomy
  - Hyposplenism
  - Splenosis
  - Splenic rupture
  - Hemosiderosis of spleen
- Reactive splenic disorders
  - Granulomatous inflammation of spleen
  - Splenic infarct
- Non-neoplastic infiltrative disorders of spleen
  - Gaucher's disease involving spleen
  - Spleen involvement in amyloidosis
- Neoplastic disorders of spleen
  - Splenic marginal zone lymphoma
  - Metastases in spleen
  - Angiosarcoma of spleen

DISORDERS OF THYMUS GLAND

- Anatomy and histology of thymus gland
  - Anatomy
  - Histology
- Thymus hyperplasia
- Immunodeficiency disorders of thymus gland
  - DiGeorge syndrome
  - Wiskott-Aldrich syndrome (X-linked disorder)
- Thymic neoplasms
  - Thymoma
- Thymic carcinoma

DISORDERS OF LYMPH NODE

LYMPH NODE ORGANIZATION

Lymph nodes are collections of lymphoid tissue widely distributed within the lymphoreticular system. Beneath the collagenous capsule of lymph node is the subareolar sinus, which is lined by phagocytic cells. Lymphocytes and antigens from surrounding tissue spaces or adjacent lymph nodes pass into the sinus via the afferent lymphatic system.

LYMPH NODE COMPARTMENTS

On histologic examination, lymph nodes show four compartments: cortex showing primary and secondary lymphoid follicles containing B cells found near capsule, paracortex containing T cells surrounding follicles, medullary region and sinuses. T cells in the cortex are associated with the interdigitating antigen-presenting cells (APCs).

ARTERIAL SUPPLY AND VENOUS DRAINAGE OF LYMPH NODE

Each lymph node has its own arterial supply and venous drainage. Lymphocytes enter the lymph node from the circulation through the specialized high endothelial venules in the paracortex. The medulla contains both T and B cells as well as plasma cells, organized into cords of lymphoid tissue. Lymphocytes can leave the lymph node through the efferent lymphatic vessel.

DEVELOPMENTAL STAGES OF HEMATOPOIETIC STEM CELLS

During intrauterine life, hematopoiesis initially occurs in the yolk sac, later takes place in the liver and spleen. After birth, hematopoiesis function is taken over by the red bone marrow. All the blood cells derived from a pluripotent hematopoietic stem cell differentiate into various lineages forming erythrocytes, megakaryocytes, neutrophils, eosinophils, basophils, lymphocytes and monocytes. The precursor cells of these lineages are known as colony-forming units (CFUs).

T CELL DEVELOPMENT

Precursor T cells reach the thymus for development through several stages of thymocytes, i.e. stage I: prothymocyte, stage II: common thymocyte, stage III: mature thymocyte and finally stage IV: post-thymic mature T cells. T cells express terminal deoxynucleotidyl transferase (TdT) in the nucleus, CD3 in the cytoplasm, CD38, and CD71 on surface of T cells. Earliest T cell surface marker is CD7. Immunophenotyping of different developmental stages of T cells is given in Table 13.1.

B CELL DEVELOPMENT

Development of B cells is confined to the bone marrow through various stages, i.e. progenitor B cell, pre-pre-B cell, pre-B cell, immature B cell, mature B cell,

Table 13.1 Immunophenotyping of different developmental stages of T cells. Adapted from flow cytometry, immunohistochemistry and molecular genetics for hematologic neoplasms

Immunophenotyping	Stage I: Prothymocyte	Stage II: Common Thymocyte	Stage III: Mature Thymocyte	Stage IV: Post-thymic Mature T Cells
TdT*	Positive	Positive	Positive	Negative
CD7	Positive	Positive	Positive	Positive
cCD3*	Positive	Positive	Positive	Positive
CD2	Positive	Positive	Positive	Positive
CD5	Negative	Positive	Positive	Positive
CD34	Positive	Negative	Negative	Negative
HLA-DR	Positive	Negative	Negative	Negative
TCR*	Negative	Negative	Positive	Positive
CD1	Negative	Positive	Negative	Negative
CD71	Positive	Negative	Negative	Negative
CD4	Negative	Positive	Positive	Positive
CD8	Negative	Positive	Positive	Positive
CD3	Negative	Negative	Positive	Negative

**TdT (terminal deoxynucleotidyl transferase); *TCR—T cell receptor (TCR ab 95%, TCR gd 5%); *cCD3 (cytoplasmic CD3)*

activated B cell and plasma cell. As the heavy chain of immunoglobulin switching takes place on B cell activation, the activated B cells express surface IgA, IgM or IgG. CD21 disappears at this stage. Plasma cells synthesize cytoplasmic immunoglobulin and express CD38, CD138, PCA-1 and PC-1 (Table 13.2).

- Lymph node consists of four compartments: (a) cortex, (b) paracortex, (c) medullary cords, and (d) sinuses.

Table 13.2 Immunophenotyping of different stages of B-lymphocytes (adapted from 2024 WHO classification of tumors of the hematopoietic and lymphoid tissues)

Progenitor B cell	TdT, HLA-DR, CD34, cCD79
↓	
Pre-pre-B cell	TdT, HLA-DR, CD34, CD19, CD10, cCD22, cCD79
↓	
Pre-B cell	TdT, HLA-DR, CD19, CD10, CD20, CD79, cCD22
↓	
Immature B cell	HLA-DR, CD19, CD20, CD79, CD21, cCD72, sIgM
↓	
Mature B cell	HLA-DR, CD19, CD20, CD21, CD22, CD79, sIgM, sIgD
↓	
Activated B cell	HLA-DR, CD19, CD20, CD22, CD79, sIgM, IgG
↓	
Plasma cell	Immunoglobulins, CD38, CD138, PCA-1, PC1

c (cytoplasmic), s (surface), TdT (terminal deoxynucleotidyl transferase), Ig (immunoglobulin)

- Cortex of a lymph node is composed of primary lymphoid follicles containing resting B cells. Antigenic stimulation of resting B cells leads to proliferation and lymphoblastic transformation, and formation of germinal center, now termed secondary lymphoid follicle.
- In the germinal center of secondary lymphoid follicles, B cells are activated by antigens resulting in proliferation. Generation of plasma cells and memory cells as well as switching of heavy chain class takes place in the germinal center of secondary lymphoid follicles. A germinal center is surrounded by a mantle zone. In the mantle zone, small lymphocytes develop into mantle cells and finally into blastoid cells. Tissue sampling and preparation of histologic slides are given in Table 13.3.

LYMPH NODE NON-NEOPLASTIC DISORDERS

Lymph node non-neoplastic disorders include acute and chronic nonspecific lymphadenitis occurring in response to a number of infectious agents or immune stimuli.

SUPPURATIVE LYMPHADENITIS

Suppurative lymphadenitis occurs in the draining lymph nodes following acute bacterial infection. The lymph nodes rapidly become enlarged due to edema and hyperemia and are tender due to distension of the capsule. Histologic examination of lymph node shows polymorphonuclear leukocytic infiltration in lymph node sinuses and stroma associated with prominent follicular hyperplasia. Infective and granulomatous lymphadenopathy are given in Table 13.4.

Table 13.3 Tissue sampling and histologic slide preparation of lymph node

Sampling of Lymph Node	Utility
Wet touch preparation	▪ Hematoxylin and eosin (fixed in formalin) ▪ Papanicolaou staining (fixed in 95% alcohol)
Air-dried touch smears	▪ Myeloperoxidase stain ▪ Nonspecific esterase ▪ FISH (fluorescence *in situ* hybridization) for cytogenetics
Rapid frozen tissue	▪ Immunohistochemistry ▪ Cytochemistry ▪ Genetic analysis
Fresh tissue in RPMI 1640 medium or saline	Flow cytometry
Sterile fresh tissue	Microbial culture
Formalin fixed paraffin-embedded sections	▪ Hematoxylin and eosin ▪ Giemsa stain ▪ Periodic acid–Schiff (PAS) stain ▪ Masson's trichrome
Thin shaved tissue	Electron microscopy (fixed in glutaraldehyde)

Table 13.4 Infective and granulomatous lymphadenopathy

Suppurative Granulomatous Lesions
▪ Cat-scratch fever (*Bartonella hensalae*)
▪ Lymphogranuloma venereum (*Chlamydia trachomatis*)
▪ Tularemia
▪ *Yersinia enterocolitica*
▪ *Listeria monocytogenes*
▪ *Corynebacterium bovis*
Necrotizing Granulomatous Lesions
▪ Mycobacterium tubercle bacilli
▪ Atypical mycobacterium tubercle bacilli
▪ Mycobacterium lepra bacilli
▪ Systemic fungal infections (e.g. *Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis, Blastomycosis dermatitidis*)
Non-necrotizing Granulomatous Lesions
▪ Sarcoidosis
▪ Berylliosis
▪ Crohn's disease
▪ Protozoal infection
▪ Lymph node draining carcinoma
▪ Reaction to foreign material

GRANULOMATOUS LYMPHADENITIS

Granulomatous lymphadenitis is characterized by formation of epithelioid granulomas in the lymph nodes.

- Caseating granulomas with central necrosis and caseation are demonstrated in *Mycobacterium tuberculosis*. Acid-fast bacilli are stained by Ziehl-Neelsen stain appearing as bright red, slender and beaded rods. *Mycobacterium leprae* and histoplasmosis are other examples of caseating lymphadenitis.
- Noncaseating granulomas are seen in sarcoidosis composed of epithelioid histiocytes with scattered multinucleated giant cells. Noncaseating epithelioid granulomas can be demonstrated in draining lymph nodes in Crohn's disease. Granulomatous lymphadenitis is shown in Fig. 13.1.

REACTIVE LYMPHOID HYPERPLASIA

Reactive lymphoid hyperplasia refers to enlargement of lymphoid follicles (principally in the cortex of the lymph node), which consist of B cells. Reactive follicular lymphoid hyperplasia of lymph nodes represents a response to infections, inflammation, or malignant tumors. Reactive hyperplasia of the secondary follicles, germinal centers, and increased plasma cells in medullary cords indicates B cell immune response. Reactive hyperplasia of the deep cortex or paracortex (diffuse hyperplasia in interfollicular region) is characteristic of T cell immune response. Patterns of reactive follicular hyperplasia of lymph node are given in Table 13.5.

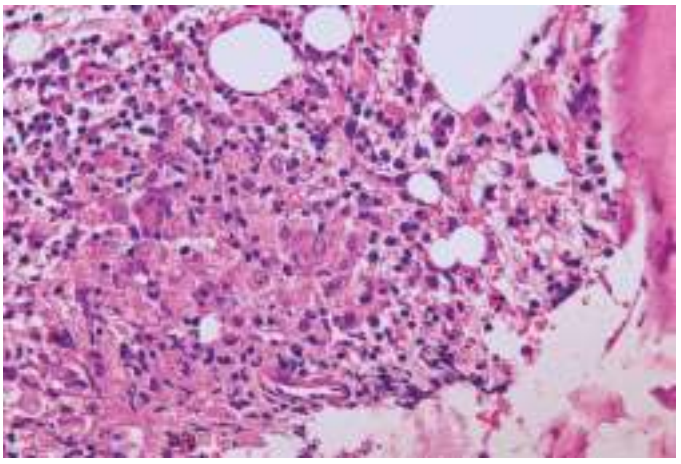

Fig. 13.1: Lymph node shows granulomatous lymphadenitis (400X).

Table 13.5 Patterns of reactive hyperplasia of lymph node

Reactive Follicular Hyperplasia of Lymph Node
▪ Nonspecific follicular hyperplasia
▪ Castleman disease
▪ Kimura disease—syphilis
▪ Cytomegalovirus lymphadenitis
▪ Toxoplasmosis
▪ Human immunodeficiency virus lymphadenitis
▪ Rheumatoid lymphadenopathy
Paracortical Expansion of Lymph Node
▪ Dermatopathic lymphadenopathy
▪ Systemic lupus erythematosus
▪ Drug-induced lymphadenopathy
▪ Viral lymphadenitis (Epstein-Barr virus, cytomegalovirus (CMV), HSV-6, varicella, adenovirus)
▪ Inflammatory pseudotumor of lymph node
▪ Autoimmune lymphoproliferative syndrome
Sinus Expansion of Lymph Node
▪ Rosai-Dorfman disease (sinus histiocytosis with massive lymphadenopathy)
▪ Langerhans' cell histiocytosis
▪ Lipogranulomatous reaction (exogenous lipid during lymphangiogram, endogenous lipid in diabetes mellitus and hyperlipidemia)
▪ Silicone lymphadenopathy
▪ Storage disorders

SINUS HISTIOCYTOSIS

Necrotic products of the malignant tumor antigens often evoke reactive changes in the lymph nodes such as enlargement and hyperplasia of the lymphoid follicles, proliferation of macrophages in the subcapsular sinuses known as sinus histiocytosis.

INFECTIOUS MONONUCLEOSIS

Infectious mononucleosis is a benign self-limited disorder caused by Epstein-Barr virus (EBV), which has an affinity for B cells. It occurs frequently in young adults.

- Circulating atypical reactive CD8+ cytotoxic T cells are characteristic of infectious mononucleosis. The disorder is marked by a number of serum anti-EBV antibodies and heterophil antibodies (heterophil agglutinins) directed against sheep erythrocytes.
- Patient presents with history of sore throat, fever, generalized lymphadenopathy, and hepatosplenomegaly. The spleen is especially susceptible to traumatic rupture. Heterophil agglutinins (antibodies) negative infectious mononucleosis is most often associated with cytomegalovirus infection.

KIKUCHI DISEASE

Kikuchi disease is a benign disorder characterized by generalized necrotizing lymphadenopathy, which affects more often young women than men. Patient presents with recurrent necrotizing lymphadenitis often associated with other systemic inflammatory disorders such as systemic lupus erythematosus, tuberculosis, HIV and parvovirus B19.

Surgical Pathology: Kikuchi Disease
Light Microscopy
Histopathologic examination of lymph node shows paracortical expansion, admixture of lymphocytes, histiocytes, widespread apoptosis of cellular infiltrate and necrosis. Neutrophils are absent (Fig. 13.2).

KIMURA'S DISEASE

Kimura's disease is characterized by angiolymphoid proliferation of lymph nodes predominantly in the head and neck region forming eosinophilic abscess involving of subcutaneous tissue and eosinophilia. Salivary gland involvement, glomerulonephritis, nephrotic syndrome, eosinophilia and increased IgE are more common in Kimura's disease than Kikuchi disease.

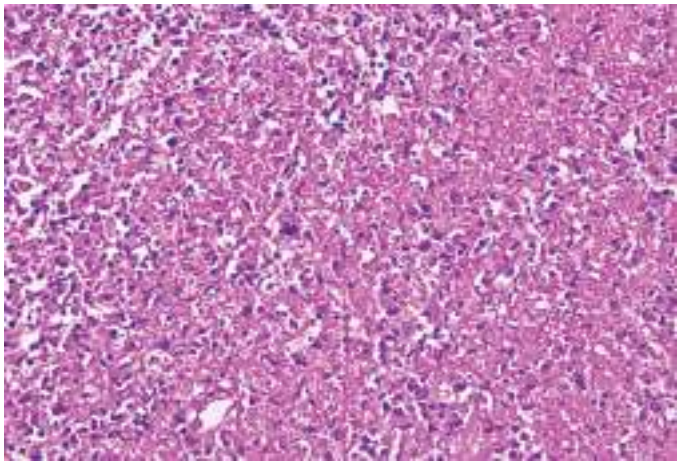

Fig. 13.2: Kikuchi disease shows necrotizing lymph node with paracortical expansion, admixture of lymphocytes, histiocytes and widespread apoptosis. However, neutrophils are absent (100X).

CASTLEMAN DISEASE

Castleman disease is also known as angiofollicular hyperplasia of lymph node or giant lymph node hyperplasia or angiomatous lymphoid hamartoma. Disorder may be localized (hyaline vascular variant and multicentric cell variant) or multicentric lymph nodes involvement in mediastinum, which most often affects adults but less prevalent in children. Castleman disease variants are given in Table 13.6.

Clinical Features

Patient presents with dyspnea and radiograph reveals widening of mediastinum due to solitary or multicentric lymph node enlargement. It may be associated with

Table 13.6 Castleman disease variants

Hyaline Vascular Variant of Castleman Disease	Multicentric Plasma Cell Variant of Castleman Disease	Multicentric Variant of Castleman Disease (IL-6 Lymphadenopathy)
Age group		
Young adults	Wide range age group	Older age group
Clinical features		
Mediastinal lymph nodes involved	▪ Abdominal lymph nodes involved ▪ Anemia, raised ESR, raised serum globulins and increased plasma cells in bone marrow	▪ Multicentric lymph nodes involved due to dysregulation of IL-6 ▪ Association with HIV/AIDS; and POEMS syndrome
Histologic features		
▪ Hyaline follicles are composed of endothelial cells and dendritic reticulum cells ▪ Mantle zone shows onion-skin appearance ▪ Interfollicular areas are vascular	▪ Follicular hyperplasia with narrow mantle zones ▪ Numerous plasma cells in interfollicular regions with obscuring lymph node architecture ▪ One-third of cases exhibit light-chain restriction	▪ Follicular hyperplasia with narrow mantle zones ▪ Numerous plasma cells in interfollicular regions with obscuring lymph node architecture ▪ About 33% of cases exhibit light-chain restriction

POEMS (polyneuropathy, organomegaly, endocrine abnormalities, monoclonal gammopathy, skin rashes) syndrome.

Kaposi sarcoma, mantle cell lymphoma, and diffuse large B cell lymphoma (DLBCL).

Castleman Disease: Histologic Variants

- Hyaline-vascular variant of Castleman disease is unicentric and asymptomatic disease. Patient presents with anemia, night sweats, weight loss, elevated sedimentation rate and hypergammaglobulinemia. Histologic examination shows increased lymphoid follicles in cortex and medulla. Follicles contain hyalinized and sclerosed blood vessels resembling white pulp of spleen.
- Plasma cell variant of Castleman disease is multicentric disease. Patient presents with generalized lymphadenopathy. Histologic examination exhibits proliferation of blood vessels surrounded by mature plasma cells. There is distension of lymph node sinuses. Children have good prognosis with corticosteroid therapy. It is associated with HHV8+ infection, with an accumulation of HHV8+ lymphocytes in mantle zone leading to dissolution of the germinal center.
- Multicentric variant of Castleman disease is an atypical lymphoproliferative disorder characterized by systemic lymphadenopathy and constitutional inflammatory symptoms. Dysregulated overproduction of interleukin-6 (IL-6) is responsible for clinical manifestations.

CERVICAL LYMPHADENOPATHY

The most common swellings in cervical region are enlarged lymph nodes caused by bacterial or viral infections, lymphomas and metastatic deposits. Inflammatory lymphadenopathy is most often painful, firm and mobile located in the midline or lateral side of neck. Delphin lymph nodes located along thyrohyoid membrane are enlarged in thyroid carcinoma. Malignant lymph nodes are painless and hard in consistency. *Leishmania donovani* (*LD*) bodies in the fine needle aspirate of lymph node are shown in Fig. 13.3. Metastatic deposits in lymph nodes are shown in Figs 13.4A to C and 13.5. Lymphadenopathy may be generalized or

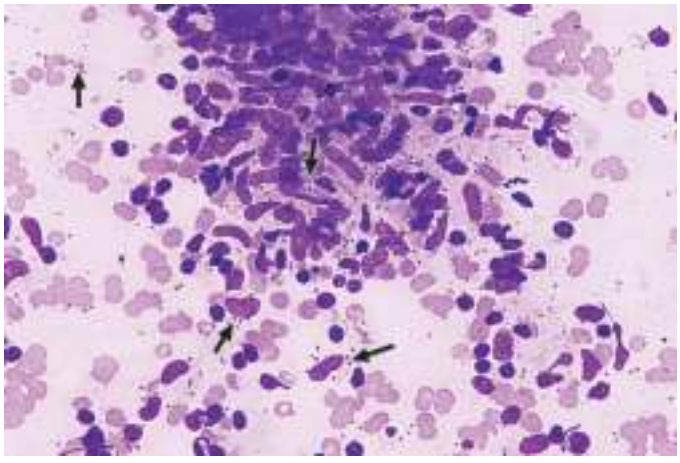

Fig. 13.3: *Leishmania donovani* bodies are demonstrated in the fine needle aspirate of lymph node. Epithelioid granuloma is also seen (arrows) (400X).

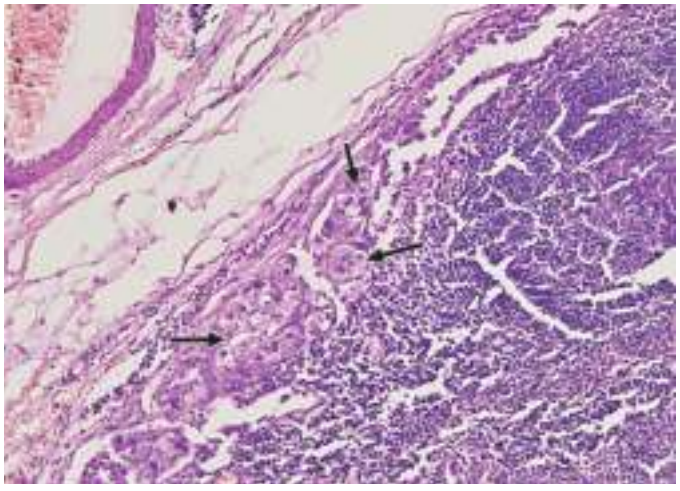

Fig. 13.4A: Metastatic deposits from carcinoma are seen in subcapsular area of lymph node (arrows) (400X).

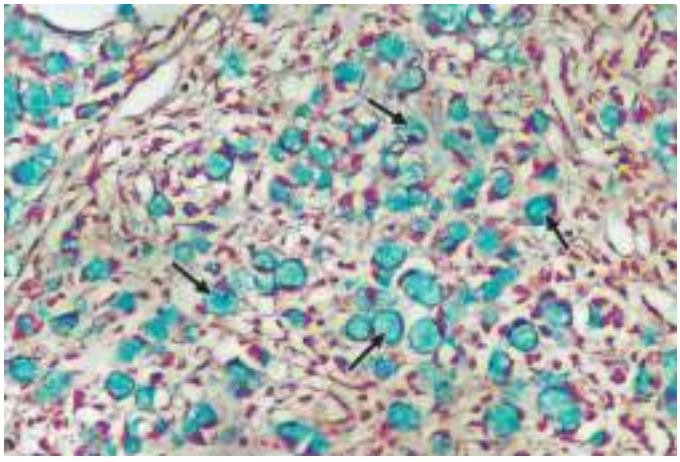

Fig. 13.4B: Metastatic deposits from adenocarcinoma to lymph node show positivity with Alcian blue (arrows) (400X).

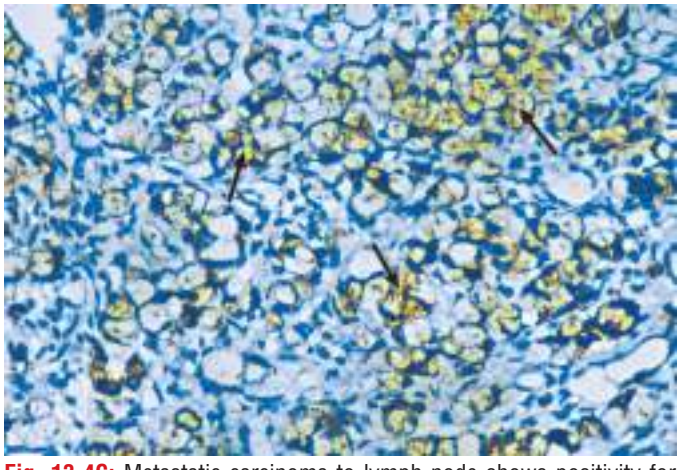

Fig. 13.4C: Metastatic carcinoma to lymph node shows positivity for cytokeratin (arrows) (400X).

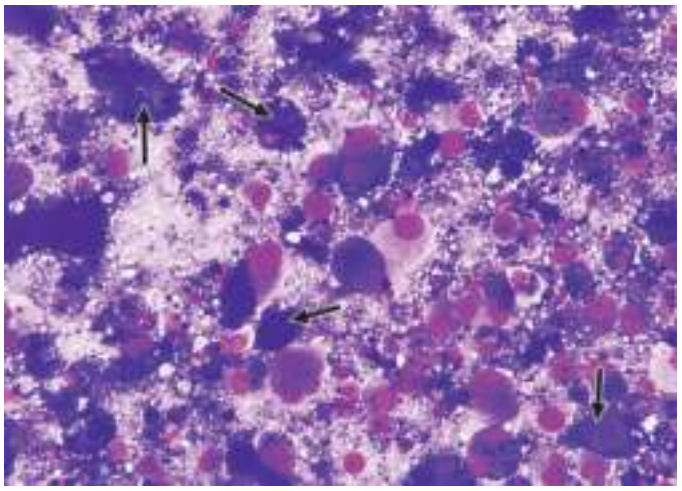

Fig. 13.5: Fine needle aspirate from lymph node shows metastatic deposits from melanoma (arrows) (400X).

localized (Table 13.7). Differential diagnosis of cervical lymphadenopathy is given in Table 13.8.

LYMPHOID NEOPLASMS

Lymphoid neoplasms arise from T or B cells and their precursor cells. Patient presents with localized lymphadenopathy characterized by formation of gross malignant tumor nodules.

- Conversely, neoplastic proliferation of lymphocytes with diffuse and systemic involvement from their inception are categorized as leukemias. Malignant lymphoid neoplasms can be divided into two major categories—Hodgkin's disease and non-Hodgkin's lymphoma.

Table 13.7 Causes of lymphadenopathy

Categories	Causes
Generalized lymphadenopathy	
Lymphomas	▪ Hodgkin's disease ▪ Non-Hodgkin's lymphoma
Leukemias	▪ Acute lymphoblastic leukemia ▪ Chronic lymphocytic leukemia
Infections	▪ Tuberculosis ▪ Brucellosis ▪ Syphilis ▪ *Toxoplasma gondii* ▪ Sarcoidosis
Autoimmune disorders	▪ Systemic lupus erythematosus ▪ Rheumatoid arthritis
Hypersensitivity reaction	Serum sickness
Lipid storage diseases	▪ Gaucher's disease ▪ Niemann-Pick disease
Drug	Phenytoin
Miscellaneous	▪ Sarcoidosis ▪ Pseudolymphoma
Localized lymphadenopathy	
Infections	Acute or chronic infections
Metastatic cancers	▪ Breast carcinoma ▪ Lung carcinoma ▪ Kidney carcinoma ▪ Head and neck carcinoma

- Non-Hodgkin's lymphoma may develop at each stage of differentiation in lymph nodes, e.g. mantle cell

Table 13.8 Differential diagnosis of cervical lymphadenopathy

Lymph Node Groups	Areas Drained by Lymph Node Group	Categories of Etiology	Differential Diagnosis
Preauricular or posterior cervical lymph nodes	Scalp and skin	▪ Infections ▪ Cancers	▪ Tubercular lymphadenitis ▪ Squamous cell carcinoma skin (head and neck regions) ▪ Hodgkin's disease ▪ Non-Hodgkin's lymphoma
Supraclavicular nodes	Gastrointestinal tract, pulmonary and genitourinary systems	▪ Infections ▪ Cancers	▪ Tubercular lymphadenitis ▪ Fungal infections ▪ Cancers of thorax, abdomen, thyroid and larynx
Submandibular or anterior lymph nodes	Oral cavity	▪ Infections ▪ Cancers	▪ Tubercular lymphadenitis ▪ Dental or upper respiratory tract infections ▪ Infectious mononucleosis ▪ *Toxoplasma gondii* ▪ Cytomegalovirus ▪ Rubella ▪ Squamous cell carcinoma of head and neck ▪ Leukemia ▪ Hodgkin's and non-Hodgkin's lymphomas

lymphoma, follicular lymphoma, nodal marginal zone lymphoma, plasma cell myeloma and lymphoplasmacytic lymphoma.

- Pre-germinal center lymphoma comprises B cell lineage CLL/small lymphocytic lymphoma and mantle cell lymphoma.
- Germinal center lymphoma is represented by follicular lymphoma, Burkitt's lymphoma and diffuse large B cell lymphoma. Lymphomas that express immunoglobulin VH (IgVH) gene mutation and intraclonal diversity are derived from germinal center.
- Post-germinal center lymphoma is represented by nodal zone B cell lymphoma, plasma cell myeloma and lymphoplasmacytic lymphoma. Lymphomas that express VH gene mutation but not intraclonal diversity is derived from post-germinal center B cell.

- Diagnostic criteria of Hodgkin's disease are demonstration of Reed-Sternberg cells in the lymph nodes. World Health Organization (WHO) classification identifies a number of Hodgkin's disease variants: classic variants (lymphocyte rich, lymphocytic depletion, nodular sclerosis and mixed cellularity) and nodular lymphocytic–histiocytic predominant. Epstein-Barr virus has been demonstrated in Reed-Sternberg cells in Hodgkin's disease.

2024 WHO CLASSIFICATION OF LYMPHOID NEOPLASMS

Revised 2024 WHO classification of hematolymphoid B cell and T cell/NK cell tumors (adapted from 5th edition, WHO classification of hematolymphoid tumors) is given in Tables 13.9A and B.

Table 13.9A Revised 2024 WHO classification of hematolymphoid B cell tumors (adapted from 5th edition, WHO classification of hematolymphoid tumors)

Tumor-like Lesions with B Cell Predominance

- Reactive B cell-rich lymphoid proliferations that can mimic lymphoma
- IgG4-related disease
- Unicentric Castleman disease
- Idiopathic multicentric Castleman disease
- KSHV/HHV8-associated multicentric Castleman disease

Precursor B Cell Lymphoblastic Leukemia/Lymphoma

- B-lymphoblastic leukemia/lymphoma, NOS
- B-lymphoblastic leukemia/lymphoma with hyperdiploidy
- B-lymphoblastic leukemia/lymphoma with hypodiploidy (hypodiploid ALL)
- B-lymphoblastic leukemia/lymphoma with iAMP21
- B-lymphoblastic leukemia/lymphoma with BCR-ABL1 fusion
- B-lymphoblastic leukemia/lymphoma with BCR-ABL1-like
- B-lymphoblastic leukemia/lymphoma with KMT2A rearrangement
- B-lymphoblastic leukemia/lymphoma with ETV6-RUNX1 fusion
- B-lymphoblastic leukemia/lymphoma with TCF3-PBX1 fusion
- B-lymphoblastic leukemia/lymphoma with IGH/IL3 fusion
- B-lymphoblastic leukemia/lymphoma with other genetic abnormalities

Mature B Cell Neoplasms

- Pre-neoplastic and neoplastic small lymphocytic proliferations
  - Monoclonal B cell lymphocytosis, CLL type
  - Monoclonal B cell lymphocytosis, non-CLL type
  - Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL)
- Splenic B cell leukemia/lymphoma
  - Hairy cell leukemia-defining mutation in BRAF V600E
  - Hairy cell leukemia-defining MAP2K1 mutations in 50% of cases
  - Splenic marginal zone lymphoma
  - Splenic diffuse red pulp small B cell lymphoma
  - Splenic B cell leukemia/lymphoma with prominent nucleoli
- Lymphoplasmacytic lymphoma
- Marginal zone lymphoma
  - Extranodal cutaneous marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT)
  - Extranodal cutaneous marginal zone lymphoma
  - Nodal marginal zone lymphoma
  - Pediatric nodal marginal zone lymphoma
- Follicular lymphoma
  - *In situ* follicular B cell neoplasia
  - Follicular lymphoma—molecular landscape, CREBBP, EZH20 and KMT20 (MML2) possibly early mutations
  - Pediatric-type follicular lymphoma
  - Duodenal-type follicular lymphoma
  - Testicular follicular lymphoma
- Cutaneous follicle center lymphoma
- Primary cutaneous follicle center lymphoma
- Mantle cell lymphoma
  - *In situ* mantle cell lymphoma
  - Leukemic non-nodal mantle cell lymphoma
- Transformation of indolent B cell lymphoma
- Large B cell Lymphoma
  - Diffuse large B cell lymphoma, NOS
  - T cell/histiocyte-rich large B cell lymphoma
  - Diffuse large B cell lymphoma/high-grade B cell lymphoma with Myc and BCL-2 rearrangements
  - ALK-positive diffuse large B cell lymphoma
  - Large B cell lymphoma with IRF4 rearrangement
  - High-grade B cell lymphoma with 11q aberrations

Contd...

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Table 13.9A Revised 2024 WHO classification of hematolymphoid B cell tumors (adapted from 5th edition, WHO classification of hematolymphoid tumors) (*Contd...*)

- Lymphomatoid granulomatosis
- EBV-positive diffuse large B cell lymphoma
- Diffuse large B cell lymphoma associated with chronic inflammation
- Fibrin-associated large B cell lymphoma
- Fluid overload-associated large B cell lymphoma
- Plasmablastic lymphoma
- Primary large B cell lymphoma of immune-privileged sites
- Primary cutaneous diffuse large B cell lymphoma
- Primary cutaneous diffuse large B cell lymphoma, leg type
- Intravascular large B cell lymphoma
- Primary mediastinal (thymic) large B cell lymphoma
- High-grade B cell lymphoma, NOS
- High-grade B cell lymphoma with Myc and BCL-2 and/or BCL-6 rearrangements

- Burkitt lymphoma

- KSHV/HHV8-associated B cell lymphoid proliferations and lymphomas
  - Primary effusion lymphoma
  - KSHV/HHV8-positive diffuse large B cell lymphoma
  - KSHV/HHV8-positive geminotropic lymphoproliferative disorder

- Lymphoid proliferations and lymphomas associated with immune deficiency and dysregulation
  - Hyperplasia arising in immune deficiency/dysregulation
  - Polymorphic lymphoproliferative disorders arising in immune deficiency/dysregulation
  - EBV-positive mucocutaneous ulcer
  - Lymphomas arising in immune deficiency/dysregulation
  - Inborn error of immunity-associated lymphoid proliferations and lymphomas

Contd...

- Hodgkin's lymphoma
  - Nodular sclerosis classic variant of Hodgkin's lymphoma
  - Lymphocyte-rich classic variant of Hodgkin's lymphoma
  - Mixed cellularity classic variant of Hodgkin's lymphoma
  - Lymphocytic-depletion classic variant of Hodgkin's lymphoma

- Plasma cell neoplasms and other diseases with paraproteins
  - Monoclonal gammopathies
    - Cold agglutin disease
    - IgM monoclonal gammopathy of undermined significance (Waldenström macroglobulinemia)
    - Non-IgM monoclonal gammopathy of undermined significance
    - Monoclonal gammopathy of renal significance
  - Diseases with monoclonal immunoglobulin deposition
    - Immunoglobulin-related AL amyloidosis
    - Monoclonal immunoglobulin deposition disease
  - Heavy chain diseases
    - μ (Mu) heavy chain disease
    - γ (Gamma) heavy chain disease
    - α (Alpha) heavy chain disease
  - Plasma cell neoplasms
    - Solitary osseous/extraosseous plasmacytoma
    - Plasma cell myeloma
    - Plasma cell neoplasm with associated paraneoplastic syndrome
      - POEM syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal plasma cell disorder)
      - TEMPI syndrome (telangiectasia, elevated erythropoietin and erythrocytosis monoclonal gammopathy, perinephric fluid collection, intrapulmonary shunting)
      - AESOP syndrome (adenopathy and an extensive skin scratch overlying plasmacytoma)

Table 13.9B Revised 2024 WHO classification of tumors of hematolymphoid T cell/NK cell tumors tumors (adapted from 5th edition, WHO classification of hematolymphoid tumors)

Tumor-like Lesions with B Cell Predominance

- Kikuchi-Fujimoto disease
- Indolent T-lymphoblastic proliferation
- Autoimmune lymphoproliferative syndrom

Precursor T Cell Neoplasm

- T-lymphoblastic leukemia/lymphoma, NOS
- Early T cell precursor lymphoblastic leukemia/lymphoma

Mature T Cell and NK Cell Neoplasm

- T-lymphocytic leukemia
- T large granular lymphocytic leukemia
- NK large granular lymphocytic leukemia
- Adult T cell leukemia/lymphoma
- Sézary syndrome
- Aggressive NK cell leukemia

Contd...

Primary Cutaneous T Cell Lymphoma

- Primary cutaneous CD4+ small or medium T cell lymphoproliferative disorder
- Primary cutaneous CD8+ lymphoproliferative disorder
- Mycosis fungoides
- Primary cutaneous CD30+ T cell lymphoproliferative disorder: lymphomatoid papulosis
- Primary cutaneous CD30+ T cell lymphoproliferative disorder: primary cutaneous anaplastic large cell lymphoma
- Subcutaneous panniculitis-like T cell lymphoma
- Primary cutaneous γ/δ T cell lymphoma
- Primary cutaneous CD8+ aggressive epidermotropic cytotoxic T cell lymphoma
- Primary cutaneous peripheral T cell lymphoma, NOS

Contd...

Table 13.9B Revised 2024 WHO classification of tumors of hematopoietic and lymphoid tissues (*Contd...*)

Intestinal T Cell and NK Cell Lymphoid Proliferations
▪ Indolent T cell lymphoma of the gastrointestinal tract (GIT)
▪ Indolent NK cell lymphoproliferative disorder of the GIT
▪ Enteropathy-associated T cell lymphoma
▪ Monomorphic epitheliotropic intestinal T cell lymphoma
▪ Intestinal T cell lymphoma, NOS
Hepatosplenic T Cell Lymphoma
Hepatosplenic T cell lymphoma
Anaplastic Large Cell Lymphoma
▪ ALK-positive anaplastic large cell lymphoma
▪ ALK-negative anaplastic large cell lymphoma
▪ Breast implant-associated anaplastic large cell lymphoma
Nodal T-Follicular Helper (TFH) Cell Lymphoma
▪ Nodal TFH cell lymphoma, angioimmunoblastic-type
▪ Nodal TFH cell lymphoma, follicular-type
▪ Nodal TFH cell lymphoma, NOS
Other Peripheral T Cell Lymphoma
▪ Peripheral T cell lymphoma, NOS
EBV-positive NK Cell/T Cell Lymphoma
▪ EBV-positive nodal T cell and NK cell lymphoma
▪ Extranodal NK cell/T cell lymphoma
EBV-positive NK Cell/T Cell Lymphoid Proliferations and Lymphomas of Childhood
▪ Severe mosquito bite allergy
▪ Hydroa vacciniforme lymphoproliferative disorder
▪ Systemic chronic active EBV disease
▪ Systemic EBV-positive T cell lymphoma of childhood

Contd...

CLL: Chronic lymphocytic leukemia, SLL: Small lymphocytic lymphoma, DLBCL: Diffuse large B cell lymphoma; MALT: Mucosa-associated lymphoid tissue; NK cell: Natural killer cell; PTLD: Post-transplant lymphoproliferative disorders. Adapted from WHO classification of tumors of hematopoietic and lymphoid tissues.

WHO Classification of Lymphoid Neoplasms

2024 WHO Classification of non-Hodgkin's Lymphomas

- High-grade non-Hodgkin's lymphoma (NHL) may involve a single lymph node, a localized group of lymph nodes, or an extranodal site.
- Low-grade NHL tends to involve multiple lymph nodes at multiple sites, whereas high-grade NHL tends to be more localized. B cell NHLs are more common than T cells. WHO classification of non-Hodgkin's lymphoid neoplasms is based on origin of NHL derived from B or T cells—precursor cells or mature cells.
- Development of lymphoid malignancies is shown in Fig. 13.6. Pathway of B cells differentiation within and without follicle center is shown in Fig. 13.7. Normal and abnormal counterparts of B cell progeny are shown in Fig. 13.8. Normal T cell progeny is shown in Fig. 13.9.

2024 WHO Classification of Hodgkin's Disease

- **Diagnostic criteria** of Hodgkin's disease are demonstration of Reed-Sternberg cells in the lymph nodes.
- World Health Organization (WHO) classification identifies a number of disease variants: classic variants (lymphocyte rich, lymphocytic depletion, nodular sclerosis and mixed cellularity) and nodular lymphocytic–histiocytic predominant.
- Differences between Hodgkin's disease and non-Hodgkin's lymphoma are given in Table 13.10.

PREDISPOSING FACTORS

Most mature B cell, T cell and NK cell non-Hodgkin's lymphoid neoplasms demonstrate recurrent genetic alterations including non-random chromosomal translocations, somatic mutations, DNA gains, or losses.

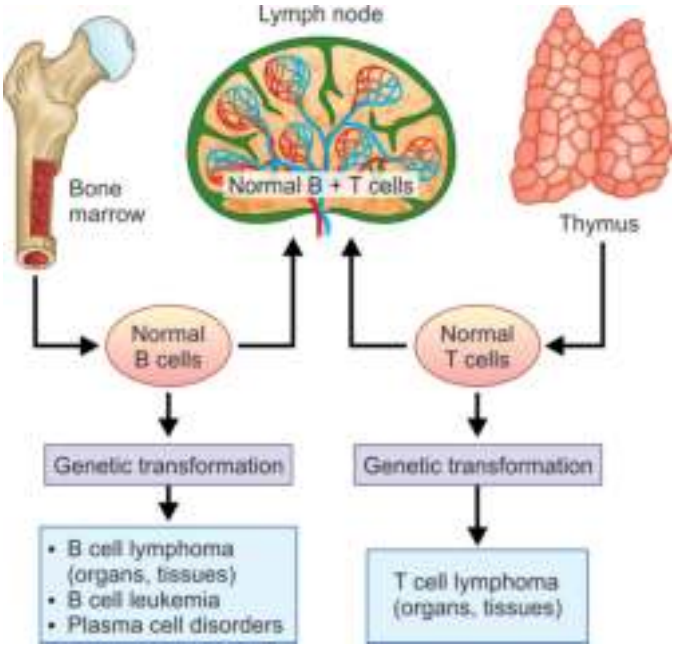


Fig. 13.6: Development of lymphoid malignancies.

Some of these genetic alterations can be preferentially involved in individual lymphoma entities. But the vast majority of genetic alterations are shared by different lymphoma subtypes.

- At the molecular level, genetic alterations can result in activation of oncogenes due to chromosomal translocations, as well as inactivation of tumor suppressor genes by chromosomal deletion or mutation.
- It is now known that the genome of certain lymphoid neoplasms can be altered by introduction of

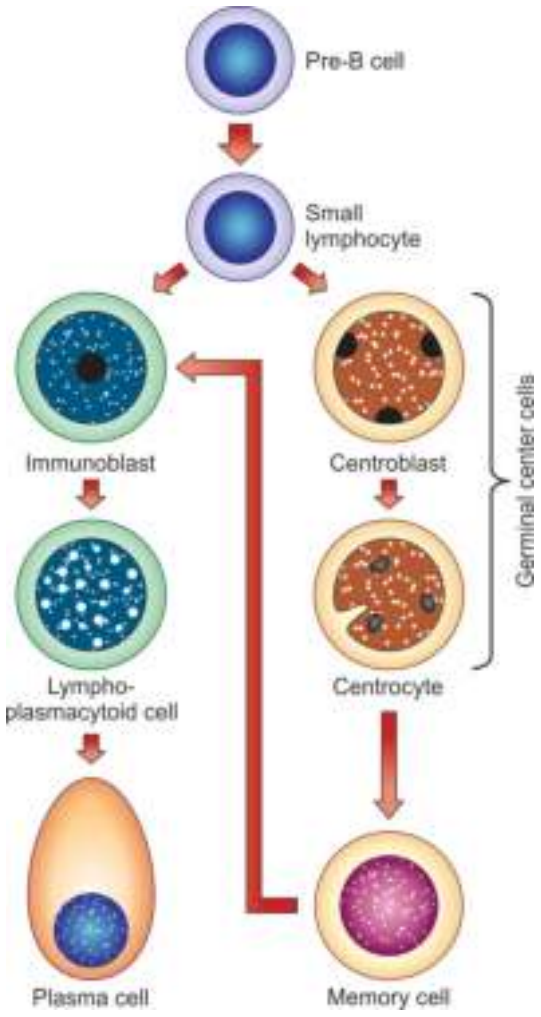


Fig. 13.7: Pathway of B cells differentiation within and without follicle center. Clones of malignant cells may arise any stage differentiation.

exogenous genes by oncogenic viruses such as Epstein-Barr virus, HHV8 and DTLV-1.

- Molecular genetic alterations are detected by cytogenetics or fluorescence *in situ* hybridization (FISH), together with histologic examination and clinical features of the disease.
- In contrast to some subtypes of NHL, no recurrent specific chromosomal translocations have been demonstrated in Hodgkin's disease.
  - Comparative genomic hybridization demonstrates recurrent gains of the chromosomal subregions on chromosomal arms 2p, 9p, and 12q and high level of amplifications on 4p16, 4q23.24 and 9p23-p24.
  - Amplification on 9p24.1 represents a recurrent genetic abnormality in nodular sclerosis variant of Hodgkin's disease.
  - Reed-Sternberg cells have been infected by Epstein-Barr virus in 40% cases of Hodgkin's disease. Human immunodeficiency virus has been associated with 100% cases of Hodgkin's disease.

Chemical Carcinogen-induced Lymphoid Neoplasms

There is increased risk for development of Hodgkin's disease and non-Hodgkin's lymphoma in patients receiving alkylating agents are such as nitrogen mustard, cyclophosphamide and chlorambucil administered to treat various cancers. Prolonged exposure to aromatic hydrocarbons such as benzene increases the risk for development of Hodgkin's disease. Chemical carcinogens associated cancers are given in Table 13.11.

Bacterial and Viruses-induced Lymphoid Neoplasms

The cause of lymphoid neoplasms is not completely understood, but there have been some associations with the development of lymphoma. Both bacterial and viral infections have been associated with lymphomas.

- For example, MALT lymphoma derived from mucosa-associated lymphoid tissue in gastric region has been associated with ***Helicobacter pylori*** infection. Antibiotic therapy can lead to regression of MALT lymphoma.
- Hepatitis C has been associated with an increased risk of marginal zone lymphomas.
- Epstein-Barr virus (EBV) is associated with Burkitt's lymphoma, primary effusion lymphoma and high-grade B cell lymphoma in 60–90 years elderly persons, Hodgkin's disease and lymphoproliferative disorders in post-organ transplant patients.
- Human immunodeficiency virus (HIV) infection has been associated with several types of aggressive B cell NHL.
- Human T cell lymphotropic virus (HTLV-1) has been associated with T cell lymphoma especially in Japan, South America, Central and West Africa. Infectious agents associated lymphomas are given in Table 13.12.

Autoimmune Disorders Associated Lymphoid Neoplasms

Patients suffering from autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis, celiac disease and Sjögren's syndrome are at great risk of development of lymphoid neoplasms. Tumor necrosis factor (TNF) inhibitors administered in autoimmune disorders increase the risk for development of lymphoid neoplasms. Immunosuppressive drugs administered in persons undergoing organ transplantation increase the risk for development of lymphoid neoplasms.

MOLECULAR DIAGNOSTICS

In lymphoid neoplasms, clonality is determined by the presence of a dominant clone with identical gene arrangement involving the immunoglobulin heavy chain (IgH) in B cell-, or T cell-derived lymphomas.

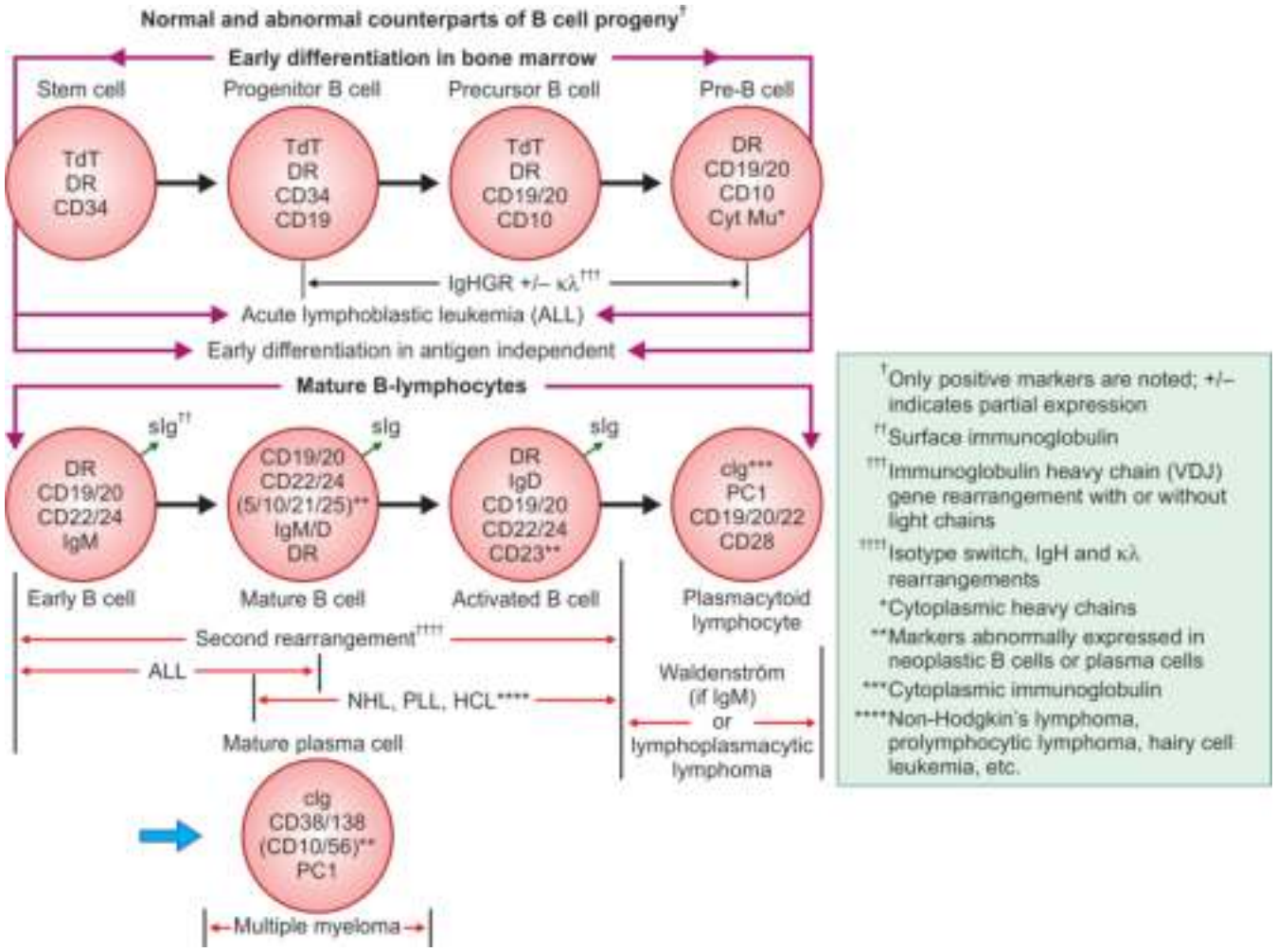


Fig. 13.8: Normal and abnormal counterparts of B cell progeny.

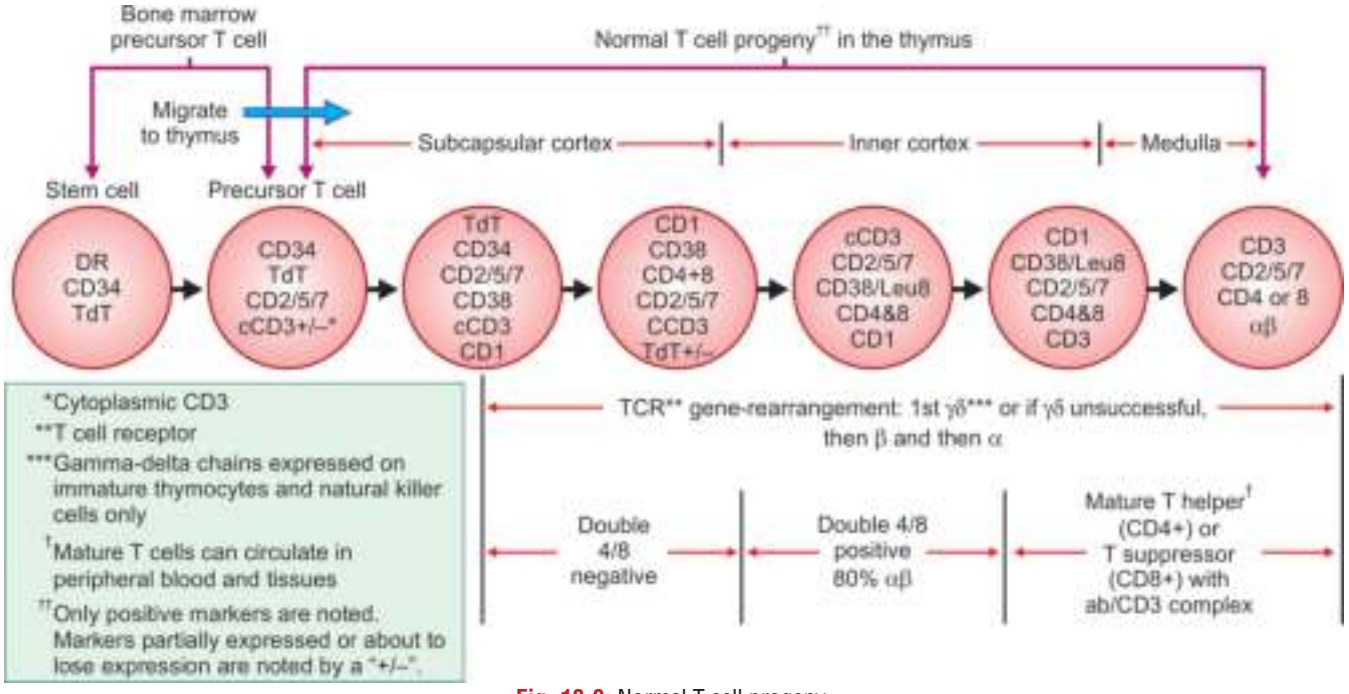


Fig. 13.9: Normal T cell progeny.

Table 13.10 Differences between Hodgkin's disease and non-Hodgkin's lymphoma

Parameters	Hodgkin's Disease	Non-Hodgkin's Lymphoma
Cell derivation	Mostly B cells	B cells (90%), T cells (10%)
Stage/grade	Begins in one lymph node then spread to other lymph nodes. In some cases, grade may be significant, but it is mostly unremarked	Systemic disease and grade influences prognosis and therapy more than stage
Source of mass	Neoplastic Reed-Sternberg cells are usually <1% of the total population of other benign inflammatory cells	Majority of the lymphoid cells are malignant
Lymph nodes involvement	Localized to single axial group of nodes (cervical, mediastinal, para-aortic lymph nodes)	Frequent involvement of multiple peripheral nodes
Gastrointestinal tract mesenteric/ Waldeyer's involvement	Rare involvement	Common involvement
Extranodal involvement	Uncommon (10% cases)	Common (40% cases)
Immune deficiency	Cell-mediated immunity impaired in all classic variants except nodular sclerosis Mycobacterial, fungal, viral and protozoal infections	▪ Humoral immunity impaired ▪ Recurrent bacterial infections
Bone marrow involvement	Common (significant)	Uncommon (bone marrow involvement occurs in some cases)
Architecture of lymph node	Effaced	Effaced
Other autoimmune disease	Uncommon	Common
Constitutional symptoms	Common	Uncommon
Chromosomal defects	Aneuploidy	Translocation and deletion
Mode of spread	Ordinarily contiguous spread	Noncontiguous spread
Treatment	Radiotherapy (mild localized lesion)	Chemotherapy and radiotherapy (systemic disorder)
Prognosis	Good	Poor

Table 13.11 Chemical carcinogens associated cancers

Chemical Carcinogens	Examples	Associated Cancers
Direct-acting chemical carcinogens associated cancers		
Alkylating agents	Nitrogen mustard, cyclophosphamide, chlorambucil, nitrosoureas, β-propiolactone, dimethyl sulphate and diepoxibutane are chemo-therapeutic agents	▪ Hodgkin's disease ▪ Non-Hodgkin's lymphoma ▪ Leukemias ▪ Ovarian carcinoma
Indirect-acting chemical carcinogens associated cancers		
Aromatic hydrocarbons	Benzene presens in crude oil (petrochemical), benz(a)anthracene and benz(a)pyrene	▪ Hodgkin's disease ▪ Acute leukemia ▪ Urinary bladder carcinoma

- The tests can be performed by polymerase chain reaction (PCR) assay and DNA sequences. Characteristics of chromosomal translocations can be detected by fluorescence *in situ* hybridization (FISH). For examples, chromosomal rearrangement t(14;18)involving B cell lymphoma 2 (BCL-2) gene is frequently demonstrated in follicular lymphoma and diffuse large B cell lymphoma (DLBCL). BCL-1 gene involvement is demonstrated in mantle cell lymphoma.

Table 13.12 Infectious agents associated lymphomas

Infectious Agent	Lymphoid Malignancy	
Viruses-associated lymphoid malignancies		
Epstein-Barr virus (EB virus)	▪ Hodgkin's disease ▪ Burkitt's lymphoma ▪ Primary diffuse large B cell lymphoma (DLBCL) of CNS	▪ Post-organ transplant lymphoma ▪ Extranodal NK/T cell lymphoma, nasal type ▪ Primary effusion lymphoma
Human T cell lymphotropic virus 1 (HTLV-1)	Adult T cell leukemia/lymphoma	
Human herpesvirus 8	▪ Primary effusion lymphoma	▪ Castle's disease
Human immunodeficiency virus (HIV)	▪ Diffuse large B cell lymphoma (DLBCL)	▪ Burkitt's lymphoma
Hepatitis C virus (HCV)	▪ Lymphoplasmacytic lymphoma	▪ Marginal zone lymphoma
Bacteria-associated lymphoid malignancies		
Helicobacter pylori	Gastric mucosa-associated lymphoid tissue (MALT) lymphoma	
Chlamydia psittaci	Orbital adnexal lymphoma	
Campylobacter jejuni	Immunoproliferative small bowel disease	
Borrelia burgdorferi	Cutaneous mucosa-associated lymphoid tissue (MALT) lymphoma	

NK cell: Natural killer cell

- **Myc rearrangement** is observed in Burkitt's lymphoma and in rare case of diffuse large B cell lymphoma (DLBCL). Finally, gene arrangement t(2;17) involving ALK is observed in ALK gene positive anaplastic large cell lymphoma.
- DNA and RNA sequencing assays are increasingly performed in the clinical settings of lymphoid neoplasms to detect clonal genetic alterations, including mutations, and copy number changes.
- Chromosomal translocations in non-Hodgkin's lymphoma (NHL) are given in Table 13.13. Correlation between protein expression status and prognosis in different non-Hodgkin's lymphoma subtypes is given in Table 13.14.

Table 13.13 Chromosomal translocations in non-Hodgkin's lymphoma (NHL)

Lymphoid Neoplasm	Chromosomal Abnormalities	Fusion Transcript, Involved Genes
B cell non-Hodgkin lymphomas (high fraction lymphomas)		
Diffuse large B cell lymphoma (DLBCL)	▪ t(14;18)(q27;q11-13) ▪ t(3;14)(q27;q32) ▪ t(2;17)(p23;q23)	▪ BCL-2; IgH (30%) ▪ BCL-6; IgH (30%) ▪ ALK; clathrin
Burkitt's lymphoma	▪ t(8;14),(q24;q32) ▪ t(2;8)(p11;q24) ▪ t(8;22)(q24;q11)	▪ c-Myc; IgH (80%) ▪ Igκ; c-Myc (10%) ▪ c-Myc; IgH-λ (10%)
Burkitt-like lymphoma	t(8;14),(32q2;q21)	c-Myc; IgH
B cell non-Hodgkin lymphomas (low fraction lymphomas)		
Follicular lymphoma	t(14;18)(q32;q21)	BCL-2; IgH
Small cell lymphocytic lymphoma/CLL	▪ t(14;19)(q32;q13) ▪ Trisomy 12,13q	BCL-3; IgH
Lymphoplasmacytic lymphoma	t(9;14)(p13;q32)	PAX5 (BSAP); IgH
Mantle cell lymphoma	t(11;14)(q13;q32)	BCL-1 (cyclin D1); IgH
Marginal zone/MALT lymphoma	▪ t(11;18)(q21;q11) ▪ t(11;14)(q22;q32)	▪ API2; MLT ▪ BCL-10; IgH

Contd...

Table 13.13 Chromosomal translocations in non-Hodgkin's lymphoma (NHL) (*Contd...*)

Lymphoid Neoplasm	Chromosomal Abnormalities	Fusion Transcript, Involved Genes
Plasma cell myeloma	▪ t(4;14)(p16;q32) ▪ t(14;16)(q32;q23) ▪ t(16;22)(q23;q11)	▪ FGFR3; IgH ▪ c-MAF; IgH ▪ c-MAF; IgH-λ
T cell lymphomas		
T-chronic lymphocytic leukemia/T-prolymphocytic leukemia	▪ Inv14(q11;q32) ▪ Trisomy 8q	▪ BCL-3 ▪ TCR gene rearrangement
Mycosis fungoides	Not applicable	TCR gene rearrangement
Angioimmunoblastic T cell lymphoma	Trisomy 3 or 5	▪ TCR gene rearrangement ▪ Epstein-Barr virus
Adult T cell leukemia/lymphoma	Not applicable	▪ TCR gene rearrangement ▪ HTLV1 integration

MALT: Mucosa associated lymphoid tissue; CLL: Chronic lymphocytic leukemia.

Table 13.14 Correlation between protein expression status and prognosis in different non-Hodgkin's lymphoma subtypes

Biomarker	Non-Hodgkin's Lymphoma	Prognosis
SOX1	Mantle cell lymphoma	Poor prognosis
Myc and BCL-2 expression	Diffuse large B cell lymphoma	Poor prognosis
CD10	Diffuse large B cell lymphoma	Good prognosis
ALK	Anaplastic large cell lymphoma	Good prognosis

STAGING OF LYMPHOID NEOPLASMS

Once the diagnosis of lymphoid neoplasm is established, the next step is to determine the extent of the disease and assigning the stage, i.e. stages I, II, III and IV. Staging is determined by computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) scan. Bone marrow trephine biopsy is frequently required to evaluate the extent of the disease, complete staging of the disease and response at the end of therapy especially in non-Hodgkin's lymphoma. Bone marrow trephine biopsy is not required in patients with early stage I classical Hodgkin's disease, who are adequately staged with CT scan, MRI scan and PET scan; as the bone marrow is not involved in early stage I.

- Stage I lymphoid neoplasm is the least advanced early stage with involvement of one lymph node region or one extranodal site, and stage IV being the most advanced stage with involvement of nodal and extranodal sites (bone, bone marrow, liver, lungs).
- Stage II lymphoid neoplasm is established when more than one nodal involvement regardless of the number and size; on one side of the diaphragm.
- Stage III lymphoid neoplasm is established when lymph node involvement regardless of the number and size; on both sides of the diaphragm.
- Stage IV lymphoid neoplasm is the most advanced stage of the disease that has started in the lymph nodes and spread to at least one body organ outside the lymphatic system (lungs, liver, bone marrow or solid bones).

Lumbar Puncture

Lumbar puncture is indicated to determine whether the lymphoid neoplasms have spread to spinal cord in patients with Burkitt's lymphoma, testicular lymphoma and aggressive B cell lymphoma involving the paranasal lymphoma. Patients with advanced stage diffuse large B cell lymphoma (DLBCL) with multiple extranodal involvement, higher level of lactic dehydrogenase (LDH) and poor performance status are at higher risk for involvement of central nervous system, who require prophylactic intrathecal chemotherapy.

Additional Tests

Before initiating chemotherapeutic agents, it is essential to analyze patient's cardiac function including the left ventricular ejection fraction. Patients with evidence of hepatitis B and C are adequately treated by administration of antiviral therapy. Patients with human immunodeficiency virus (HIV) infection

should be treated to decrease their viral load and to restore CD4+ helper T cell counts before initiation of chemotherapy.

TREATMENT

A definite cure is the goal for patients with Hodgkin's disease, Burkitt's lymphoma, diffuse large B cell lymphoma (DLBCL), anaplastic large T cell lymphoma, in addition to several common lymphoid neoplasms presented in localized stage. It is essential to improve survival and/or the quality of life in advanced stage of follicular lymphoma (FL) and mantle cell lymphoma (MCL).

Pretreatment Evaluation

Pretreatment evaluation is essential to know the extent of disease before initiating treatment. Pretreatment evaluations must include complete medical history with presence or absence of B symptoms such as fever, night sweats, weight loss; complete physical examination with particular attention to superficial lymph nodes, Waldeyer's ring, size of liver and spleen.

- The extension of the disease is established by CT scan, MRI scan and PET scan and bone marrow trephine biopsy. Head region CT scan or brain MRI and lumbar puncture are performed to evaluate spinal cord involvement by Burkitt's lymphoma, testicular lymphoma and aggressive B cell lymphoma.
- Various laboratory tests must include complete blood counts, erythrocyte sedimentation rate, serum lactic dehydrogenase, hepatitis B, hepatitis C, HIV testing and β_2-microglobulin.
- Multigated acquisition (MUGA) scan or echocardiogram is essential prior to initiation of anthracyclines containing regimes.

Cytotoxic Drugs and Radiotherapy

Combined chemotherapy and radiotherapy represent backbone of treatment for most lymphoid neoplasms. The addition to chemotherapy of the anti-20 monoclonal antibody rituximab has significantly improved the outcome of patients suffering from B cell non-Hodgkin's lymphoma. Splenectomy may represent a treatment option in some cases of lymphoid neoplasms as with spleen involvement.

- Alkylating agents such as chlorambucil, cyclophosphamide, melphalan, dacarbazines and bendamustine are administered to treat the patients with lymphoid neoplasms.
- Other cytotoxic drugs can be used to treat these patients, which include anthracyclines (doxorubicin), glycopeptides (bleomycin), antimetabolites (methotrexate), vinca alkaloids (vincristine, vinblastine), purine analogues (fludarabine) and topoisomerase inhibitors (etoposide).
- Administration of combined corticosteroids and chemotherapeutic drug produce both lympholytic and antiemetic effects.
- Chemotherapeutic agents may produce bone marrow failure, infertility, myelodysplasia, cardiomyopathy, pulmonary fibrosis, peripheral neuropathy, renal failure and peripheral neuropathy.
- Lymphoid neoplasms are highly radiosensitive. Radiation therapy is used in patients with follicular lymphoma.

Hematopoietic Stem Cell Transplantation

High-dose chemotherapy followed by allogenic or autologous hematopoietic stem cell transplantation can be curative in some forms of chemotherapy-sensitive relapsed lymphomas. Hematopoietic stem cells (HSCs) obtained from peripheral blood are infused after administration of high doses chemotherapy-induced bone marrow aplasia.

HODGKIN'S DISEASE

HODGKIN'S DISEASE: OVERVIEW

Hodgkin's disease (HD) is a malignant neoplasm of lymph node. It is important to determine whether the patient has only a single lymph node region or multiple lymph node regions involved, or extranodal involvement. After therapy, about 5% of patients develop myelodysplastic syndromes, acute myelogenous leukemia, or carcinomas, particularly of the lung. Hodgkin's disease has bimodal age distribution with a peak at 15–40 years and second small peak during seventh decade. Classification of Hodgkin's disease according to revised 2024 WHO classification of tumors of hematopoietic and lymphoid tissues is given in Table 13.15.

DIAGNOSTIC CRITERIA OF HODGKIN'S DISEASE

Diagnostic criteria of Hodgkin's disease include demonstration of neoplastic Reed-Sternberg cells in the lymph nodes, which are derived from B cells admixed with inflammatory cells in background as a result of cytokines synthesis such as IL-1, IL-4, IL-5, IL-6, TNF-α, GM-CSF,

Table 13.15 Classification of Hodgkin's disease according to revised 2024 WHO classification of tumors of hematopoietic and lymphoid tissues

Hodgkin's Disease: Histologic Subtypes
▪ Nodular lymphocyte predominant Hodgkin disease
▪ Classic variants of Hodgkin's disease • Nodular sclerosis classic variant of Hodgkin's disease • Lymphocyte-rich classic variant of Hodgkin's disease • Mixed cellularity classic variant of Hodgkin's disease • Lymphocyte-depletion classic variant of Hodgkin's disease

Adapted from 2024 WHO classification of tumors of hematopoietic and lymphoid tissues.

and TGF-β. Reed-Sternberg cells develop as a result of genetic rearrangement and constitute 1–5% of cell population. Background lymphocytes are usually T cells in Hodgkin's disease.

Reed-Sternberg Cells

Mystery of the origin of Reed-Sternberg cells in Hodgkin's disease was solved by molecular studies that relied on single cell of Reed-Sternberg cells, coupled with amplification of RNA and genomic DNA by polymerase chain reaction (PCR) technique, which established that Reed-Sternberg cell arises from germinal center or post-germinal center B cells. Reed-Sternberg cells usually have clonal somatically mutated IgG rearrangements indicative of B cell origin. Some reports suggest that classic Reed-Sternberg cells can be of T cell origin, where T cell markers are expressed on neoplastic Reed-Sternberg cells.

Growth and Survival of Reed-Sternberg Cells

Growth and survival of classic Reed-Sternberg cells are closely related to the activation of the nuclear factor kappa B (NF-κB) transcription factor-signaling pathway. The consecutive nuclear activity of NF-κB can prevent both apoptosis and promote cell proliferation. Epstein-Barr (EB) virus, which is found in the Reed-Sternberg cells expresses viral proteins such as latent membrane protein 1 (LMP1) leading to NF-κB activation.

Role of Epstein-Barr Virus in Pathogenesis of Hodgkin's Disease

Approximately 40% cases of Hodgkin's disease (classic or lymphocytic depletion) are associated by Epstein-Barr virus detected in Reed-Sternberg cells, which plays an important role in the pathogenesis of Hodgkin's disease and protects Reed-Sternberg cells from apoptosis and CD8+ cytotoxic T cells, which would ordinarily destroy the neoplastic Reed-Sternberg cells. It may cause mutation in the TP53 tumor suppressor gene. Epstein-Barr virus is demonstrated by EBV-LMP1 immunohistochemistry or EBV encoded early RNA (EBER) by fluorescence *in situ* hybridization.

Pathogenesis of Hodgkin's disease is shown in Fig. 13.10. Cellular interactions in the Hodgkin's disease and Reed-Sternberg cells microenvironment are shown in Fig. 13.11.

Pathology Pearls: Reed-Sternberg Cell Variants

- **Classical Reed-Sternberg Cells:** Classical Reed-Sternberg cells (RS cells) are large and binucleated or bilobed, with two halves often appearing as mirror images of each other resembling owl eyes from prominent nucleoli. Classical Reed-Sternberg cells measure 20–50 μm in diameter with amphophilic cytoplasm. Nuclear membrane is thick and sharply defined. These RS cells are seen in mixed cellularity variant of Hodgkin's disease (Fig. 13.12A and B).
- **Mononuclear Reed-Sternberg Cells:** Mononuclear Reed-Sternberg cells may be seen in any type of Hodgkin's disease but are encountered in mixed cellularity Hodgkin's disease (Fig. 13.13A and B).
- **Pleomorphic Reed-Sternberg Cells:** Pleomorphic Reed-Sternberg cells are larger than other type of Reed-Sternberg cells, which contain hyperchromatic large nuclei. Reed-Sternberg cells are seen in lymphocytic depletion variant of Hodgkin's lymphoma (Fig. 13.14A and B).
- **Lacunar Reed-Sternberg Cells:** Lacunar Reed-Sternberg cells are predominantly seen in the nodular sclerosis variant of Hodgkin's disease. Lacunar RS cells have more delicate folded or solitary nuclei surrounded by abundant pale cytoplasm in histological sections that can retract during processing (Fig. 13.15A and B).
- **Lymphocytic–Histiocytic Reed-Sternberg Cells:** Lymphocytic–histiocytic (L&H) Reed-Sternberg cells are sometimes called popcorn cells seen in nodular lymphocyte predominant Hodgkin's disease. Popcorn cells have bubbly outline of nucleus (Fig. 13.16A and B).

Reed-Sternberg Cells versus Reed-Sternberg-like Cells

Reed-Sternberg cells should be differentiated from Reed-Sternberg-like multinucleated cells in lymph node. Megakaryocyte can simulate closely in hematoxylin and eosin stained sections. Megakaryocytes are strongly positive for factor VIII related antigen and CD61.

- Immunoblasts in infectious mononucleosis are morphologically similar to Reed-Sternberg cells.
- Reed-Sternberg-like cells are demonstrated in peripheral T cell lymphoma, anaplastic large cell lymphoma, infectious mononucleosis and reactive viral lymphadenitis.

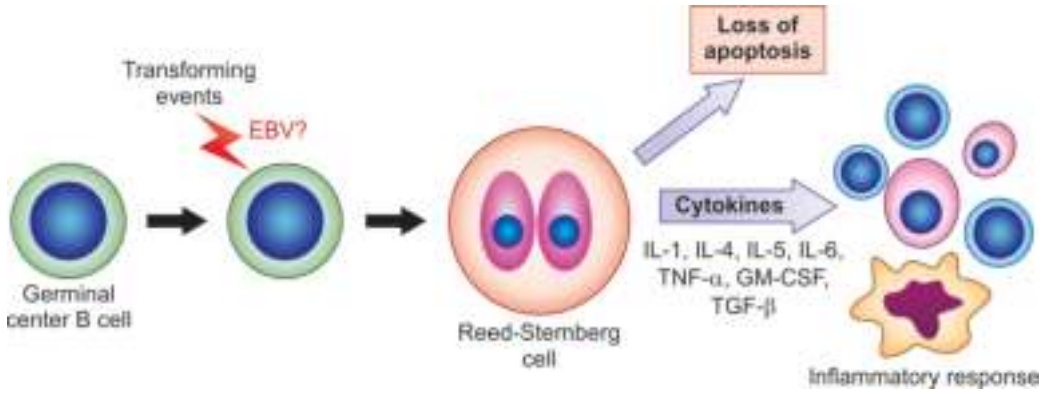


Fig. 13.10: Pathogenesis of Hodgkin's disease.

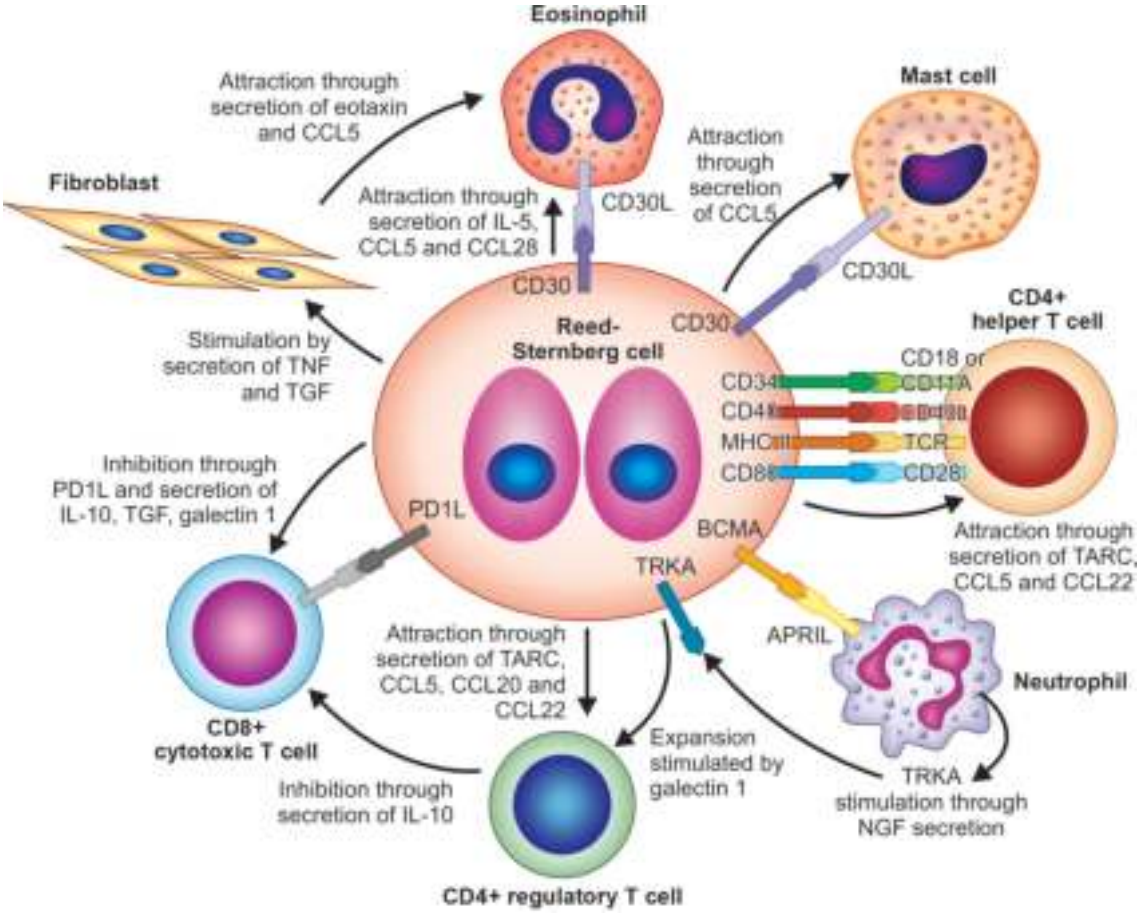


Fig. 13.11: Cellular interactions in the Hodgkin's disease and Reed-Sternberg cells microenvironment.

- Differences between Reed-Sternberg cells and Reed-Sternberg-like cells are given in Table 13.16.

CLINICAL FEATURES

Patient with Hodgkin's disease presents with painless lymphadenopathy most often in upper half of body in cervical or mediastinal regions, but occasionally in the axilla or inguinal-femoral region.

- **Constitutional symptoms** such as fever, night sweats, and weight loss are observed in 40–50% of cases. Pruritus is present in some cases. Ingestion of alcohol may cause pain at involved sites.
- Patients with Hodgkin's disease are categorized into two grades: grade B with one or more of these symptoms and grade A lacking these symptoms.
- On clinical examination, the lymph nodes are enlarged discrete, rubbery, painless and mobile.

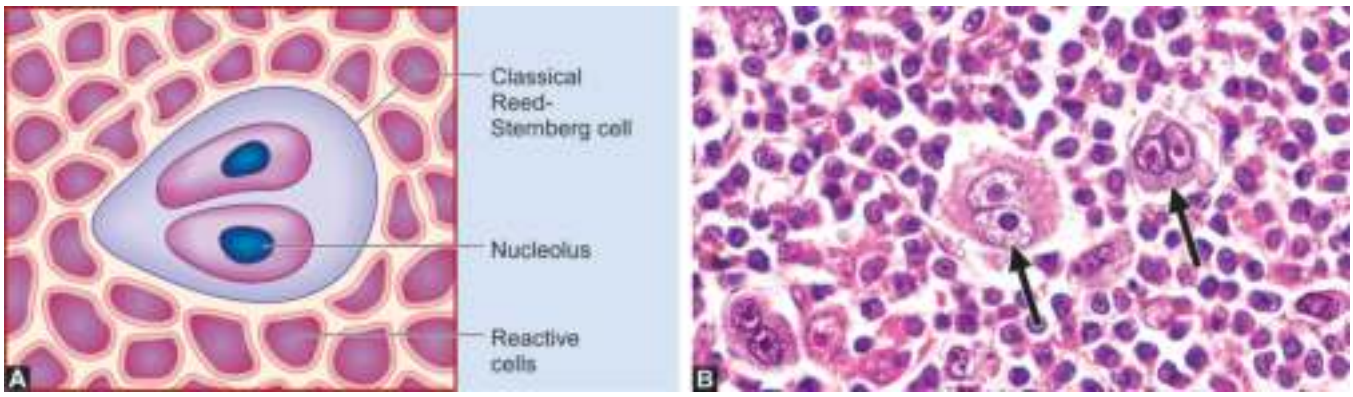


Fig. 13.12A and B: Classical Reed-Sternberg cells (arrows).

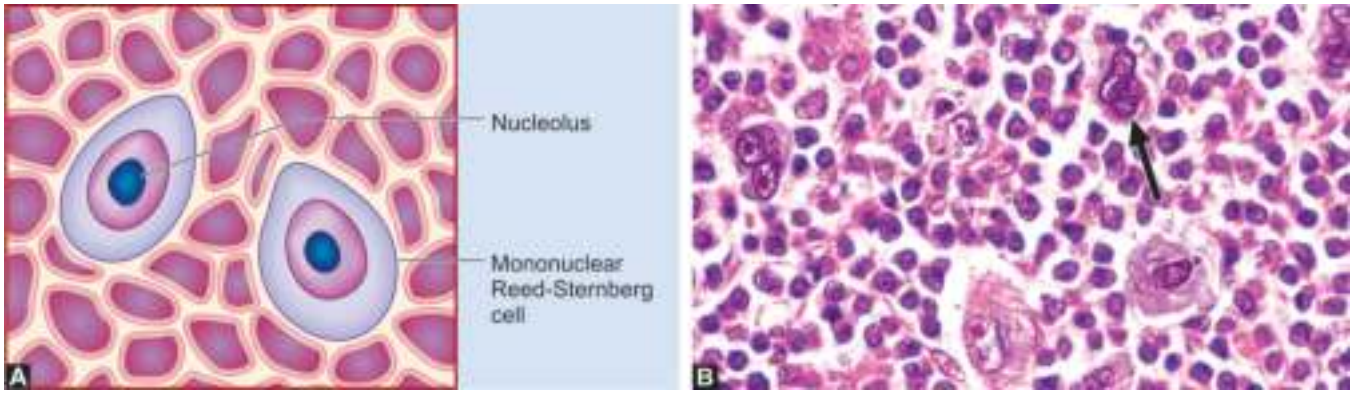


Fig. 13.13A and B: Mononuclear Reed-Sternberg cells (arrow).

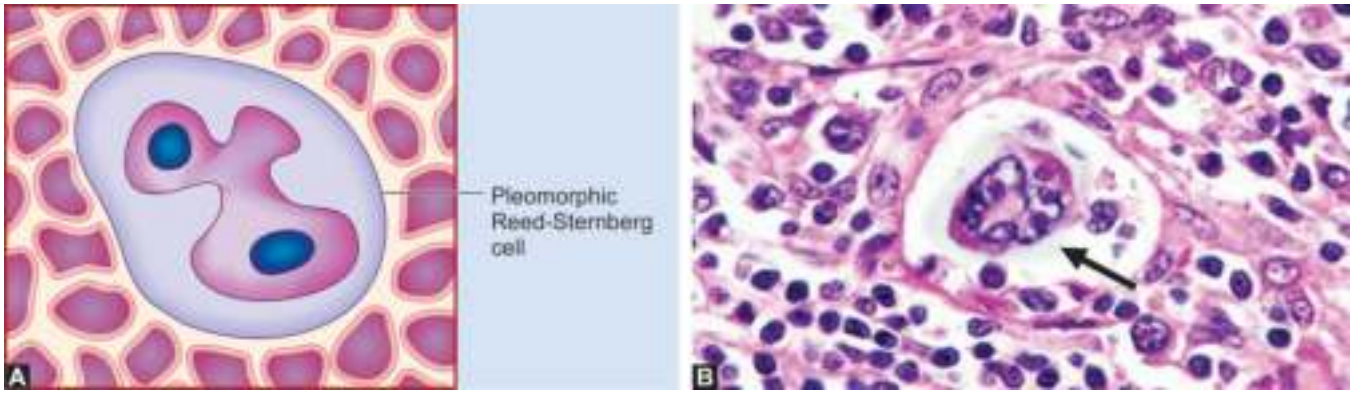


Fig. 13.14A and B: Pleomorphic Reed-Sternberg cells (arrow).

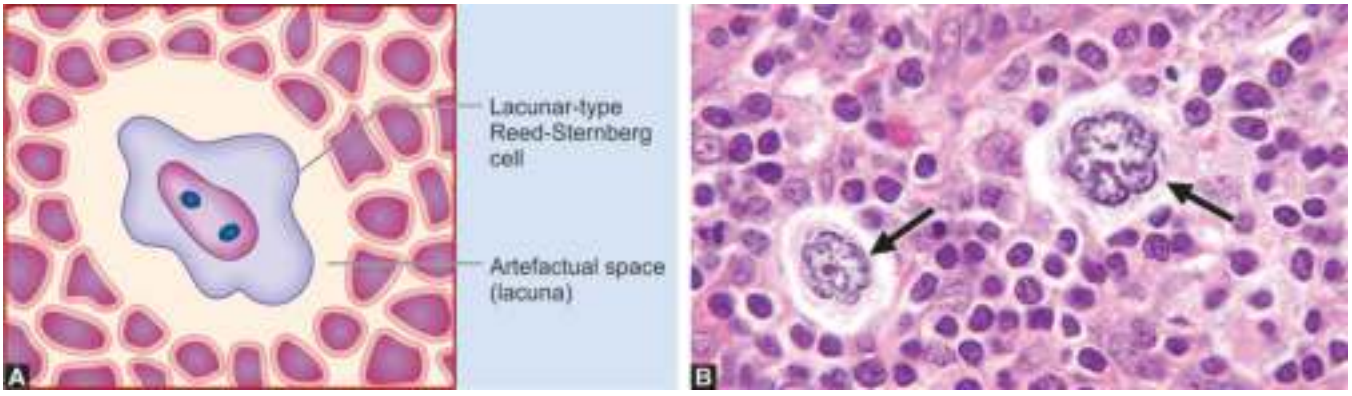


Fig. 13.15A and B: Lacunar-type Reed-Sternberg cells (arrows).

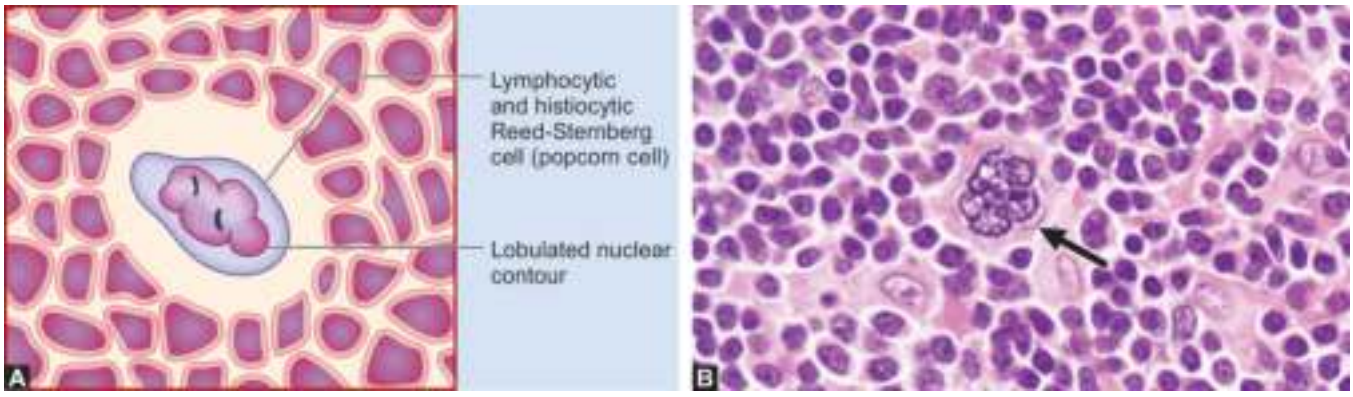


Fig. 13.16A and B: Lymphocytic–histiocytic Reed-Sternberg cells (arrow).

Table 13.16 Differences between Reed-Sternberg cells and Reed-Sternberg-like cells

Parameters	Reed-Sternberg Cells	Reed-Sternberg-like Cells
Cell morphology	Reed-Sternberg cells are present in Hodgkin's disease	Reed-Sternberg-like cells are present in infectious mononucleosis, peripheral T cell lymphoma and anaplastic large cell lymphoma
Nucleolus	Acidophilic, regular with clear halo and more centrally placed	Basophilic, irregular and adjacent to nuclear membrane
Cytoplasm	Usually acidophilic and variable	Amphophilic with usual strong pyroninophilic
Surrounding cells	Lymphocytes and histiocytes	Mononuclear immunoblasts and plasmacytoid cells

CHEST RADIOGRAPHS

Chest radiographs demonstrate mediastinal lymphadenopathy in 40% of cases, with associated lower cervical lymphadenopathy in Hodgkin's disease.

MODE OF SPREAD

Hodgkin's disease spreads via direct extension, lymphatic and hematogenous routes.

- **Direct extension:** Direct extension of Hodgkin's disease involves adjacent lymph nodes, skin, skeletal muscle, while mediastinal lymph nodes invade large vessels, lung and chest wall in nodular sclerosis variant of Hodgkin's disease.
- **Lymphatic route:** Initially Hodgkin's disease involves other lymph nodes via lymphatic route in continuous and predictable fashion.
- **Hematogenous route:** Later, Hodgkin's disease spreads via the blood and involves the spleen, liver and bone marrow. Spleen is practically always involved. Liver is involved if splenic hilum and retroperitoneal lymph nodes are involved. Other organs like lung, GIT, skin and CNS may also be involved. Bone marrow is involved in lymphocytic depletion variant of Hodgkin's disease. Bone marrow involvement is often asymptomatic but may produce pain with vertebral osteoblastic lesions (ivory vertebrae) and, rarely, osteolytic lesions and compression fracture.

CLINICAL STAGING

Clinical staging of Hodgkin's disease is most important in determining extent of disease, therapeutic modalities and prognosis. It is done by radiological techniques.

- Chest radiograph is low cost method for diagnosis and surveillance of Hodgkin's disease. It is useful in detection of mediastinal mass.
- Ultrasonography determines the size of the lymph nodes, in chest and abdomen and involvement of liver and spleen. CT scan is standard imaging technique for thoracic examination in Hodgkin's disease patients. It is useful for determination of sites on initial involvement as well as extent of Hodgkin's disease. It helps in classification of early stage patients.
- Whole body FDG-PET scan using fluorodeoxyglucose-18 is a sensitive indicator of Hodgkin's disease. It is recommended when other diagnostic modalities are inconclusive.
  - FDG-PET scan most accurately demonstrates the correct pretreatment stage in Hodgkin's disease compared with high-dose contrast-enhanced computed tomography (CECT), which tends to understage or over-stage of Hodgkin's disease.
  - FDG-PET scan is able to distinguish viable/active tumor cells from fibrosis or necrosis in a residual mass after treatment.

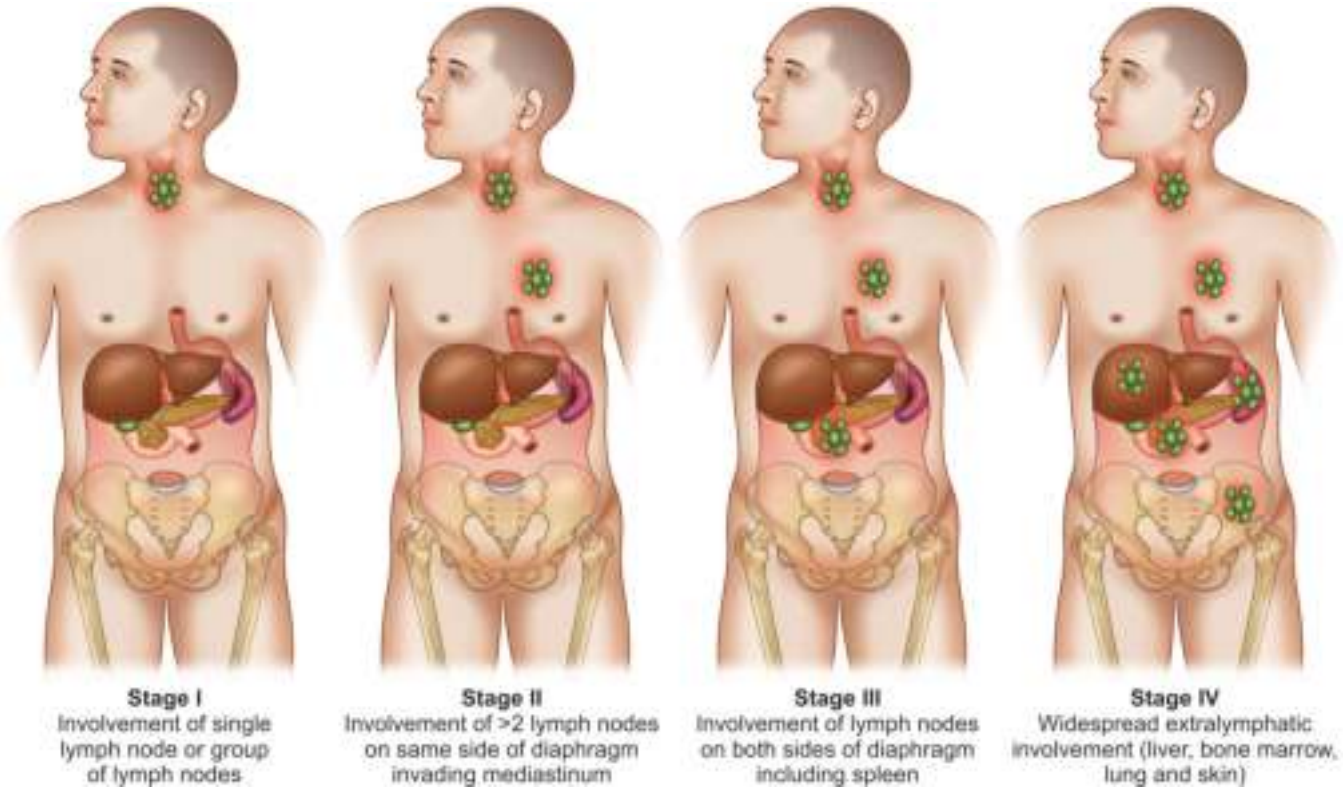


Fig. 13.17: Staging of Hodgkin's diseases.

Table 13.17 Modified Ann Arbor staging system of Hodgkin's disease by Costwold

Stage	Organs Involved
Stage I	Single lymph node or lymphoid structures (spleen, thymus, Waldeyer's ring)
Stage II	Two or more lymph nodes on the same sides of the diaphragm
Stage III	▪ Lymph nodes involved on both sides of the diaphragm ▪ Stage III_1 (with or without splenic, hilar, celiac or portal nodes) ▪ Stage III_2 (with para-aortic, iliac or mesenteric nodes)
Stage IV	Involvement of extranodal site(s) beyond those designated E

The stages can also have a designation of 'A' for asymptomatic or 'B' for constitutional symptoms (weight loss >10%, drenching sweats, fever).

Ann Arbor Staging System

Ann Arbor staging system is the basis for most Hodgkin's disease staging based on the degree of dissemination, involvement of extralymphatic sites, and presence or absence of systemic signs such as fever. It is an essential part of the diagnostic evaluation of patients with Hodgkin's disease. Although grading of histopathologic variants of Hodgkin's disease roughly correlates with clinical behavior, prognosis is better predicted by staging. Modified Ann Arbor staging system of Hodgkin's disease by Costwold is shown in Fig. 13.17 and Table 13.17.

LABORATORY DIAGNOSIS

Hematologic abnormalities may be caused by extensive bone marrow involvement by Hodgkin's disease. Hypersplenism may appear, but mainly in patients with marked splenomegaly. Elevated serum alkaline phosphatase levels usually indicate bone marrow or liver involvement or both.

Laboratory Diagnosis of Hodgkin's Disease
Indicators of Active Disease
Increase in leukocyte alkaline phosphatase, serum haptoglobin, ESR, serum copper, and other acute phase reactants usually reflect active Hodgkin's disease.
Anemia and Iron Parameters
Patient most often develops microcytic-hypochromic anemia in advanced Hodgkin's disease. Defective iron reutilization is characterized by low serum iron, low iron-binding capacity, and increased bone marrow iron.

Total and Differential WBCs

Slight-to-moderate polymorphonuclear leukocytosis may be present in Hodgkin's disease. Lymphocytopenia may occur early and become pronounced with advanced Hodgkin's disease. Eosinophilia is present in about 20% of patients, and thrombocytosis may be observed.

Erythrocyte Sedimentation Rate

The erythrocyte sedimentation rate is commonly elevated in patients with active Hodgkin's disease and sometimes may be the only evidence that the disease has been inadequately treated or that clinical recurrence is imminent.

Bone Marrow Smear Examination

- Bone marrow involvement by Hodgkin's disease can be demonstrated by the usual bone marrow aspirate technique and examination of marrow smears.
- Bone marrow involvement is focal, often associated with fibrosis. It may be found with increasing frequency as the Hodgkin's disease becomes more widespread with systemic symptoms and often associated with an elevated serum alkaline phosphatase, radiologic evidences of bone involvement and unexplained pancytopenia.

Preferred Lymph Node Biopsy Sites in Hodgkin's Disease

- Hodgkin's disease is very rare in the absence of lymphadenopathy.
- Lymph node biopsy must be taken from cervical region, axillary region, splenic hilum and retroperitoneal region.
- Biopsy specimens then can be obtained from bone marrow, liver, or other parenchymal tissue.
- Mesenteric and inguinal lymph nodes are usually spared, hence should not be biopsied for histological diagnosis. Excised lymph node should be fixed in 10% buffered formalin or Zenker's fixative.

Gross Morphology

Cut surface of involved lymph node is uniform. Capsule is rarely involved, hence not invading surrounding adipose tissue.

Light Microscopy

- Sections stained with hematoxylin and eosin are examined by light microscopy. Hodgkin's disease can be definitively diagnosed by lymph node biopsy that reveals characteristic Reed-Sternberg cells in a characteristic histologic setting.
- On histologic examination, one finds difficulty in differentiating Hodgkin's disease from granulocytic leukemia and large cell lymphoma, hence imprint smears are helpful in differentiating these entities.

Immunophenotyping

- Reed-Sternberg cells in classic variants of Hodgkin's disease express B cell markers such as CD15 (Leu M-1), CD30 (Ki-1) and PAX5.
- On the other hand, nodular lymphocytic–histiocytic variant of Hodgkin's disease shows negativity for CD15 (Leu M-1), CD30 (Ki-1) and PAX5, CD20, CD45 and epithelial membrane antigen (EMA).
- CD30 is expressed on Reed-Sternberg cells of Hodgkin's disease, immunoblasts in infectious mononucleosis, peripheral T cell NHL, embryonal carcinoma and pancreatic carcinoma. Reed-Sternberg cells in Hodgkin's disease show positivity for CD15 (Leu M-1) as shown in Fig. 13.18A.
- Reed-Sternberg cells in Hodgkin's disease show positivity for CD30 (Ki-1) as shown in Fig. 13.18B.

DIFFERENTIAL DIAGNOSIS

Hodgkin's disease may be difficult to differentiate from lymphadenopathy caused by infectious mononucleosis, toxoplasmosis, cytomegalovirus, NHL, or leukemia. The clinical picture can also be simulated by lung carcinoma, sarcoidosis, tuberculosis, and various diseases in which splenomegaly is the predominant feature.

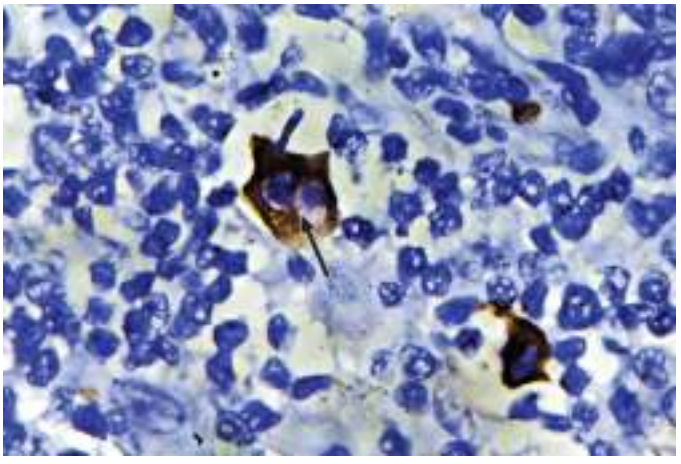


Fig. 13.18A: Reed-Sternberg cells in Hodgkin's disease show positivity for CD15 (Leu M-1) (arrow).

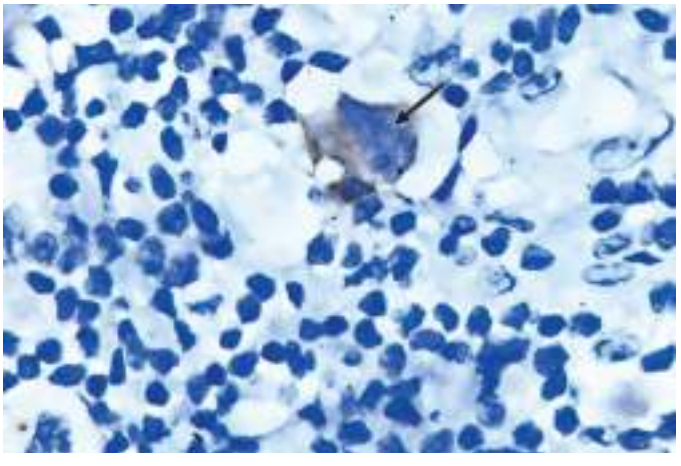


Fig. 13.18B: Reed-Sternberg cells in Hodgkin's disease show positivity for CD30 (Ki-1) positive (arrow).

HODGKIN'S DISEASE: MORPHOLOGIC SUBTYPES

There are two morphologic subtypes of Hodgkin's disease: classical Hodgkin's disease (lymphocyte-rich, lymphocyte depletion, nodular sclerosis and mixed cellularity) and nodular lymphocytic–histiocytic-predominant variant with or without histiocytes and Hodgkin's disease. Nodular sclerosis, mix cellularity and nodular lymphocyte predominant variants of Hodgkin's disease arise from B cells. Rest Hodgkin disease variants originate from T cell. Morphologic subtypes of Hodgkin's disease are given in Table 13.18. Immunophenotyping features of classic variant of Hodgkin's disease are given in Table 13.19. Histologic

Table 13.18 Morphologic subtypes of Hodgkin's disease

Hodgkin's Disease Variant	Immunophenotyping	Reed-Sternberg Cells	Prognosis
Classic variants of Hodgkin's disease			
Lymphocyte-rich classic variant of Hodgkin's disease (B cell markers)	▪ CD30+ (Ki-1) specific marker ▪ CD15+ (Leu M-1)	Popcorn Reed-Sternbeg cells (lymphocytic–histiocytic cells)	Good prognosis
Lymphocyte-depletion classic variant of Hodgkin's disease (B cell markers)	▪ CD30+ (Ki-1) specific marker ▪ CD15+ (Leu M-1)	Many Reed-Sternbeg cells with diffuse fibrosis	Worst prognosis
Nodular sclerosis classic variant of Hodgkin's disease (B cell markers)	▪ CD30+ (Ki-1) specific marker ▪ CD15+ (Leu M-1) ▪ CD20+ ▪ CD79a+ ▪ CD5+ ▪ CD3+ ▪ IgM+ ▪ IgD+	Lacunar Reed-Sternbeg cells with bands of collagen fibers	Best prognosis
Mixed cellularity classic variant of Hodgkin's disease (B cell markers)	▪ CD30+ (Ki-1) specific marker ▪ CD15+ (Leu M-1)	Classic Reed-Sternbeg cells with heterogeneous population of reactive cells	Commonest variant in India with fair prognosis
Nodular lymphocytic–histiocytic predominant variant of Hodgkin's disease with or without benign histiocytes (L&H cells)			
Nodular lymphocytic–histiocytic predominant with L&H cells (pan-B markers)	▪ CD19+ ▪ CD20+ ▪ CD22+ ▪ CD45RA+ ▪ CD45RB (LCA)+ ▪ CD74+ ▪ CDw75+ ▪ EMA+	Lymphocytic–histiocytic cells (L&H cells)	Good prognosis

All are classic variants of Hodgkin's lymphoma except nodular lymphocytic–histiocytic predominant variant.

Table 13.19 Immunophenotyping features of classic variant of Hodgkin's disease

Cell Type	Immunophenotyping	Expression
Reed-Sternberg cells		
Reed-Sternberg cells immunophenotyping	▪ CD15 (Lu M-1) ▪ CD30 (Ki-1)	▪ Positive ▪ Positive
Cellular environment		
T cells (Th2)	CD3, CD4	Positive
Eosinophils	CD16	Positive
Histiocytes	CD68	Positive
Plasma cells (variable)	CD138	Positive
Neutrophils (variable)	CD15	Positive

Table 13.20 Histologic variants of classic Hodgkin's disease (HD)

Characteristics	Nodular Sclerosis HD	Mixed Cellularity HD	Lymphocyte-rich HD	Lymphocyte-depletion HD
Origin	B cells	B cells	B cells	B cells
Frequency	Most frequent type (75%)	Overall second most frequent (25%)	Uncommon (6%)	Uncommon (4%)
Age group	Young age group	Young adults and older persons (more than 55 years), bimodal peak	Young age group	Older age group
Sex predilection	Female predominance	Males predominance	Males predominance	Males predominance
EB virus association	No association	Associated with EB virus	No association	Associated with EB virus
Clinical features	Mediastinal mass, lower cervical, supraclavicular lymphadenopathy	Lower cervical and supraclavicular lymph nodes	Axillary lymphadenopathy (mediastinal uncommon)	Patient is associated with HIV infection having dis-seminated disease
Light microscopy	Lacunar Reed-Sternberg cells with bands of collagen fibers divide the lymph node in multiple nodules	Classic and mononuclear Reed-Sternberg cells admixed with many reactive cells	A few popcorn Reed-Sternberg cells	Many Reed-Sternberg cells with diffuse fibrosis
Immunophenotyping	**Reed-Sternberg cells:** CD30 (Leu M-1) and CD15 (Ki-1)	**Reed-Sternberg cells:** CD30 (Leu M-1) and CD15 (Ki-1)	**Reed-Sternberg cells:** CD30 (Leu M-1), CD15 (Ki-1) and CD20	**Reed-Sternberg cells:** CD30 (Leu M-1) and CD15 (Ki-1)
Stage	Most cases are in stage I or II	Most cases are in stage III or IV (>50%)	Most cases are in stage I or II	Most cases are in stage III or IV
Prognosis	Best prognosis	Fair prognosis	Good prognosis	Worst prognosis

variants of classic Hodgkin's disease are given in Table 13.20.

LYMPHOCYTE-RICH CLASSIC VARIANT OF HODGKIN'S DISEASE

Lymphocyte-rich classic variant of Hodgkin's disease constitutes about 5–10% of patients, which is a slow-growing disease associated with excellent prognosis. It is more common in men than women under 35 years of age. There is an association of Epstein-Barr virus (EBV) infection in 40% of cases.

Clinical Features

Patient usually presents with solitary enlarged painless lymph node usually in cervical or inguinal regions, persistent fatigue, fever, night sweats, weight loss, severe itching and painful lymph node after drinking alcohol. Lymphocyte-rich classic variant of Hodgkin's disease never involves liver, spleen and bone marrow except when it changes to more aggressive histologic pattern. The clinical course is moderately aggressive.

Surgical Pathology: Lymphocyte-rich Classic Variant of Hodgkin's Disease

Light Microscopy

Lymphocyte-rich classic variant of Hodgkin's disease is composed of mature lymphocytes admixed with Reed-Sternberg cells, i.e. popcorn or elephant's foot cells which have excessively lobulated nuclei.

Immunophenotyping

- Reed-Sternberg cells have same immunophenotyping as in other subtypes of classic Hodgkin's lymphoma.
- These show positivity for T cell markers such as CD30 (Leu M-1) and CD15 (Ki-1).
- Reed-Sternberg B cells are constantly negative for pan-B markers such as CD19, CD20, CD22, 45RA, 45RB (LCA), CD74, CDw75, CD20 or CD45.
- Immunophenotyping of lymphocyte-rich classic variant of Hodgkin's disease is given as follows:

Markers	Expression
▪ CD30 (Leu M-1)	▪ Positive
▪ CD15 (Ki-1)	▪ Positive

Prognosis

Lymphocyte-rich classic variant of Hodgkin's disease is slow growing and associated with best prognosis.

LYMPHOCYTE-DEPLETION CLASSIC VARIANT OF HODGKIN'S DISEASE

Lymphocyte-depletion classic variant of Hodgkin's disease is least common constituting 4% of Hodgkin's disease with worst prognosis. It is most often associated with Epstein-Barr virus. It usually affects elderly persons associated with HIV infection.

Clinical Features

The lymphocyte-depletion classic variant of Hodgkin's disease is usually not diagnosed until the disease is widespread and involving liver (60%), bone marrow (40%), spleen and retroperitoneal lymph nodes. Advanced stage and systemic symptoms are frequent. Pancytopenia is occasionally caused by bone marrow involvement in these cases.

Surgical Pathology: Lymphocyte-depletion Classic Variant of Hodgkin's Disease

Light Microscopy

Lymphocyte-depletion classic variant of Hodgkin's disease can easily be confused with non-Hodgkin's lymphoma. It should only be diagnosed when anaplastic large cell lymphoma has been excluded. On histologic examination, it may show any of two patterns: diffuse fibrosis and reticular pattern.

- **Diffuse fibrosis pattern:** Lymph node shows diffuse fibrosis, few lymphocytes and polypoid Reed-Sternberg cells. Diffuse fibrosis is demonstrated by nonbirefringent collagen fibers in polarizing microscopy.
- **Reticular pattern:** Lymph node shows reticular pattern with sheets of bizarre Reed-Sternberg cells and areas of necrosis. Reticular pattern needs to be differentiated from large cell NHL.

Immunophenotyping

Immunophenotyping of lymphocyte-depletion variant of Hodgkin's disease is mentioned below:

Markers	Expression
▪ CD30 (LeuM-1)	▪ Positive
▪ CD15 (Ki-1)	▪ Positive

Prognosis

Lymphocyte-depletion classic variant of Hodgkin's disease has the worst prognosis among all the variants of Hodgkin's disease.

NODULAR SCLEROSIS CLASSIC VARIANT OF HODGKIN'S DISEASE

Nodular sclerosis classic variant of Hodgkin's disease constitutes 60–75% of all cases, which involves B cells (all other types of Hodgkin's disease involve T cells). Presence of large bundles of collagenous connective tissue separates the lymph node into nodules. Most cases in the stage I or II.

Age and Sex Predilection

Nodular sclerosis classic variant of Hodgkin's disease usually affects adolescents and young adults between 20 and 30 years of age. Females are more affected than males.

Clinical Features

Patient presents with lymphadenopathy in upper mediastinum (50%), supraclavicular or lower cervical regions. There is rarely an association with Epstein-Barr virus (EBV) infection. Patient may have recurrent laryngeal paralysis due to compression of recurrent laryngeal nerves. Neurologic pain follows nerve root compression. Lymphadenopathy in thorax compresses respiratory tract (trachea and bronchi) resulting in cough, chest pain and severe dyspnea.

Laboratory Diagnosis

Hematologic findings are anemia, leukocytosis, and an elevated erythrocyte sedimentation rate.

Surgical Pathology: Nodular Sclerosis Classic Variant of Hodgkin's Disease

Gross Morphology

- Lymph node is enlarged, firm, fleshy with formation of irregular nodules separated by bands of firmer fibrotic tissue.
- Fibrosis in lymph node is caused by cytokines synthesized by neoplastic lacunar cells, i.e. IL-5 (attracting eosinophils), IL-4, TNF-α and GM-CSF.

Light Microscopy

- Lymph node from nodular sclerosis classic variant of Hodgkin's disease shows broad bands of pink-staining birefringent collagen fibrous tissue on polarizing microscopy (Fig. 13.19).
- There is presence of numerous lacunar Reed-Sternberg cells and reactive cells. Lacunar RS cells contain more delicate folded or solitary nuclei surrounded by abundant clear or pale eosinophilic cytoplasm in histologic sections that can retract during processing. It is an artifact of formalin fixation.

- Cytokines synthesized by Reed-Sternberg cells are currently believed to cause progressive attraction of T cells, histiocytes, plasma cells and eosinophils.
- Nodular sclerosis of Hodgkin's disease has cellular phase and syncytial phase. Nodular sclerosis classic variant of Hodgkin disease in syncytial phase is more aggressive in clinical behavior.

Immunophenotyping

- Reed-Sternberg cells have same immunophenotyping as in other subtypes of classic Hodgkin's disease. Reactive component consists of small lymphocytes bearing phenotype of mantle B cells.
- B cells show positivity for CD30 (Leu M-1), CD15 (Ki-1), CD20, CD79a, CD5, CD3, IgM and IgD.
- Immunophenotyping of nodular sclerosis classic variant of Hodgkin's disease is mentioned as under:

Markers	Expression
CD30 (Leu M-1)	Positive
CD15 (Ki-1)	Positive
CD20	Positive
CD79a	Positive
CD5	Positive
CD3	Positive
IgM	Positive
IgD	Positive

Prognosis

The prognosis of nodular sclerosis classic variant of Hodgkin disease is relatively good. Patients respond to locoregional treatment with radiation and have an excellent prognosis. Cell-mediated immunity is often reduced, as evidenced by anergy (absence of normal immune response to a particular antigen) by skin testing.

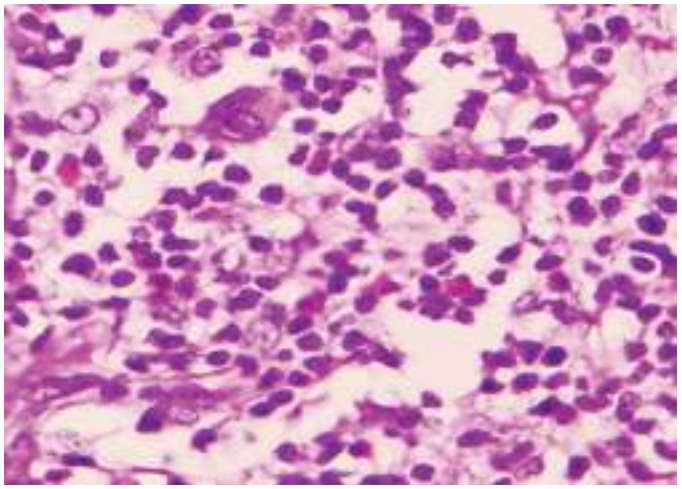


Fig. 13.19: Nodular sclerosis classic variant of Hodgkin's disease (400X).

MIXED CELLULARITY CLASSIC VARIANT OF HODGKIN'S DISEASE

Mixed cellularity classic variant of Hodgkin's disease constitutes 5–25% of all cases, which is most common in India. It is associated with EBV infection in 70% of cases. Although >50% of cases progress to stage III or IV, yet prognosis is still good.

Age and Sex Predilection

Mixed cellularity classic variant of Hodgkin's disease most often affects elderly men and women. It may also affect immunocompromised children.

Clinical Features

Patient presents with multiple painless, rubbery discrete lymph node groups. Spleen, liver and bone marrow may be involved. Reed-Sternberg cells synthesize powerful cytokines such as IL-1, IL-6 and TNF-α, which cause local pain, fever, night sweats and weight loss exceeding 10% of body weight. Pruritus indicates progression of the disease. Mixed cellularity classic variant of Hodgkin's disease—patients have moderately aggressive clinical course but disease is curable.

Surgical Pathology: Mixed Cellularity Classic Variant of Hodgkin's Disease

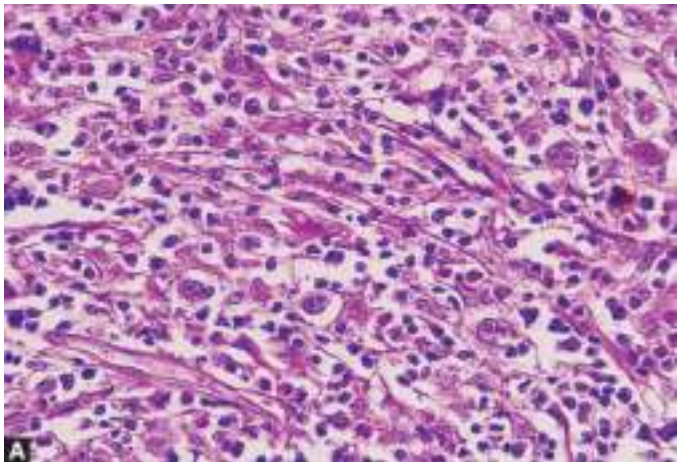
Gross Morphology

- Lymph nodes are enlarged with diffuse involvement.
- Cut surface is fleshy, gray tan without well-defined nodules or fibrosis.

Light Microscopy

- Histologic examination of lymph node in mixed cellularity classic variant of Hodgkin's disease shows classic Reed-Sternberg cells admixed with reactive inflammatory cells such as eosinophils, plasma cells, and small lymphocytes, histiocytes in the background. Cytokine IL-5 synthesized by Reed-Sternberg cells and exotoxin released by fibroblasts participate in chemotaxis of eosinophils.
- Patches of necrosis and fibrosis may be present.
- Presence of noncaseating epithelioid granulomas in Hodgkin's disease indicates good prognosis is shown in Fig. 13.20A and B.
- Classic Reed-Sternberg cells constitute 1–10% of total population of cells, which are large and binucleated or bilobed, with two halves often appearing as mirror images of each other resembling owl eyes. Reed-Sternberg cells measure 20–50 μm in diameter with amphophilic cytoplasm.
- Nuclear membrane is thick and sharply defined. Mononuclear Reed-Sternberg cells are also encountered in mixed cellularity disease, which synthesize IL-5, IL-4, TNF-α and GM-CSF.

Immunophenotyping	
▪ Reed-Sternberg cells have same immunophenotyping as in other subtypes of classic Hodgkin's disease. ▪ Reed-Sternberg cells show positivity for B cell markers such as CD30 (Leu M-1) and CD15 (Ki-1), which are constantly negative for pan-B markers such as CD19, CD20, CD22, 45RA, 45RB (LCA), CD74, CDw75 or CD45. ▪ Immunophenotyping of mixed cellularity classic variant of Hodgkin's disease is shown as under.	
Markers	**Expression**
▪ CD30 (Leu M-1) ▪ CD15 (Ki-1)	▪ Positive (100%) ▪ Positive (75–80%)



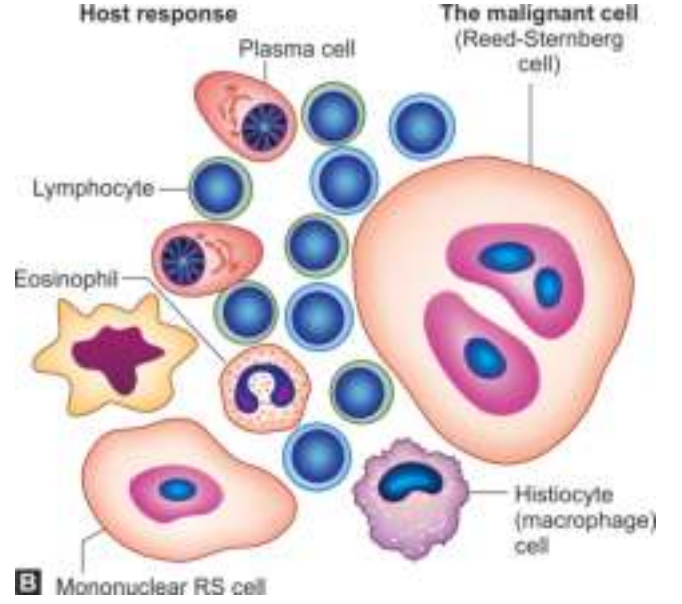


Fig. 13.20: Mixed cellularity classic variant of Hodgkin's disease. (A) Histologic examination of lymph node shows numerous classic Reed-Sternberg (RS) cells admixed with reactive inflammatory cells such as eosinophils, plasma cells, lymphocytes and histiocytes including epithelioid cell granulomas (400X), (B) mononuclear RS cells.

NODULAR LYMPHOCYTIC–HISTIOCYTIC PREDOMINANT VARIANT OF HODGKIN'S DISEASE

Nodular lymphocytic–histiocytic predominant variant is distinct entity from classic Hodgkin's disease. It constitutes 5% of all Hodgkin's disease cases. It most often affects young males involving cervical or axillary adenopathy with long history. Rarely, mediastinal or bone marrow may be involved. It has indolent behavior, but higher recurrence rate than classic Hodgkin's disease.

Clinical Features

Patients with stage I/II have excellent prognosis with 10-year survival of 80–90% cases. Recurrence is frequent and associated with progressive transformation of germinal centers with infiltration by small lymphocytes. Approximately 3–5% transform to diffuse large B cell lymphoma. Bone marrow involvement is associated with aggressive disease.

Surgical Pathology: Nodular Lymphocytic–Histiocytic Predominant Variant of Hodgkin's Disease	
Gross Morphology	
Lymph node architecture is completely effaced with formation of vague nodules of small lymphocytes.	
Light Microscopy	
▪ Nodular lymphocytic–histiocytic-predominant variant of Hodgkin's disease is composed of small B cells with or without accompanying population of benign appearing histiocytes. ▪ Eosinophils, plasma cells and foci of fibrosis are scanty or absent. Endothelium of postcapillary venules may be prominent.	
Immunophenotyping	
Reed-Sternberg cells of nodular lymphocytic–histiocytic variant of Hodgkin's disease show positivity for pan-B markers such as CD19, CD20, CD22, 45RA, 45RB (LCA), CD74, CDw75 and EMA. These RS cells are constantly negative for B cell markers such as CD15 (Ki-1) and CD30 (Leu M-1).	
Markers	**Expression**
▪ CD19 ▪ CD20 ▪ CD22 ▪ 45RA ▪ 45RB (LCA) ▪ CD74 ▪ CDw75 ▪ EMA	▪ Positive ▪ Positive ▪ Positive ▪ Positive ▪ Positive ▪ Positive ▪ Positive ▪ Positive

NON-HODGKIN'S LYMPHOMAS

NON-HODGKIN'S LYMPHOMAS: OVERVIEW

Non-Hodgkin's lymphomas (NHLs) arise from lymphoid cells or other cells native to lymphoid tissue. They originate most frequently within lymph nodes or in other lymphoid areas. Tumor involvement of the para-aortic lymph nodes is frequent. The seriousness of non-Hodgkin's lymphoma depends on which type of cell has undergone mutation, and how quickly it replicates. Revised 2024 WHO classification of non-Hodgkin's lymphoid neoplasms is based on origin from B or T cells: precursor lymphoid neoplasms and mature B cell neoplasms (*refer* to Table 13.17). Origin of non-Hodgkin's lymphoid neoplasms derived from B and T cells is shown in Fig. 13.21A and B.

NON-HODGKIN'S LYMPHOMAS: GRADES

The disease is sometimes described by the behavior of its cells: low-grade, intermediate grade and high-grade non-Hodgkin's lymphoma (Table 13.21).

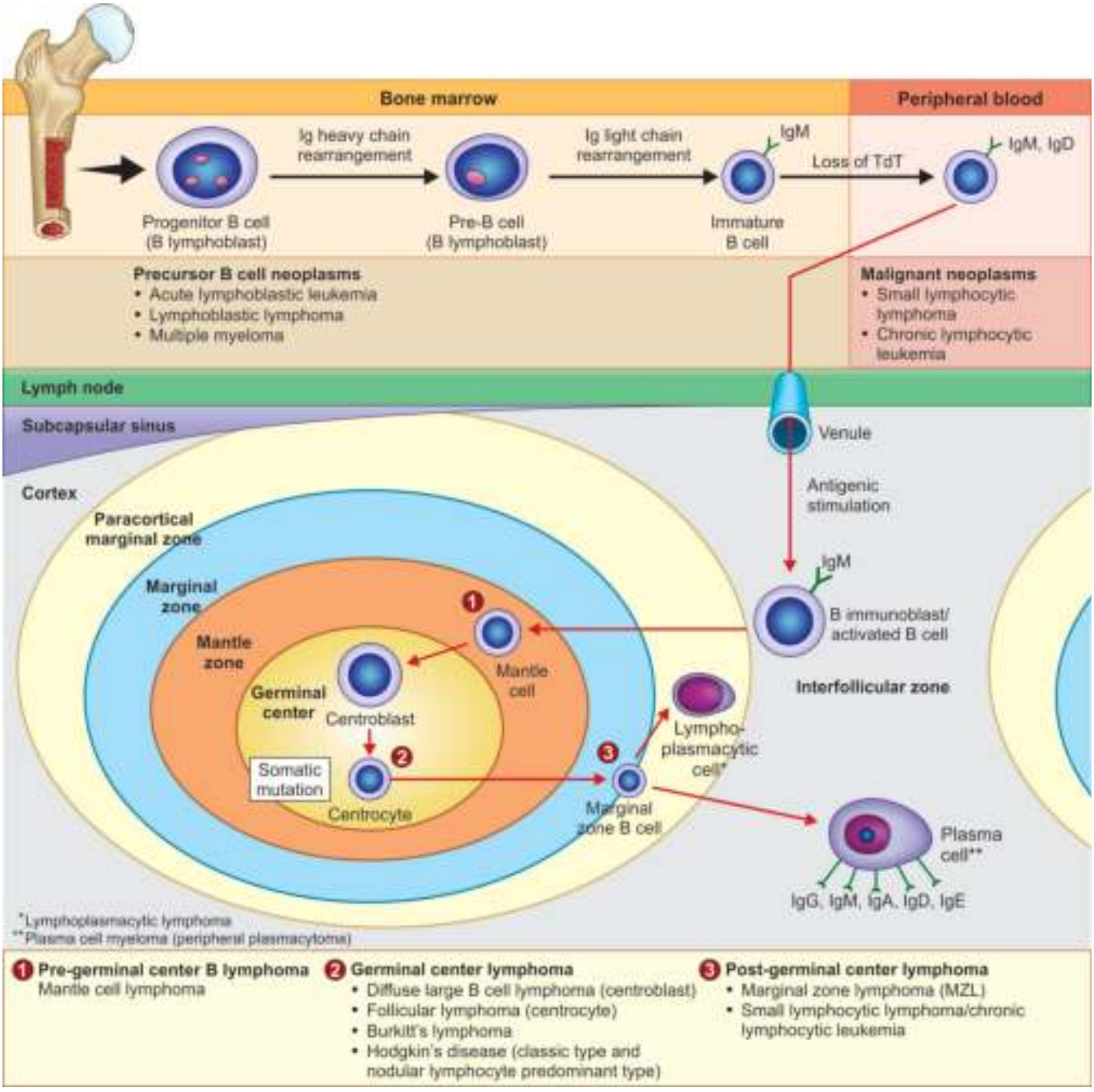


Fig. 13.21A: Origin of B cell derived non-Hodgkin's lymphoid neoplasms.

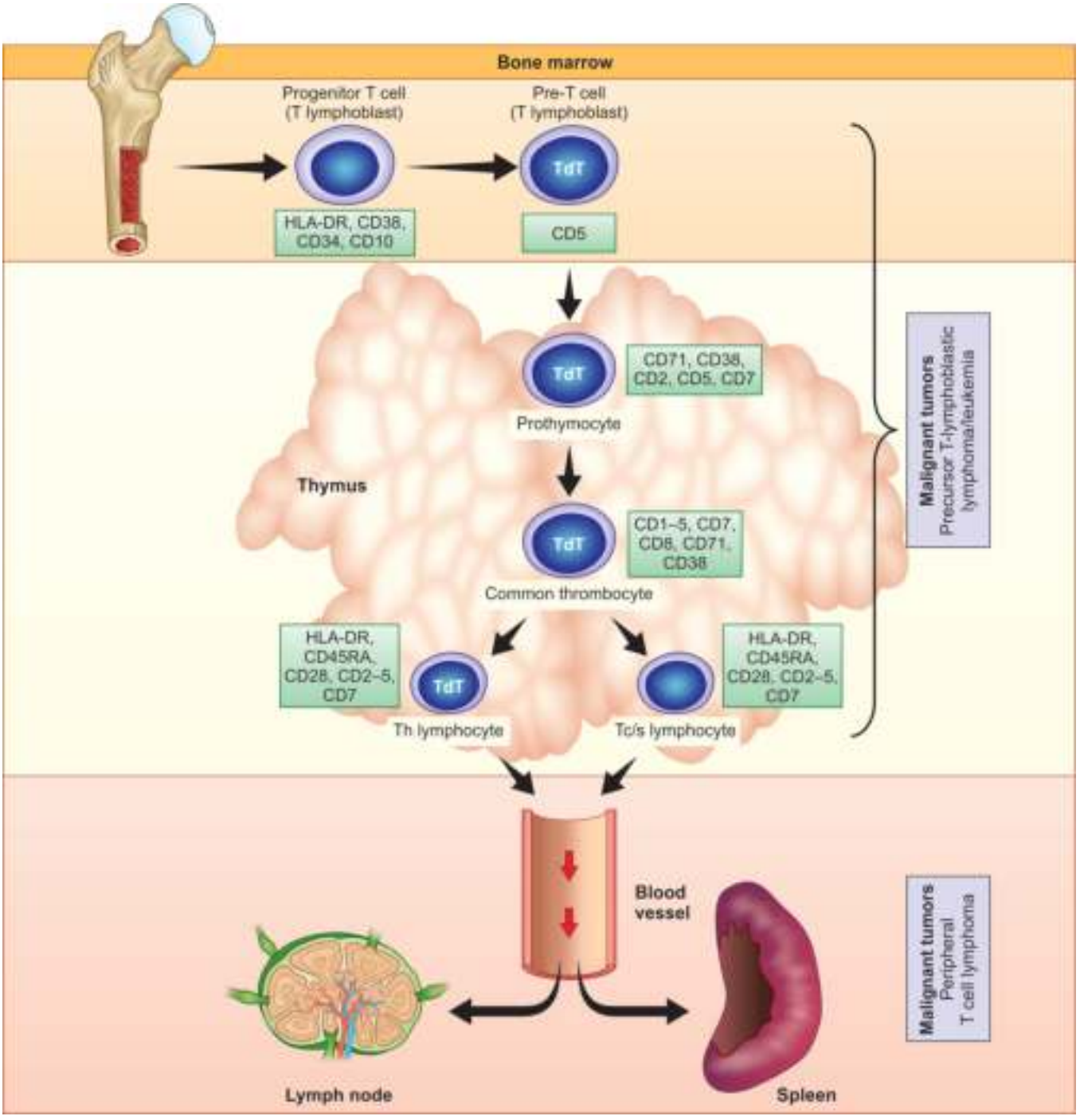


Fig. 13.21B: Origin of T cell-derived non-Hodgkin's lymphoid neoplasms.

- Low-grade NHLs tend to involve multiple lymph nodes at multiple sites, whereas high-grade NHLs tend to be more localized. Low-grade NHLs are slow growing and responsive to treatment.
- Intermediate-grade NHLs are rapidly growing, aggressive but responsive to treatment.
- High-grade NHLs are rapidly growing, aggressive and more localized but may be resistant to treatment.

APPROACH TO NHL WORK UP

Antigen markers useful in delineating and sub-classifying non-Hodgkin's lymphoid malignancies are given in Table 13.22. The work up of NHLs is done by evaluation of disease-specific markers such as cyclin D1 and anaplastic large lymphoma kinase 1 (ALK-1), which can only be evaluated by immunophenotyping on tissue sections.

Table 13.21 Updated Kiel classification of non-Hodgkin's lymphoma (adapted from flow cytometry, immunohistochemistry and molecular genetics of hematologic neoplasms)

Grades of NHL	Histologic Subtypes (B Cell Origin)	Histologic Subtypes (T Cell Origin)	Clinical Course
Low-grade NHL*	**Lymphocytic NHLs** ▪ Chronic lymphocytic leukemia ▪ Prolymphocytic leukemia ▪ Hairy cell leukemia **Lymphoplasmacytic/plasmacytoid (immunocytoma)** ▪ Plasmacytic NHL ▪ Centroblastic-centrocytic (follicular ± diffuse or diffuse) NHL ▪ Mantle cell (centrocytic) NHL ▪ Monocytoid NHL ▪ Marginal zone cell NHL	**Lymphocytic NHLs** ▪ Chronic lymphocytic leukemia ▪ Prolymphocytic leukemia ▪ Small cell NHL ▪ Cerebriform NHL **Mycosis fungoides/Sézary syndrome** ▪ Lymphoepithelioid (Lennet NHL) ▪ Angioimmunoblastic NHL ▪ T-zone NHL ▪ Pleomorphic, small cell (HTLV-1 ±) NHL	▪ Low-grade NHLs are slow growing neoplasms and responsive to therapy. ▪ Low-grade NHL may transform to high-grade NHL
High-grade NHL	▪ Centroblastic NHL ▪ Immunoblastic NHL ▪ Burkitt's lymphoma ▪ Large cell anaplastic NHL (Ki-1 ±)	▪ Pleomorphic, medium sized and large cell NHL (HLTV-1 ±) ▪ Immunoblastic NHL (HTLV-1 ±) ▪ Large cell anaplastic NHL (Ki-1 ±)	High-grade NHLs are rapidly growing neoplasms, which may be resistant to therapy. Intermediate grade NHLs are rapidly growing neoplasms, but responsive to therapy

**Low-grade or indolent NHL may change to a more aggressive form later. Intermediate NHLs are rapidly growing, aggressive but responsive to treatment.*

Table 13.22 Antigen markers used in subclassification of non-Hodgkin's lymphoma

Examples of Markers	Cellular Distribution
Leukocyte marker	
CD45 (known as common leukocyte antigen)	All leukocytes
Markers of immaturity	
TdT (terminal deoxynucleotidyl transferase, a specialized DNA polymerase)	Nuclear expression in pre-B and pre-T lymphoblasts
CD34	Pluripotent hematopoietic stem cells (HSCs) and progenitor cells of many lineages
CD10 (CALLA—common acute lymphoblastic leukemia antigen)	Bone marrow pre-B cells and mature follicular center B cells (ALL, B cell lymphoma)
CD22	Pre-B cells (B cell lymphoma)
Cytoplasmic μ heavy chains	B cells in bone marrow
Primary B cell associated markers	
CD19	Bone marrow pre-B cells, mature B cells (not on plasma cells) (B-ALL, B cell lymphoma, CLL)
CD20	Bone marrow pre-B cells, mature B cells (not on plasma cells) (B-ALL, B cell lymphoma, CLL)
CD79a	Bone marrow pre-B cells, mature B cells and plasma cells (B cell lymphoma)
CD22 (transmembrane molecule)	Bone marrow pre-B cells (cCD22) and mature B cells (B cell lymphoma)
Markers helpful in sub-classifying mature B cell lymphomas	
CD5	T cells, small subsets of B cells, neoplastic CLL and mantle cell lymphoma cells (T-ALL, CLL, T and B cell lymphoma)
CD10	Immature B cells in germinal centers and mature granulocytes (ALL, B cell lymphoma)

Contd...

Table 13.22 Antigen markers used in subclassification of non-Hodgkin's lymphoma (*Contd...*)

Examples of Markers	Cellular Distribution
CD11c	Monocytes/macrophages, natural killer cells, granulocytes, subsets of B and T cells (AML)
CD23	Activated mature B cells (B cell lymphoma, chronic lymphocytic leukemia)
CD38	Mature B cells and plasma cells (multiple myeloma)
CD43	All leukocytes except resting B cells, also demonstrated in B cell lymphoma, T cell lymphoma
BCL-6	Germinal center lymphocytes in normal lymph node
BCL-2	T cells and normal mantle B cells, also expressed in aberrantly B cell lymphomas
Cyclin D1 (cell regulatory protein)	Rearranged through t(11;14) in mantle cell lymphoma, weakly expressed in hairy cell leukemia and plasma cell dyscrasias
CD138 (Syndecan 1)	Multiple myeloma, but also present in carcinomas
Markers of clonality	
κ or λ immunoglobulins	Multiple myeloma
Primarily T cell and NK cell associated markers	
CD1a	Thymocytes and Langerhans' cell histiocytosis (T cell lymphoma, T-ALL)
CD2	T cells in thymus and peripheral blood and NK cells (T-ALL, T cell lymphoma)
CD3	Lineage specific marker for T cells and cytoplasmic form expressed in NK cells
CD5	All T cells and subsets of B cells (T-ALL, B cell lymphoma, CLL)
CD7	All T cells and subsets of myeloid precursor cells (T-ALL, T cell lymphoma)
CD8	Cytotoxic T cells, subset of peripheral T cells, thymocytes and NK cells
CD16	NK cells and granulocytes (NK cell disorders)
CD56	NK cells, subset of T cells and multiple myeloma (NK cell disorders)
TIA is a cytoplasmic granule-associated RNA binding protein	NK cells and cytotoxic T cells
Flow cytometry	
κ or λ immunoglobulins	B cell lymphoid neoplasms, clonality of T cell neoplasms can be demonstrated by molecular or genetic analysis
Southern blot analysis	
Clonal immunoglobulin heavy chain (IgH) or TCR rearrangements	Southern blot targets TCRb detects additional band in B cell and T cell malignancies (normal T cells rearrange TCR genes such as α, β, γ and δ)
PCR (polymerase chain reaction)	
TCRg gene	T cell lymphoid neoplasms
Cytogenetic analysis	
Conventional karyotyping	Acute leukemias
FISH (fluorescence *in situ* hybridization)	B cell, T cell and NK cell lymphoid neoplasms

IMMUNOPHENOTYPING

Immunophenotying in NHLs plays important role in those cases, who lack diagnostic cytologic and histomorphologic features.

- Immunophenotying can distinguish a benign lesion from malignant one; and thus achieve definite diagnosis. It plays important role in differential diagnosis, subclassification and prediction of prognosis in various non-Hodgkin's lymphomas, e.g. low-grade lymphomas such as small lymphocytic lymphoma, chronic lymphocytic leukemia, mantle cell lymphoma, follicular lymphoma, hairy cell leukemia and various types of marginal zone B cell lymphoma.

- Availability of **CD23** monoclonal antibody facilitates the distinction between small lymphocytic lymphoma and mantle cell lymphoma.
  - Mantle cell lymphoma and chronic lymphocytic leukemia/small lymphocytic lymphoma (SLL/CLL) share many histomorphologic and immunophenotyping features.
  - Typically, SLL/CLL is CD23+, whereas mantle cell lymphoma is CD23–.
- Prognosis of certain non-Hodgkin's lymphomas is analyzed by assessing quantitative proliferation-associated antigens, such as Ki-67 and proliferating cell nuclear antigen (PCNA). Presence of high percentages of activated antigens, e.g. CD25, CD38, CD71 and human leukocyte antigen (HLA) has poor prognosis.
- Diagnostic criteria of hematolymphoid neoplasms by flow cytometry include restrictive of Ig light chain, loss of surface Ig in B cells, coexistence of two different cell lineage markers on the same cell population, expression of numerous cell markers in large number of cells, selective loss of one or more cell lineage antigens and analysis of T cell receptor Vb repertoire.

PRECURSOR B CELL AND T CELL NHLs

Precursor B and T cell leukemias/lymphomas include precursor B cell lymphoblastic leukemia/lymphoblastic lymphoma, precursor T cell lymphoblastic leukemia/lymphoblastic lymphoma; and myeloid and lymphoid neoplasms with FGFR1 abnormalities.

PRECURSOR B CELL LYMPHOBLASTIC LEUKEMIA/LYMPHOMA

Precursor B cell lymphoblastic leukemia and B cell lymphoblastic lymphoma are essentially one disease process. Patient with involvement of blood and bone marrow is designated precursor B cell lymphoblastic leukemia, if the bone marrow contains >25% lymphoblasts. On the other hand, patient with nodal or extranodal disease especially bone marrow is designated as precursor B cell lymphoblastic lymphoma. Disease predominantly affects children below the age of six years and occasional adult patient. Common sites of involvement are skin, bone and lymph nodes. Central nervous system and testes are common sites of relapse.

Clinical Features

Patient presents with multiple skin nodules, bone mass or soft tissue mass and lymphadenopathy. Recurrent infections, anemia, bleeding are related to pancytopenia.

Molecular Genetic Alterations

- Majority of cases of precursor B cell lymphoblastic leukemia/lymphoma demonstrate clonal, but not mutated, rearrangements of the immunoglobulin genes.
- About 66% of cases demonstrate clonal immunoglobulin heavy chain (IgH) rearrangements; and remaining cases show clonally rearranged IgH and light chain genes.
- About 50% of cases demonstrate t(12;21) and hyperploidy, usually with trisomy of chromosomes 4 and 8. These patients have a favorable prognosis. Philadelphia chromosomal abnormality is demonstrated in a small number of cases.

Surgical Pathology: Precursor B Cell Lymphoblastic Leukemia/Lymphoma

Light Microscopy

- Morphologically percursor B cell lymphoblastic leukemia/lymphoma and percursor T cell lymphoblastic leukemia/lymphoma are indistinguishable.
- In precursor B cell lymphoblastic leukemia/lymphoma, lymph node involved is composed of medium-sized blast cells with round or convoluted nuclei and small or inconspicuous nucleoli. Neoplastic cells may either uniform or varying degrees of anisocytosis. Mitotic figures are usually demonstrated.
- Tumor cells infiltrate lymph nodes in the manner of a leukemia, leaving reticulin architecture partially intact. Residual reactive germinal centers may be present.
- Tumor cells infiltrate in the capsule and around blood vessels often show 'Indian file pattern'.

Immunophenotyping

Most reliable B cell markers expressed on precursor B cell lymphoblastic lymphoma are CD19, cytoplasmic CD22, and cytoplasmic CD79a. It also shows positivity for CD10, CD34, PAX2, CD43 and TdT.

Markers	Expression
CD19	Positive
CD22	Positive
CD79a	Positive
CD10	Positive
CD34	Positive (50%)
PAX2	Positive (sensitive marker)
CD43	Positive (sometimes)
TdT	Positive (nuclear staining)
CD45	Negative
CD20	Negative
CD3	Negative

Table 13.23 Distinction of precursor B cell lymphoblastic leukemia/lymphoma from precursor T cell lymphoblastic leukemia/lymphoma

Features	Precursor B Cell Lymphoblastic Leukemia/Lymphoma	Precursor T Cell Lymphoblastic Leukemia/Lymphoma
Frequency	More common	Less common
Age group	Children below 6 years	Adolescents
Organs involved	▪ Skin, bone and lymph nodes ▪ Central nervous system and testes are commonly involved in the course of disease during relapse	▪ Anterior mediastinum, lymph nodes liver, spleen and skin ▪ Central nervous system and testes are commonly involved in the course of disease during relapse
Clinical features	▪ Multiple skin nodules, bone or soft tissue tumors and lymphadenopathy ▪ Recurrent infections, anemia, bleeding related to pancytopenia	Symptoms related to mediastinal obstruction and pleural effusion, involvement of lymph nodes, liver, spleen and skin. Central nervous system and testes are commonly involved in the course of disease during relapse. Patient with involvement of blood and bone marrow is designated T cell lymphoblastic leukemia, if the bone marrow contains more than 25% lymphoblasts
Molecular genetics	▪ Rearrangements of the immunoglobulin genes involving heavy and light chains ▪ Chromosomal translocation t(12;21) and hyperploidy, usually with trisomy of chromosomes 4 and 8 in 50% of cases with favorable prognosis	▪ Clonal rearrangement of one or more of the T cell receptor gene ▪ Loss of chromosome 9p results in loss of tumor suppressor gene CDCK2A. About 25% of cases show dysregulation of hematopoiesis due to involvement of TAL-1 gene
Light microscopy	▪ Morphologically B cell lymphoblastic lymphoma and T cell lymphoblastic lymphoma are indistinguishable ▪ Lymph node involved is composed of medium-sized blast cells with round or convoluted nuclei and small or inconspicuous nucleoli. Neoplastic cells are either uniform or varying degrees of anisocytosis. Mitotic figures are usually demonstrated	▪ Morphologically B cell lymphoblastic lymphoma and T cell lymphoblastic lymphoma are indistinguishable ▪ Lymph node involved is composed of medium-sized blast cells with round or convoluted nuclei and small or inconspicuous nucleoli. Neoplastic cells are either uniform or varying degrees of anisocytosis. Mitotic figures are usually demonstrated
Immunophenotyping	Positivity for CD19, CD22, CD79a, CD10, CD34, PAX2, CD43, TdT	Positive for CD3 and CD99 (most reliable), CD1a, CD2, CD4, CD5, CD7, CD8, CD34

PRECURSOR T CELL LYMPHOBLASTIC LEUKEMIA/ LYMPHOMA

Precursor T cell lymphoblastic leukemia/lymphoma is less common than precursor B cell lymphoblastic leukemia/lymphoma. Majority of patients of precursor T cell lymphoblastic leukemia/lymphoma present with solid tumor without evidence of leukemia. It occurs in children and young adults with male predominance. Distinction of precursor B cell lymphoblastic leukemia/lymphoma from precursor T cell lymphoblastic leukemia/lymphoma is given in Table 13.23.

Clinical Features

Precursor T cell lymphoblastic leukemia/lymphoma involves anterior mediastinum resulting in mediastinal obstruction and pleural effusion. It may also involve lymph nodes, liver, spleen and skin. Central nervous system and testes are commonly involved in the course of disease during relapse. Patient with involvement of blood and bone marrow is designated precursor T cell lymphoblastic leukemia, if the bone marrow contains >25% lymphoblasts.

Surgical Pathology: Precursor T Cell Lymphoblastic Leukemia/Lymphoma

Light Microscopy

- Morphologically precursor B cell lymphoblastic leukemia/lymphoma and T cell lymphoblastic leukemia/lymphoma are indistinguishable.
- In precursor T cell lymphoblastic leukemia/lymphoma, lymph node involved is composed of medium-sized lymphoblasts with round or convoluted nuclei and small or inconspicuous nucleoli. Lymphoblasts are either uniform or having variable sizes. Mitotic figures are usually demonstrated.
- Lymphoblasts infiltrate lymph nodes in the manner of leukemia, leaving reticulin architecture partially intact. Residual reactive germinal centers may be present.
- Lymphoblasts infiltrate in the capsule and around blood vessels often show 'Indian file pattern'.

Immunophenotyping

Precursor T cell lymphoblastic leukemia/lymphoma shows variable expression of CD1a, CD2, CD4, CD5, CD7, CD8 and CD34. CD3 and CD99 are the most reliable lineage markers, which indicate the precursor nature of the tumor cells.

Markers	Expression
▪ CD1a	▪ Positive
▪ CD2	▪ Positive
▪ CD4	▪ Positive
▪ CD5	▪ Positive
▪ CD7	▪ Positive
▪ CD8	▪ Positive
▪ CD34	▪ Positive
▪ CD3 and CD99	▪ Positive (most reliable marker)

Molecular Genetic Alterations

Precursor T cell lymphoblastic leukemia/lymphoma most often demonstrate clonal rearrangement of one or more of the T cell receptor gene. About 33% of cases demonstrate translocation between the TCR genes and a number of the oncogenes. Loss of chromosome 9p results in loss of tumor suppressor gene CDCK2A. About 25% of cases show dysregulation of hematopoiesis due to involvement of TAL-1 gene.

MYELOID AND LYMPHOID NEOPLASMS WITH FGFR1 ABNORMALITIES

Myeloid and lymphoid neoplasms with FGFR1 abnormalities are pluripotent hematopoietic stem cell neoplasms and also known as 8p11 myeloproliferative syndrome. Patient presents initially with T cell lymphoblastic lymphoma of lymph node and most often accompanied by eosinophilia and myeloproliferative neoplasm. Involved lymph node shows nemerous eosinophils. Disorder may affect 3–85 years of age and is characterized by translocation or insertion of fibroblast growth factor receptor 1 (FGFR1) gene at chromosome 8p11.

MATURE B CELL NON-HODGKIN'S LYMPHOMA

Mature B cells are characterized by the synthesis, expression and sometimes secretion of immunoglobulin molecules.

- Diversity of immunoglobulin molecules is achieved by rearrangement of immunoglobulin genes by somatic mutations.
- Analysis of the immunoglobulin genes provides a valuable means for the detection and subdivision of mature B cell lymphomas.
- Classification of B cell lymphoid neoplasms based on clinical presentation according to 2024 World Health Organization is given in Table 13.24. Lymphoid neoplasms derived from B cell and T cell are given in Table 13.25. Chromosomal abnormalities in B cell derived non-Hodgkin's lymphoma (NHL) are given in Table 13.26. Characteristic features of various B cell leukemias/lymphomas are given in Table 13.27.

Table 13.24 Classification of B cell lymphoid neoplasms based on clinical presentation according to revised 2024 WHO criteria. Adapted from flow cytometry, immunohistochemistry, and molecular genetics for hematologic neoplasms

Categories	Organs Involved	Histologic Type of B Cell Lymphoid Neoplasms
Predominantly disseminated lymphoma/leukemia	▪ Bone marrow with or without peripheral blood ▪ Lymph nodes ▪ Spleen	▪ Chronic lymphocytic leukemia ▪ Hairy cell leukemia ▪ Splenic marginal zone lymphoma ▪ Plasma cell myeloma
Primary extranodal lymphomas	▪ Stomach ▪ Eye and ocular adnexa ▪ Skin ▪ Lung ▪ Salivary glands ▪ Breasts ▪ Thyroid gland	▪ Extranodal marginal zone B cell lymphoma of mucosa-associated lymphoid tissue (MALT)
Predominantly nodal lymphomas	▪ Lymph nodes ▪ Bone marrow ▪ Liver ▪ Spleen ▪ Peripheral blood	▪ Follicular lymphoma ▪ Mantle cell lymphoma ▪ Nodal marginal zone B cell lymphoma

Table 13.25 Lymphoid neoplasms of precursor B and T cells

Disorder	Origin	Age Group	Clinical Features	Genotype	Immunophenotyping
B cell acute lymphoblastic leukemia or lymphoma	Precursor B cells in bone marrow	Children	Recurrent infections, anemia and bleeding related to pancytopenia	Diverse chromosomal translocation t(12;21) involving RUNX1 and ET16	CD19, CD79a, CD22, CD10, CD22, CD24 and PAX5
T cell acute lymphoblastic leukemia or lymphoma	Precursor T cells in thymus	Adolescents	Mass in thymus, variable bone marrow involvement with aggressive clinical course	Diverse chromosomal translocation, NOTCH mutations in 50–70% of cases	TdT, CD1a, CD2, CD3 (specific), CD4, CD5 and CD8

Table 13.26 Chromosomal abnormalities in B cell derived non-Hodgkin's lymphomas

Histologic Variant of Lymphoma	Cytogenetic Abnormality	Oncogene	Juxtaposed Gene
Burkitt's lymphoma* (tropical and nontropical)	▪ t(8;14) ▪ t(8;22) ▪ t(2;8)	▪ c-Myc ▪ c-Myc ▪ c-Myc	▪ Ig heavy chain ▪ Ig κ chains ▪ Ig λ chains
Centroblastic/centrocytic lymphoma	t(14;18)	BCL-2	Ig heavy chain
Centrocytic (intermediate cell) mainly lymphoma in mantle zone	t(11;14)	BCL-2 (PRAD 1)	Ig heavy chain
Small lymphocytic B cell lymphoma	Trisomy 12	–	–
B cell chronic lymphocytic leukemia	t(14;19)	BCL-3	Ig heavy chain
Centroblastic/centrocytic large cell type lymphoma	t(3;22)	–	Ig λ chains

**In Burkitt's lymphoma, t(8q24) is always involved.*

Table 13.27 Characteristic features of various B cell leukemias/lymphomas

Disease	Origin	Clinical Presentation	Morphology	Immunophenotyping	Genetics
Chronic lymphocytic leukemia/small lymphocytic leukemia	Naïve B cell or memory B cells	Weakness, autoimmune hemolytic anemia, enlargement of lymph nodes, liver and spleen	Small mature appearing lymphocyte cells involving bone marrow	Bright staining (CD5, CD19), weak staining (CD20, CD22, CD23, CD19, IgD, CD79a, CD43)	▪ Favorable prognosis t(13q14) ▪ Poor prognosis (deletion of 17p and TP53), trisomy 12, deletions (11q22–23) and rearrangement of IgH (50–60%)
Prolymphocytic leukemia	B cells in marginal zone of lymph node	Marked splenomegaly without lymphadenopathy, rapidly rising lymphocyte count (>100 × 10^9/L)	**Peripheral blood smear:** Medium-sized cells with round nucleus, moderately condensed chromatin, and prominent central nucleolus	CD20 (bright), CD19, IgM, CD22, CD79a, FMC7, CD23 typically absent, CD5 present in 30% of cases	Complex karyotypes are common, abnormalities of TP53 in ~50%, IgH clonally rearranged
Hairy cell leukemia	Memory B cell	Predominantly middle-aged men, splenomegaly, pancytopenia with monocytopenia	Hairy cells in peripheral blood, bone marrow virtually always involved, and spleen infiltration (red pulp)	CD19, CD20, CD22 (bright), CD79a, CD11c (bright), CD25 (bright), CD103, TRAP, DBA in tissue sections	Activating FRAF mutations, rearrangement of IgH
Splenic marginal zone B cell lymphoma	B cell in spleen	Splenomegaly, sometime associated with autoimmune thrombocytopenia or anemia, peripheral lymphadenopathy uncommon	**Peripheral blood smear:** Small- to medium-sized cells with polar villi (villous lymphocytes) **Spleen:** Both white pulp and red pulp infiltration	CD19, CD20, CD79a, IgM and IgD, no expression of CD10, CD5, CD23, and CD43	Allelic loss of chromosome 7q21–32 in 40% of cases, trisomy 3 in rare cases, IgH clonally rearranged
Extranodal marginal zone B cell lymphoma (MALT lymphoma)	Naïve B cell	GI tract most common site of involvement	Small lymphocytes with ample cytoplasm	CD19, CD20, CD79a, IgM, no expression of CD5, CD23, CD10	t(11;18), t(14;18), t(14;18) creating API2-MALT1, BCL10-IgH fusion genes, respectively

Contd...

Table 13.27 Characteristic features of various B cell leukemias/lymphomas (*Contd...*)

Disease	Origin	Clinical Presentation	Morphology	Immunophenotyping	Genetics
Nodal marginal zone B cell lymphoma	B cell	Localized or generalized lymphadenopathy	Marginal zone and interfollicular areas infiltrated by centrocyte-like B cells, monocytoid B cells, or small lymphocytes; plasma cell differentiation may be present	CD19, CD20, CD79a, IgM (no expression of CD5, CD23, CD10)	The translocations associated with extranodal MZL are not detected; IgH clonally rearranged
Follicular lymphoma	Germinal center B cell	Widespread peripheral and central lymphadenopathy, infiltration in liver (portal tract), spleen (white pulp) and bone marrow (85%)	Neoplastic cells with variable morphology forming closely packed neoplastic follicles	CD19, CD20, CD22, CD79a, BCL-6, BCL-2, CD10	t(14;18) (q23;q21) → rearrangement of BCL-2 gene leading to overexpression of the BCL-2 protein and survival advantage of malignant cells; IgH clonally rearranged
Mantle cell lymphoma	Naïve B cell	Lymphadenopathy, most common extranodal site is the GI tract (multiple lymphomatous polyposis)	Small neoplastic lymphocytes with nuclei showing clefts	CD19, CD20, CD5, FMC-7, CD43, cyclin-D1 (lack of expression of CD23, CD10, BCL-6)	t(11;14) creating cyclin D1-IgH fusion gene
Diffuse large B cell lymphoma (DLBCL)	Germinal center or post-germinal center B cell	Rapidly enlarging, often symptomatic mass, often disseminated disease	Variable morphology (centroblastic, immunoblastic, anaplastic cells with round to oval nucleus, prominent nucleoli and frequent mitosis	CD19, CD20, CD22, CD79a, CD10, BCL-6	Diverse chromosomal rearrangement, BCL-6 (30%), BCL-2 (10%) and Myc (5%)
Mediastinal (thymic) large B cell lymphoma	B cell in thymus	Mostly women in their third to fifth decade, large anterior mediastinal mass, sometimes with impending superior vena cava syndrome	Large cells with associated delicate interstitial fibrosis causing compartmentalization, possible thymic remnants, biopsy samples often small and obscured by profuse sclerosis and crush artifact	CD19, CD20, CD30 (weak), IgH, and HLA class I and II expression is often absent	IgH clonally rearranged; hyperdiploid karyotype gains in chromosome 9p
Burkitt's lymphoma	Germinal center B cell	African variant (EB virus-related tumor in maxilla, mandible), Western variant (abdominal lymph nodes around ileocecal region in children), and lymphoma in HIV patients	Medium-sized cells with basophilic vacuolated cytoplasm and regular nuclei with several small nucleoli, tangible-body macrophages imparting "**starry-sky**" appearance	CD19, CD20, CD22, CD38, CD10, CD79a, BCL-6, Ki-67 index (100%)	IgH clonally rearranged; t(8;14) in most cases, t(2;8) and t(8;22) rare

- Mature B cell non-Hodgkin's lymphomas may be categorized into those with low growth fraction (e.g. B-CLL/SLL, lymphoplasmacytic lymphoma, mantle cell lymphoma, marginal zone lymphoma and follicular lymphoma) and high-grade fraction (e.g. diffuse large B cell lymphoma and Burkitt's lymphoma). A proportion of low-fraction mature B cell non-Hodgkin's lymphomas may transform into high-fraction mature B cell non-Hodgkin's lymphoma. Low-fraction mature B cell

non-Hodgkin's lymphomas demonstrate alterations in genes controlling apoptosis. On the other hand, high-fraction mature B cell non-Hodgkin's lymphomas show abnormalities of genes involving proliferation control.

- Immunophenotyping plays important role in analysis of lineage markers for the identification of B cells (e.g. CD20, CD79a) on paraffin-embedded tissues. CD79a is the most reliable marker, expressed on B cells from pre-B cell stage of maturation through to plasma cells. CD5 is expressed on B-CLL/SLL and primary mediastinal large B cell lymphoma. Transcription factor BCL-6 is expressed in the nuclei of follicle center cell-derived lymphomas.

BURKITT'S LYMPHOMA

Burkitt's lymphoma is a highly aggressive neoplasm derived from germinal B cell. It most often involves maxilla, mandible and abdominal organs. Patient responds to aggressive chemotherapy. Upon initiation of chemotherapy, patient may develop 'tumor lysis syndrome' due to rapid tumor cell death.

- Burkitt's lymphoma is divided into three subtypes: (a) African endemic Burkitt's lymphoma, (b) Western sporadic Burkitt's lymphoma, and (c) immunodeficiency-associated Burkitt's lymphoma, with identical cytomorphologic and histopathologic features but different clinical and gross anatomical characteristics. Burkitt's lymphoma is composed of monomorphic small to medium blast cells with basophilic cytoplasm containing lipid droplets/vacuoles, numerous apoptotic bodies, starry-sky macrophages and proliferative index 100%.
- The characteristic c-Myc gene translocation t(8,14) in Burkitt's lymphoma to Ig locus (usually IgH on chromosome 14) and BCL rearrangements are commonly demonstrated. Combination of morphology, genetic analysis or immunophenotyping can be used as the gold standard for diagnosis.

Clinical Features

Patient most often presents with bulky disease and high tumor burden due to the short doubling time of the neoplasm. Upon initiation of chemotherapy, patient may develop tumor lysis syndrome due to rapid tumor cell death. In the course of disease, few patients may present with Burkitt's cell leukemia with involvement of peripheral blood, bone marrow and central nervous system.

Clinical Pearls: Burkitt's Lymphoma: Variants

African Endemic Burkitt's Lymphoma

- African endemic Burkitt's lymphoma affects children with median age seven years with male predominance. It is strongly associated with Epstein-Barr virus in Central African children.
- Patient presents with bulky disease and high tumor burden due to the short doubling time of Burkitt's lymphoma in the maxilla or mandible. Gastrointestinal tract, kidneys, liver, pancreas, retroperitoneum, gonads, breast, endocrine glands and brain may be involved. Leukemic manifestations are rare. Burkitt's cell leukemia most often involves central nervous system in the early course of disease.
- Epstein-Barr virus activates oncogene (c-Myc). It stimulates proliferation of B cells. It increases opportunity for translocation t(8;14) of N-RAS gene.

Western Sporadic Burkitt's Lymphoma

- Western sporadic Burkitt's lymphoma affects children and young adults with male predominance. It is not strongly associated with Epstein-Barr virus.
- Sites of involvement are ileocecal region, omentum, gonads, kidneys, thyroid gland, salivary glands, female breasts, Waldeyer's ring and peripheral lymph nodes. Leukemic manifestations are uncommon.
- Patient presents with intra-abdominal tumor in ileocecal region.

Immunodeficiency-associated Burkitt's Lymphoma

- Immunodeficiency-associated Burkitt's lymphoma mainly occurs in the early stages of HIV-infected persons before CD4+ helper T cells count fall.
- Patient may present with nodal or extranodal disease. Lymph nodes are frequently involved in immunodeficiency associated Burkitt's lymphoma.
- Adjacent organs and tissues may be compressed and/or infiltrated by tumor cells.

Molecular Genetic Alterations

Burkitt's lymphoma is associated with t(8;14) in 75% of cases, t(2;8) in 5% of cases and t(8;22) in 10% of cases. Epstein-Barr virus promotes polyclonal B cell proliferation, which increases risk translocation (8;14). Epstein-Barr virus activates c-Myc proto-oncogene located on chromosome 8, which is transposed to the site adjacent to the immunoglobulin heavy chain (IgH) locus on chromosome 14. Pathogenesis of Burkitt's lymphoma is shown in Fig. 13.22.

- Proximity of regulatory sequences of the immunoglobulin heavy chain gene results in increased expression of the c-Myc gene. This chromosomal rearrangement is the basis of malignant transformation of lymphocyte in Burkitt's lymphoma.

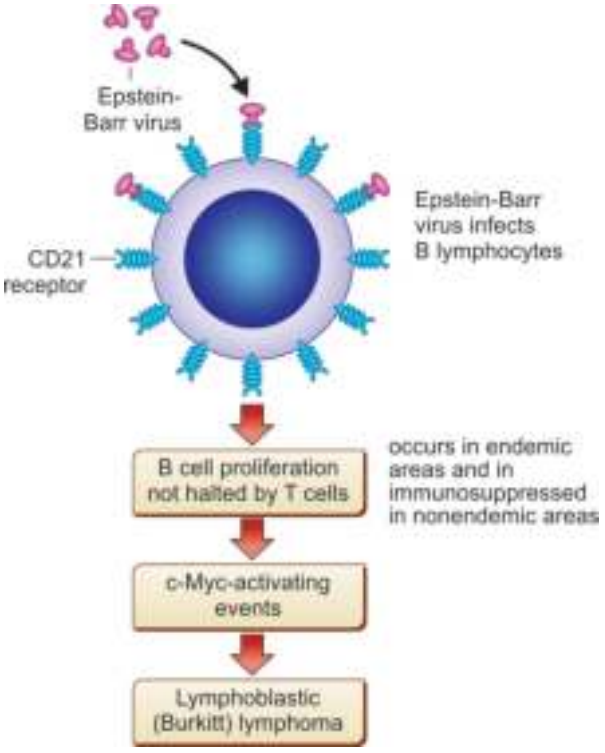


Fig. 13.22: Pathogenesis of Burkitt's lymphoma.

- In endemic areas, chromosomal translocation t(8;14) occurs in the pre-B cell, but in nonendemic areas at later stage of B cell development. Burkitt lymphoma associated with t(8;14) is shown in Fig. 13.23.

Surgical Pathology: Burkitt's Lymphoma

Gross Morphology

- Organs involved in Burkitt's lymphoma are replaced by masses with a fish-flesh appearance.
- Cut surface of tumor mass usually shows areas of hemorrhage and necrosis.
- Adjacent organs or tissues are compressed and/or infiltrated by tumor mass.
- Lymph node involvement is rare in endemic and sporadic variants of Burkitt's lymphoma.
- Lymph nodes are most frequently involved in immunodeficiency-associated Burkitt's lymphoma.
- Uninvolved lymph nodes are surrounded by tumor mass.

Light Microscopy

- Lymph node in Burkitt's lymphoma is composed of medium-sized tumor cells showing a diffuse monotonous pattern of growth.
- Tumor cells appear to be cohesive but often show squarred-off borders of retracted cytoplasm in formalin embedded tissue sections. Nuclei of tumor cells are round with finely clumped chromatin containing multiple basophilic and usually containing lipid vacuoles, better demonstrated in imprint preparations or fine needle aspiration cytology.
- Tumor has extremely high-proliferative rate with many mitotic figures. High rate of spontaneous tumor cell death by apoptosis. Cellular debris of tumor cells is cleared by numerous non-neoplastic macrophages, whose scattered appearance termed "Starry-sky appearance".
- It is worth mentioning the "Starry-sky appearance" can be seen in any lymphoid tumor with high proliferative rate (Fig. 13.24).

Immunophenotyping

- Tumor cells in Burkitt's lymphoma express moderate to strong membrane IgM with light chain restriction, B cell antigens such as CD19, CD22, CD79a and PAX5 and germinal centre markers (e.g. CD10 and BCL-6). CD43 and CD77 are also frequently positive.
- Most cases of Burkitt's lymphoma have strong expression of Myc protein.
- Proliferate rate of tumor is very high showing strong positivity for Ki-67 in all cases. Immunophenotyping of Burkitt's lymphoma is mentioned below.

Markers	Expression
Surface membrane IgM with light chain restriction	Positive
CD19 (B cell antigen)	Positive
CD22 (B cell antigen)	Positive
CD79a (B cell antigen)	Positive
PAX5 (B cell antigen)	Positive
CD10 (germinal center marker)	Positive
BCL-6 (germinal center marker)	Positive
CD10	Positive
CD77	Positive
Myc protein	Positive
Ki-67	Positive

Prognosis

Burkitt's lymphoma is highly-aggressive but potentially curable. Even patients with advanced stage disease with bone marrow and central nervous system involvement can be cured with high intensity chemotherapy.

DIFFUSE LARGE B CELL LYMPHOMA

Diffuse large B cell lymphoma (DLBCL) is a high-grade neoplasm with diffuse growth pattern derived from germinal center or post-germinal center B cell.

- Tumor cells are more than twice the size of small lymphocytes. BCL-2 gene rearrangements are often demonstrated, suggesting a potential germinal center origin.
- Disease may arise in *de novo*, or may represent transformation of pre-existing low-grade B cell lymphoma, and occur at all ages; with median age in seventh decade.

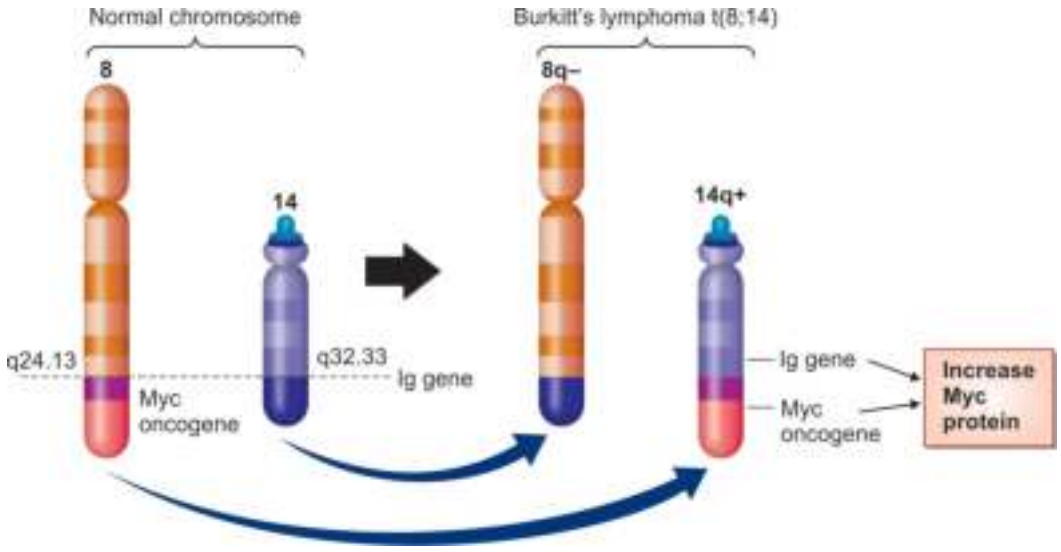


Fig. 13.23: Burkitt's lymphoma showing t(8;14) in which c-Myc proto-oncogene located on chromosome 8 is transposed to a site adjacent to the immunoglobulin heavy chain locus on chromosome 14.

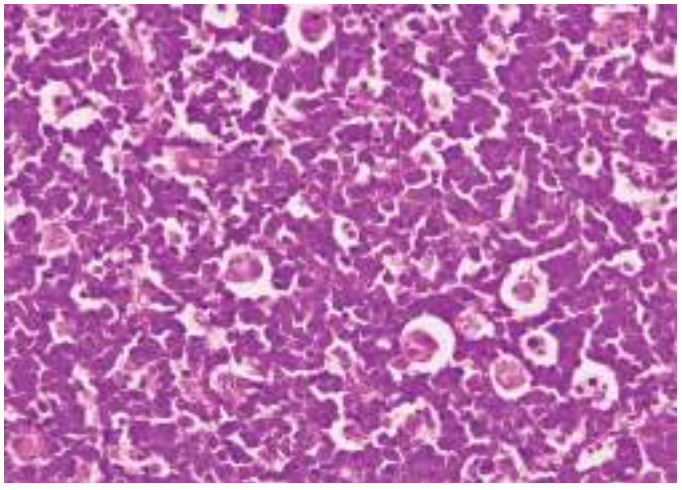


Fig. 13.24: Burkitt's lymphoma. Tumor is composed of medium-sized cells with round nuclei with fine clumped chromating and cellular debris showing starry-sky appearance (400X).

- Involvement of lymph nodes occurs in 60% of cases and extranodal sites occur in 40% of cases. Diffuse large B cell lymphoma has three histologic variants, i.e. centroblastic, immunoblastic and anaplastic subtypes. Three histologic variants of diffuse large B cell lymphoma (DLBCL) are given in Table 13.28.

Age Group Predilection

Diffuse large B cell lymphoma, not otherwise specified constitutes 25–35% of adult non-Hodgkin's lymphoma. It most often affects elderly persons in seventh decade of life. However, it may also occur in children and young adults.

Pathogenesis

Pathogenesis of DLBCL, not otherwise specified remains unknown, which usually arises *de novo* referred to as primary, but can also represent transformation of a less aggressive non-Hodgkin's lymphoma referrd to as secondary DLBCL.

- Diffuse large B cell lymphoma (DLBCL) may arise by transforming low-grade B cell large cell lymphoma (Richter syndrome).

Table 13.28 Three histologic variants of diffuse large B cell lymphoma (DLBCL)

Parameters	Centroblastic Variant of DLBCL	Immunoblastic Variant of DLBCL	Anaplastic Variant of DLBCL
Architecture	Diffuse	Diffuse	Diffuse in some cases
Sinus involvement	Obliteration	Obliteration	Distension
Cell population	▪ Centroblasts (10–100%) admixed with centrocytes ▪ Cytoplasm scanty ▪ Nucleus vesicular with several nucleoli near nuclear membrane	▪ Centroblasts (10%) admixed with plasmablasts with plasmacytoid features ▪ Cytoplasm moderate to abundant with deep staining ▪ Nucleus solitary centrally placed with prominent nucleus	▪ Anaplastic cells ▪ Cytoplasm to abundant with deep staining ▪ Nucleus large with prominent nucleus
Mitotic activity	Low-proliferative index	High-proliferative index	High-proliferative index

- Richter syndrome is characterized by rapid onset of fever, abdominal pain, and progressive lymphadenopathy and hepatosplenomegaly. Richter syndrome is aggressive and refractory to therapy, with a mean survival of 2 months.
- Follicular lymphoma, marginal zone lymphoma or nodular lymphocyte predominant Hodgkin's lymphoma (NLPHL) can transform to DLBCL.

Molecular Genetic Alterations

Diverse chromosomal aberrations are seen in 30% of cases of DLBCL. These tumors show activation of BCL-2 gene, amplification of cREL, t(14;18), point mutations and DNA breakage of BCL-6 regulatory regions.

Localization of DLBCL Disease

Patients may present with lymph nodal disease in 60% of cases. In 40% of cases, disease is confined to extranodal sites such as gastric region and ileocaecal region, bone, spleen, testes, liver, Waldeyer's ring, thyroid gland, salivary glands, kidneys and adrenal glands.

- DLBCL involving the kidneys and adrenal glands is associated with an increased risk for metastasizing to central nervous system. Bone marrow involvement occurs late in 10–25% of cases.
- Recent studies suggest that fluorodeoxyglucose (FDG) positron emission tomography (PET), i.e. **(FDG-PET)** is a sensitive diagnostic imaging technique for detecting bone marrow involvement. If FDG-PET is negative, routine staging by bone marrow trephine biopsy is not required.

Surgical Pathology: Diffuse Large B Cell Lymphoma

Light Microscopy

Three common histologic variants of DLBCL have been recognized: centroblastic, immunoblastic, anaplastic variants.

- The tumor cells in DLBCL are large 4–5 times diameter of small lymphocytes. These have round to oval nucleus, with either 2–3 nucleoli located adjacent to the nuclear membrane or a single nucleolus centrally placed and moderate pale or basophilic cytoplasm. Mitoses are frequent.
- These are arranged in diffuse pattern. More anaplastic tumors may contain multinucleated Reed-Sternberg-like cells with large, inclusion-like nucleoli, and these may be termed immunoblastic lymphoma. Light microscopy of DLBCL is shown in Fig. 13.25A and B.

DLBCL: Subtypes

DLBCL subtypes include histiocyte-rich large B cell lymphoma, primary DLBCL of the CNS, primary cutaneous DLBCL of leg, and EBV positive DLBCL of the elderly.

Differential Diagnosis

DLBCL should be differentiated from metastatic carcinomas. The presence of cell surface monoclonal immunoglobulin by immunohistochemistry would help to confirm this lesion as a malignant lymphoma.

Immunophenotyping

- Tumor cells of DLBCL typically express pan-B cell markers such as CD19, CD20, CD22, CD79a and PAX-5. But tumor cells may lack one or more of these markers. DLBCL shows positivity for CD20 (Fig. 13.26A and B).
- Surface as well as cytoplasmic immunoglobulin most often IgM followed by IgG and IgA can be demonstrated in 50–75% of cases.
- Expression of CD10, BCL-2, BCL-6, IRF-4/MUM-1 varies in tumor cells.
- Tumor cells are negative for TdT.
- The tumor cells often express CD19, CD20, CD22 and CD79a.
- Most express surface immunoglobulin. Expression of CD10, BCL-6, and IRF4/MUM1 varies in tumor cells.
- DLBCL shows positivity for BCL-2 (Fig. 13.27).

Markers	Expression
CD19	Positive
CD20	Positive
CD22	Positive
CD79a	Positive
PAX-5	Positive
Surface and cytoplasmic immunoglobulin (IgM followed by IgG and IgA)	Positive

Clinical Features

Patient with DLBCL most often presents with a rapidly tumor mass involving single lymph node or multiple lymph nodes or extranodal organs/tissues.

- Almost 50% of cases have stage I or II disease. In the initial stage of the disease PET/CT imaging technique helps in reducing the percentage of cases to advanced stage.
- Patients may have localized symptoms depending on the site of extranodal involvement. Patient presents with rapidly growing single large nodal mass confined to local region. It has greater propensity to be extranodal disseminating to various organs than the low-grade NHL.
- DLBCL can be associated with immunocompromised state and another with Kaposi sarcoma herpesvirus.

Immunodeficiency Associated Diffuse Large B Cell Lymphoma

Immunodeficiency associated diffuse large B cell lymphoma occurs in end stage of HIV infection, severe

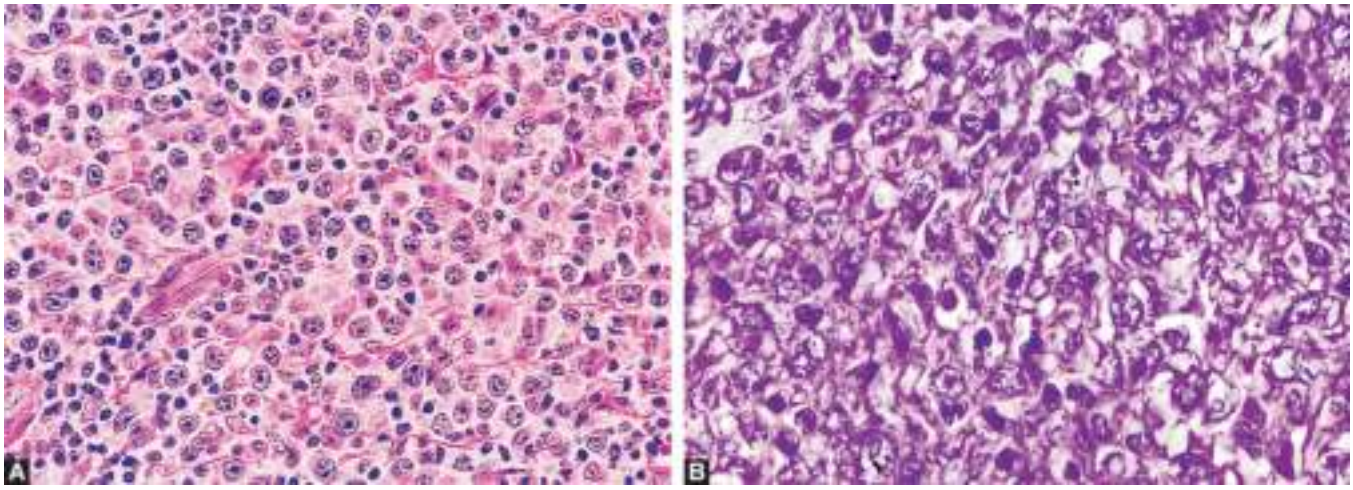


Fig. 13.25A and B: Diffuse large B cell lymphoma (DLBCL) shows large lymphoid cells with round to oval nuclei and 2–3 nucleoli arranged in diffuse pattern (400X).

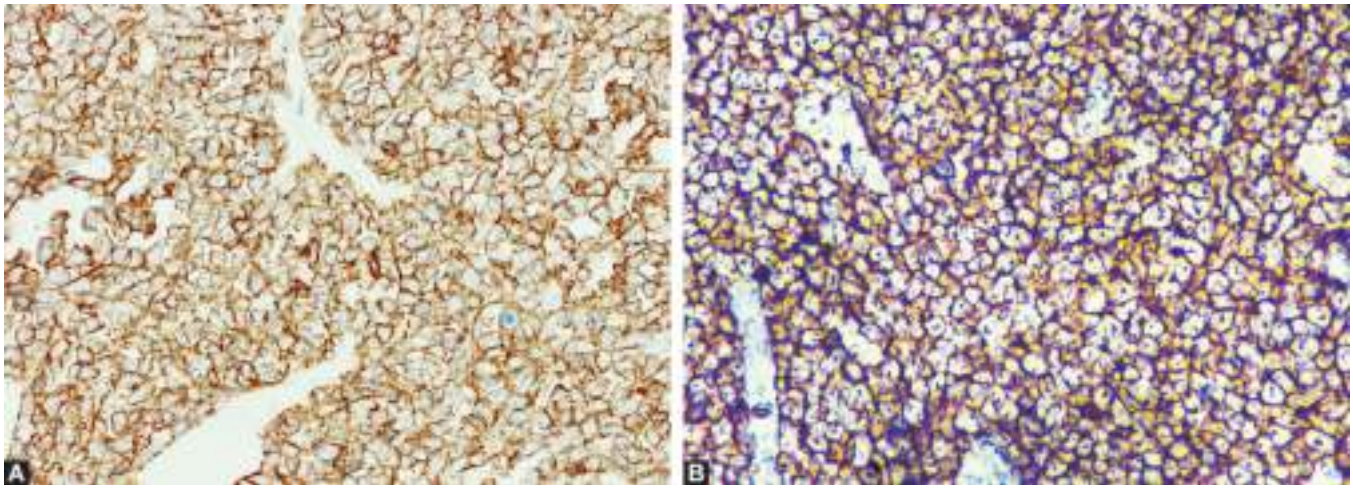


Fig. 13.26A and B: Diffuse large B cell lymphoma (DLBCL) shows positivity for CD20 (400X).

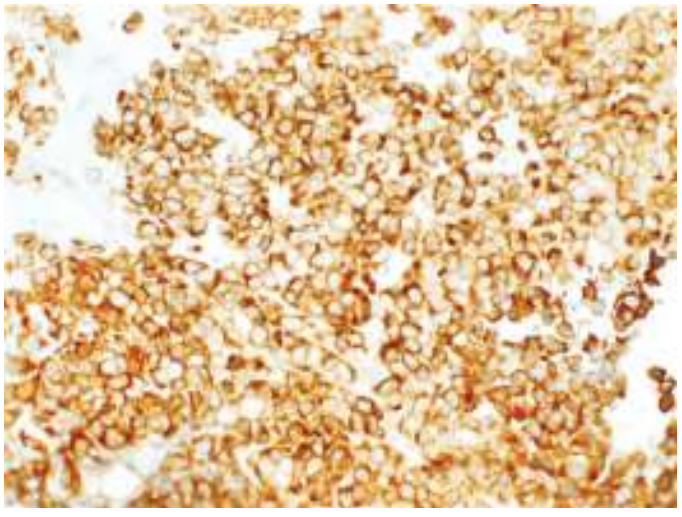


Fig. 13.27: Diffuse large B cell lymphoma (DLBCL) shows positivity for BCL-2 (400X).

combined deficiency and organ transplantation (bone marrow or solid organs). Tumor B cells are infected with Epstein-Barr virus, which may play critical role in its pathogenesis.

Body Cavity Diffuse Large B Cell Lymphoma

Patient with body cavity diffuse large B cell lymphoma develops malignant pleural or ascitic effusions especially in advanced HIV infection. The tumor cells are infected with KSHV (most often) or HHV8 in cases, which may play role in its pathogenesis.

CHRONIC LYMPHOCYTIC LEUKEMIA/SMALL LYMPHOCYTIC LYMPHOMA

Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) arises from naïve B cell or post-

germinal center memory B cell. About 10% of cases transform to a diffuse large B cell lymphoma. An autoimmune hemolytic anemia appears in about one-sixth of CLL/SLL cases.

- **Chronic lymphocytic leukemia:** A diagnosis of CLL is made if bone marrow and peripheral blood are primarily involved. Peripheral blood smear examination shows atypical small lymphocytes (smudge cells due to fragile chromatin), autoimmune hemolysis and thrombocytopenia in a minority.
- **Small lymphocytic lymphoma:** If the tumor cells give rise to lymphadenopathy or solid tumor masses, the term small lymphocytic lymphoma is used. Both disorders most often affect elderly persons.
- **Molecular genetics:** Chromosomal translocations are rare in CLL/SLL. Chromosomal aberrations occur such as trisomy 12, deletions of 11q, 13q, and 17p. In some cases, immunoglobulin genes are somatically hypermutated, and there may be a small immunoglobulin 'spike' in the serum.
- **Organs involved:** Tumor cells of CLL/SLL infiltrate many organs. Liver (portal tracts), spleen (white and red pulp), and lymph nodes may become enlarged, although organ function is often not markedly impaired.

Surgical Pathology: Chronic Lymphocytic Leukemia/ Small Lymphocytic Lymphoma

Light Microscopy

- Architecture of lymph node is diffusely obliterated.
- Tumor cells completely surround benign germinal center and form inverse follicular pattern.
- Tumor cells may completely surround benign follicles as third layer and form marginal zone pattern.
- Tumor cells are mature appearing lymphocytes measuring 6–12 μm in diameter replacing the lymph node and extending through the capsule of the lymph node and into the surrounding adipose tissue.
- Tumor cells are small round containing irregular nuclei with condensed chromatin and scanty cytoplasm. There is scant mitotic activity.
- Tumor cells are admixed with prolymphocytes with prominent mitotic activity. Presence of prolymphocytes is pathognomonic for CLL/SLL (Fig. 13.28).

Immunophenotyping

The tumor cells of chronic lymphocytic leukemia/small lymphocytic lymphoma express surface immunoglobulin and pan-B cell markers such as CD19, CD20, CD22, CD5, CD79a, CD23 and CD43. Chronic lymphocytic leukemia/small lymphocytic lymphoma shows positivity for CD5 (Fig. 13.29) and positivity for CD23 (Fig. 13.30).

Markers	Expression
IgM (either κ or λ light chain)	Positive
IgG (either κ or λ light chain)	Positive
CD19	Positive
CD20	Positive
CD22	Positive
CD5	Positive
CD79a	Positive
CD23	Positive
CD43	Positive

Neoplastic cells are negative for CD11c, CD10, FMC7 and CD79b.

Prognosis

Mean survival rate of chronic lymphocytic leukemia/small lymphocytic lymphoma is 4–6 years. The presence of deletions of 11q and 17p correlates with higher stage disease and worse prognosis.

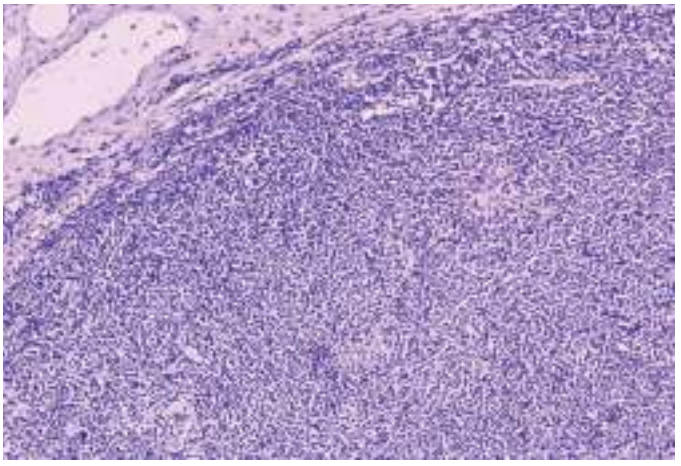


Fig. 13.28: Chronic lymphocytic leukemia/small lymphocytic lymphoma. Lymph node architecture is obscured. It is composed of mature appearing lymphocytes surrounding benign germinal center. Tumor cells contain irregular nuclei with condensed chromatin (100X).

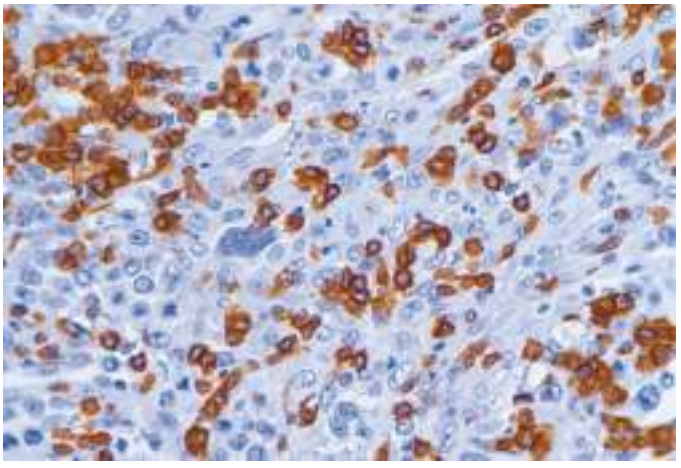


Fig. 13.29: Chronic lymphocytic leukemia/small lymphocytic lymphoma shows positivity for CD5.

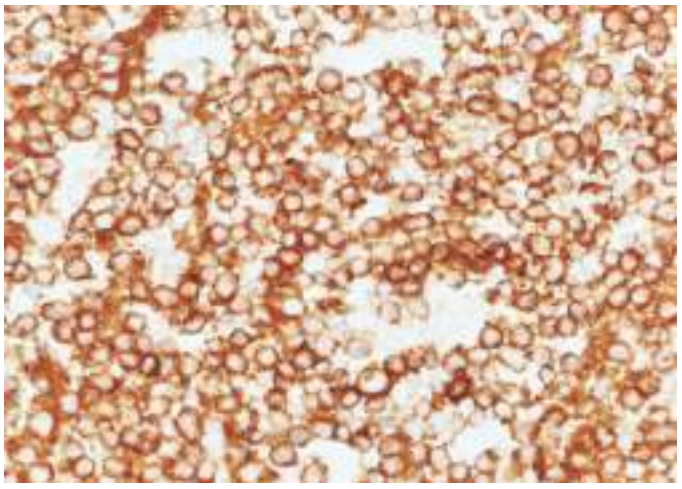


Fig. 13.30: Chronic lymphocytic leukemia/small lymphocytic lymphoma shows positivity for CD23.

FOLLICULAR LYMPHOMA

Follicular lymphoma (FL) is the commonest subtype of the low-grade non-Hodgkin's lymphoma derived from follicle center B cells, which is slow-growing tumor with fatal outcome. Tumor is composed of variable mixture of centroblasts and centrocytes randomly distributed and forming partially follicular pattern. It is most prevalent in Western world especially USA and less common in Africa and Asia.

- Follicular lymphoma involves lymph nodes, bone marrow, peripheral blood, spleen and Waldeyer's tonsillar lymphatic ring. It most often affects in sixth and seventh decades of life and usually presents with stage III or stage IV disease.
- Approximately 30–50% cases of follicular lymphoma may transform to diffuse large B cell lymphoma (DLBCL). Primary follicular lymphomas of the gastrointestinal tract and skin are clinically different from nodal non-Hodgkin lymphomas.
- Four histologic variants of follicular lymphoma include: *in situ* follicular neoplasia, duodenal type of follicular lymphoma, testicular follicular lymphoma and diffuse pattern of follicular lymphoma. Chemotherapy may be administered to control the disease, but it is rarely curative.

Molecular Genetic Alterations

Mutations affecting histone-modifying genes are crucial in pathogenesis of follicular lymphoma.

- **Physiologic state:** Normally '**cytochrome c**' leaving mitochondria and entering into the cytosol activates caspases initiating apoptosis. Protein products of antiapoptotic genes prevent **cytochrome c** from leaving mitochondria. BCL-2 gene family produces gene products that prevent mitochondrial leakage of 'cytochrome c' (signal for apoptosis).
- **Pathologic state:** Chromosomal translocation t(14;18) causes overexpression of BCL-2 protein product, which prevents apoptosis of B cells leading to B cell follicular lymphoma and chronic lymphocytic leukemia. Lymphoma cells do not die but accumulate in the lymph nodes, bone marrow, and the blood circulation. All follicular lymphomas also express BCL-6, a transcriptional repressor that regulates development of germinal center B cell.

Localization of Disease

Follicular lymphoma predominantly involves lymph nodes in the peripheral, thoracic and abdominal regions, bone marrow (tumor cells present in paratrabecular pattern), peripheral blood, spleen and Waldeyer's tonsillar lymphatic ring. Tumor cells may infiltrate portal tracts of liver, splenic white pulp, soft tissue, gastrointestinal tract, breast and ocular adnexa.

Clinical Features

Majority of patients with follicular lymphoma have widespread disease at diagnosis including peripheral and central lymphadenopathy in thoracic and abdominal region, and splenomegaly. Bone marrow involvement is demonstrated in 40–70% of cases. Involvement of central nervous system, testes and gastrointestinal tract is uncommon. Approximately 15–25% of cases are in stage I at the time of diagnosis. Despite widespread disease, patients most often remain asymptomatic. The disease follows chronic relapsing clinical course. Overall median survival of patients is 3–5 years. However, majority of the patients cannot be cured.

Imaging Techniques

CT and MRI tecniques are used to assess degree of lymphadenopathy and extent of disease. Fluro-deoxyglucose (FDG)-positron emission tomography (PET), i.e. FDG-PET imaging is done only in more aggressive metabolically active lymphomas and in identifying patients with higher risk of progression of the disease.

Staging of Disease

Stage of follicular lymphoma is now determined by Cotswold's classification, modification of the Ann Arbor staging system. Bone marrow trephine biopsy is essential to assess involvement of bone marrow. Bone marrow aspiration has lower yield due to dificulty in aspiration from the paratrabecular lymphoid aggregates.

Surgical Pathology: Follicular Lymphoma

Gross Morphology

- Cut surface of lymph node involved shows a vaguely nodular pattern.
- The neoplastic follicles most often have a bulging appearance. However, reactive follicular hyperplasia can exhibit the same pattern.
- Spleen involved in follicular lymphoma displays uniform expansion of white pulp, usually with no evidence of involvement of red pulp.

Light Microscopy

- Lymph node architecture is effaced in follicular lymphoma. It shows numerous irregularly shaped lymphoid follicles in cortex and medulla with loss of corticomedullary distinction and giving nodular appearance. Tumor cells invade the lymph node capsule and extend into the surrounding adipose tissue.
- Tumor is composed of varying degrees of centrocytes and centroblasts. Centrocytes have clefted nuclei with clumped chromatin and no nucleoli. Centroblasts have open nuclei with nucleoli located against the nuclear membrane.
- Growth patterns of (a) follicular >75% follicles, (b) mixture of diffuse and follicles 25–75%, and (c) minimally follicular with <25% follicular pattern. Histology of follicular lymphoma is shown in Figs 13.31 and 13.32.
- According to revised 2024 World Health Organization, grading of follicular lymphoma is based on the fact that prognosis worsens with increase in number of centroblasts.
  - Grade I: Centroblasts 0–5/high power field.
  - Grade II: Centroblasts 6–15/high power field.
  - Grade IIIa: Centroblasts admixed with centrocytes >15/high power field.
  - Grade IIIb: Centroblasts admixed with centrocytes >15/high power field (high-grade with worst prognosis).

Histochemistry

- There is considerable variation in histologic appearances within follicular lymphoma. Follicles may be highlighted by reticulin stain and immunohistochemistry.
- Sclerosis is a common feature of follicular lymphoma especially in retroperitoneal lymph node. Eosinophilic PAS positive extracellular amorphous material may be demonstrated in reactive and neoplastic follicles obscuring the neoplastic cells.
- Signet ring follicular lymphoma is composed of PAS negative clear cytoplasmic inclusions.
- Follicular lymphoma with plasmacytic differentiation shows plasma cell differentiation within follicles and interfollicular compartments.
- Marginal zone differentiation in follicular lymphoma is most often demonstrated in the spleen.
- Infarction of the lymph nodes may occur in follicular lymphoma.
- Composite follicular lymphoma may show areas of higher grade or diffuse growth pattern.

Immunophenotyping

- Tumor cells of follicular lymphoma are usually positive for surface IgM with or without IgG, IgA or IgD.
- Tumor cells express B cell-associated antigens such as CD19, CD20, CD22 and CD79a.
- Tumor cells are most often positive for BCL-2, BCL-6 and CD10. These are negative for CD5 and CD43.
- CD10 expression is stronger in the follicles rather than in the interfollicular tumor cells.
- BCL-2 overexpression is the hallmark of follicular lymphoma in 85–90% of grade I and II of follicular lymphoma.
- Other germinal center markers such as LMO2, GOET1 and HGAL also known as GCET2 are positive. But these are not required for routine diagnosis.

Markers	Expression
Surface IgM	Positive
CD10 (CALLA)	Positive
CD19	Positive
CD20	Positive
CD22	Positive
CD79a	Positive
BCL-2	Positive
BCL-6	Positive
LMO2	Positive
GOET1	Positive
HGAL (also known as GCET2)	Positive

Translocation t(14; 18) is demonstrate in follicular lymphoma.

Mann and Berard Grading of Follicular Lymphoma

Follicular lymphomas are graded as I, II and III based on number of centroblasts (Table 13.29). Comparison between follicular reactive hyperplasia and follicular lymphoma is given in Table 13.30.

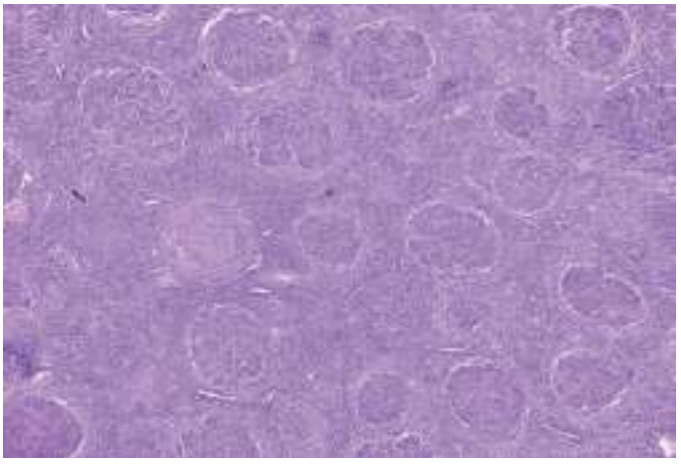


Fig. 13.31: Follicular lymphoma shows numerous irregularly shaped lymphoid follicles in cortex and medulla with loss of corticomedullary distinction (100X).

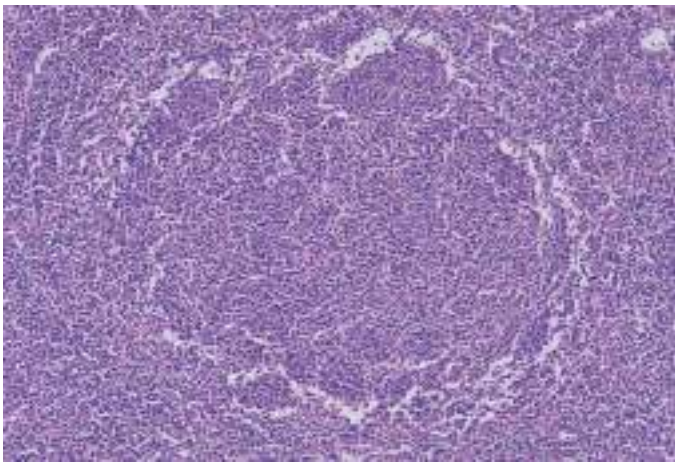


Fig. 13.32: Follicular lymphoma shows lymphoid follicle comprises small cleaved cells with scanty cytoplasm (200X).

Table 13.29 Mann and Berard grading of follicular lymphoma based on number of centroblasts/histopathological findings

Grade	Histologic Findings
Grade I	Centroblasts (0–5/high power fields)
Grade II	Centroblasts (6–15/high power fields)
Grade III	Centroblasts (>15/high power fields)

Prognosis

- Prognosis of follicular lymphoma is closely related to the disease at diagnosis. Follicular lymphoma prognostic index (FLPI) is useful predictive outcome.
- FLPI uses hemoglobin concentration, survival age >60 years, increased serum lactate dehydrogenase, Ann Arbor stage III/IV disseminated disease, and >4 involved lymph nodes.
- Genetic profile may provide additional information.
- Transformation of follicular lymphoma to diffuse large B cell lymphoma is demonstrated in 25–35% of cases.

Table 13.30 Comparison between follicular reactive hyperplasia and follicular lymphoma

Feature	Follicular Reactive Hyperplasia	Follicular Lymphoma
Epidemiology		
Age group	Younger persons	Elderly persons
Histological features		
Lymph node architecture	Maintained	Effaced
Follicle density	Normal	Increased back to back follicles
Location of follicles	Cortex	Cortex and medulla
Follicles size and shape	Pleomorphic follicles	Monomorphic follicles
Follicles demarcation	Sharply demarcated	Poorly demarcated
Follicle confinement	Within lymph node	May be seen in perinodal tissue
Mantle zone of lymphocytes around follicles	Well defined	Thin or absent
Subcapsular sinus obliteration	Absent	Present
Distribution of centroblasts and centrocytes	Zonal distribution	Random distribution
Centroblasts forming component of follicle	Forms large component of the follicle	Forms minority of follicles
Morphology of centroblasts and centrocytes	Typical morphology	Serpiginous, multinucleated and multilobated centroblasts may be present in the follicles and in interfollicular zones
Cellular polarization	Present	Absent
Tingible body macrophages containing apoptotic debris	Usually prominent	Usually absent
Mitosis	Frequent	Few
Atypical cells between follicles	Absent	May be present
Immunohistochemistry and molecular techniques		
Immunohistochemical marker BCL-2 in follicle center cells	Negative	Positive
Ig light chain restriction in follicle center cell	Ig light chain restriction absent	Ig light chain restriction present
Rearrangement of Ig/BCL-2 genes	Absent	Present and demonstrated by Southern blot or polymerase chain reaction

MANTLE CELL LYMPHOMA

Mantle cell lymphoma (MCL) is derived from B cell, which gives rise to a characteristic mantle zone expansion resulting in nodular pattern. MCL is most often composed of monomorphic small- to medium-sized lymphoid cells with oval or angulated nuclei frequently exhibit small clefts in >95% of patients. MCL is very aggressive, but incurable in majority of cases.

- MCL constitutes 3–10% of non-Hodgkin's lymphomas. Middle-aged to elderly persons with a median age of 60 years are affected. It occurs more in males than in females.
- MCL is an aggressive malignancy that manifests in middle-aged to elderly male patients as stage III or IV disease involving lymph nodes, bone marrow, peripheral blood, spleen, Waldeyer's ring and gastrointestinal tract (lymphomatous polyposis). Central nervous system involvement is commonly demonstrated at the time of diagnosis.
- MCL is characterized by chromosomal translocation of t(11;14) (q13;q32), which brings cyclin D1 gene on chromosome 11 under promoter influence of the immunoglobulin heavy chain gene on chromosome 14. Cyclin D1 gene, together with cyclin-dependent kinase-4 (CDK4) phosphorylates retinoblastoma protein (pRB), releasing transcription factors that permit transition from the G1 to the S phase of the cell cycle.
- Lymph nodes are most often involved in mantle cell lymphoma. Spleen and bone marrow involvement with or without blood are also important sites. Extranodal sites involved include gastrointestinal tract, Waldeyer's ring, lungs and pleura.
- Central nervous system involvement is commonly demonstrated at the time of diagnosis.
- Majority of mantle cell lymphoma (MCL) patients present with lymphadenopathy in stage III or stage IV disease, hepatomegaly, splenomegaly and bone marrow involvement. Extranodal involvement is common in the presence of extensive lymphadenopathy. Peripheral blood involvement is common and demonstrated by **flow cytometry** in almost all cases.

Surgical Pathology: Mantle Cell Lymphoma

Light Microscopy

- Mantle cell lymphoma is composed of small cells with irregular and intended nuclear contours.
- There are two histologic variants of mantle cell lymphoma—blastoid (classic form) and anaplastic (pleomorphic form).
  - Blastoid form may be accompanied by involvement of blood, bone marrow and spleen, hence termed mantle cell leukemia.
  - Anaplastic mantle cell lymphoma is characterized by a higher mitotic index and proliferative rare and poor survival.
- There is presence of hyalinized blood vessels and scattered epithelioid histiocytes sometimes resembling starry-sky appearance (Figs 13.33 and 13.34).

Immunophenotyping

- Tumor cells of mantle cell lymphoma express intense surface IgG/IgD with restriction of κ or λ.
- Tumor cells show positivity for BCL-2, CD5, FMC7 and CD43. These are sometimes positive for IRF4 and MUM1.
- Nuclear cyclin D1 is expressed in >95% of cases of mantle cell lymphoma.
- SOX11 positivity with monoclonal antibody is demonstrated in >90% of cases.
- Tumor cells are negative for CD10, CD25, CD23 and CD11c.

Markers	Expression
Surface IgG/IgD	Positive
BCL-2	Positive
CD5	Positive
FMC7	Positive
CD43	Positive
IRF4	Positive (some cases)
MUM1	Positive (some cases)
Nuclear cyclin D1	Positive (>95% of cases)
CD10	Negative
CD25	Negative
CD23	Negative
CD11c	Negative

Translocation t(11; 14) creating cyclin D1–IgH fusion gene is demonstrated in mantle cell lymphoma.

- **Prognosis of disease:** Median survival rate is 3–5 years in mantle cell lymphoma. Vast majority of patients do not respond to newer therapeutic modalities.

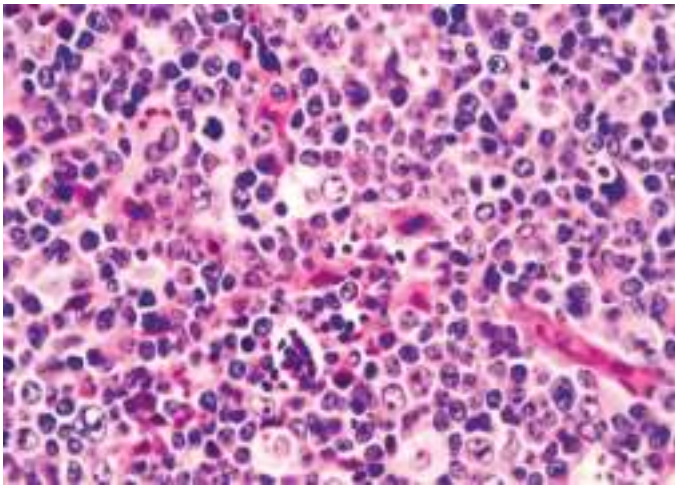


Fig. 13.33: Mantle cell lymphoma (MCL). Tumor is composed of small cells with irregular and indented nuclear contours (400X).

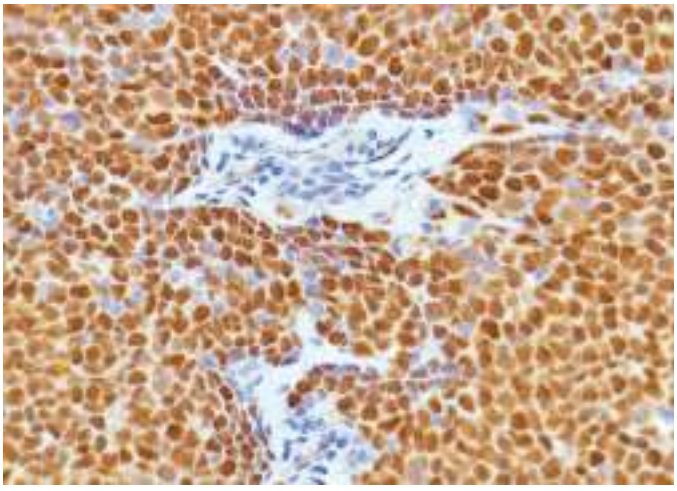


Fig. 13.34: Mantle cell lymphoma shows positivity for cyclin D1.

A high proliferative rate (mitotic or Ki-67 indices) is associated with shorter overall survival. High Ki-67 proliferative index (>30%) is currently accepted cut off point.

MARGINAL ZONE LYMPHOMA

There are three histologic variants of marginal zone lymphoma: nodal type, extranodal mucosa-associated lymphoid tissue (MALT) type and splenic type.

Nodal Marginal Zone Lymphoma

Nodal marginal zone lymphoma (NMZL) is a primary nodal B cell neoplasm without evidence of extranodal or splenic disease, which arises from mature B cells in marginal zone of lymph node.

- **Epidemiology:** Nodal marginal zone lymphoma (NMZL) accounts for <2% of all lymphoid neoplasms, which most often occurs in adults. Males and females are equally affected. Recently, NMZL has been detected in hepatitis C virus infection and autoimmune disorders.
- **Localization of disease:** NMZL most often involves peripheral lymph nodes. But it can involve bone marrow and occasionally the peripheral blood.
- **Clinical features:** Majority of patients present with asymptomatic localized or generalized lymphadenopathy especially in cervical region. About 10–25% of cases are symptomatic. Bone marrow involvement occurs in 33% of cases.

Surgical Pathology: Nodal Marginal Zone Lymphoma

Light Microscopy

- Lymph nodes demonstrate proliferation of lymphoid cells, that surround reactive follicles and expand into the interfollicular areas.
- Tumor cells are composed of variable numbers in marginal zone (centrocyte-like and monocytoid cells), B cells, plasma cells and transformed B cell.
- Bone marrow involvement is most often interstitial or nodular, with an intertrabecular or paratrabecular distribution.

Immunophenotyping

- Most cases of NMZLs express pan-B cell markers. CD43 is expressed in 20–75% of cases. CD5 may be expressed in 30% of cases. CD5 expression is observed in 17% of cases with disseminated cases.
- BCL-2 overexpression is demonstrated in most of the cases.
- Expression of MNDA and IRTA1 are observed in 75% of cases.
- CD23 may be expressed in 29% of cases.

Markers	Expression
CD43	Positive (20–75% of cases)
CD23	Positive (30% of cases)
CD5	Positive (30% of cases)
BCL-2	Positive (almost all cases)
MNDA	Positive (75% of cases)
TRTA1	Positive (75% of cases)
Cyclin D1	Negative

Extranodal Marginal Zone Lymphoma of Mucosa-associated Lymphoid Tissue

Extranodal marginal zone lymphoma of MALT most often involves gastric mucosa, which arises from mature B cells in marginal zone of lymph node. Patient develops extranodal marginal zone lymphoma of MALT often arises in *Helicobacter pylori* associated chronic gastritis, Hashimoto's thyroiditis and sialadenitis with Sjögren's syndrome.

- **Epidemiology:** Extranodal marginal zone lymphoma of MALT accounts for about 10% of all B cell lymphomas. It most often occurs in adults with median age in seventh decade. Both men and women are equally affected.
- **Localization of disease:** Extranodal marginal zone lymphoma of MALT occurs in gastric region in 50% of cases. Other common sites include the eyes, ocular adnexa, skin, salivary glands, breast and thyroid gland.
- **Molecular genetic alterations:** Trisomy 18, t(11;18), t(1;14), latter create MALT1-IAP2 and BCL-10 IgH fusion genes, respectively.
- **Clinical features:** Majority of patients present with stage I or II disease. Extranodal involvement occurs in 25–40% of cases. Bone marrow involvement is higher in non-gastric extranodal marginal zone lymphoma of MALT than extranodal marginal zone lymphoma of gastric MALT.

Surgical Pathology: Extranodal Marginal Zone Lymphoma of MALT

Light Microscopy

- Extranodal marginal zone lymphoma of MALT shows small- to medium-sized round to irregular lymphocytes admixed with plasma cells present in the marginal zone of lymphoid follicles. Some of tumor cells show plasmacytoid appearance.
- Tumor cells tend to invade gastric epithelium as small nests producing lymphoepithelial lesions.

Immunophenotyping

Tumor cells of extranodal marginal zone lymphoma are positive for CD20, CD79a, CD21 and CD35. Tumor cells are negative for CD10, CD23, and CD11c.

Markers	Expression
CD20	Positive
CD79a	Positive
CD21	Positive
CD35	Positive

- **Prognosis of disease:** Extranodal marginal zone lymphoma of MALT has **indolent course** and is slow to disseminate. Antibiotic therapy induces remission in *Helicobacter pylori* associated gastric MALT lymphoma, whereas cases with t(11;18) are resistant to antibiotic therapy. Tumor may be surgically excised.

Splenic Marginal Zone Lymphoma

Splenic marginal zone lymphoma (SMZL) is a specific low-grade small B cell lymphoma. It is characterized by splenomegaly, moderate lymphocytosis with villous morphology involving various organs, especially bone marrow. It has relatively indolent course in most of the cases except blastic form with aggressive behavior.

- Therapeutic options include treatment abstention, splenectomy, splenic irradiation, and chemotherapy.
- Mild neutropenia ($<1 \times 10^9$/L) occurs due to a combination of splenic sequestration and bone marrow infiltration in 5% of cases.

PLASMABLASTIC LYMPHOMA

Plasmablastic lymphoma is a very aggressive neoplasm, which arises from B cell that corresponds to the differentiation stage between B immunoblasts and plasma cell. It was initially described in the oral cavity in association with HIV infection. It may also occur in lymph nodes and extranodal sites.

- **Epidemiology:** Plasmablastic lymphoma most often occurs in adults with immunodeficiency state due to HIV infection. It may also occur in the settings of iatrogenic immuosuppresion in autoimmune diseases and organ transplantation.
- **Localization of disease:** Plasmablastic lymphoma most often presents as a mass in the extranodal regions of head and neck, especially in the oral cavity. The gastrointestinal tract is the most common next site after oral cavity.
  - Other extranodal sites of involvement occurs in skin, bone, genitourinary tract, nasal cavity, central nervous system, paranasal sites, liver, lungs and orbits.
  - Nodal involvement is demonstrated in <10% of cases, especially in post-transplantation cases.
- **Clinical features:** Patient presents with disseminated disease in stage III and stage IV with bone marrow involvement in 75% of HIV positive patients. About 25% of patients develop plasmablastic lymphoma with apparent immunodeficiency state.

Surgical Pathology: Plasmablastic Lymphoma

Light Microscopy

- Plasmablastic lymphoma is composed of diffuse and cohesive proliferation of neoplastic cells resembling immunoblasts and cells with more obvious plasmacytic differentiation, which resemble plasma cell myeloma.
- Tumor cells are large with vesicular nuclei, centrally located nucleoli, abundant cytoplasm and perinuclear halo.
- Mitotic figures are abundant. Apoptic cells and tingible body macrophages may be present.
- In monomorphic plasmablastic lymphoma, morphology of tumor cells is most often seen in the setting of HIV infection and located in the oral cavity, nasal region and paranasal sinuses.
- Cases with plasmablastic lymphoma most often occur in the nodal as well as extranodal sites.

Immunophenotyping

- Tumor cells express plasma cell phenotype, Which show positivity with CD138, CD38, VS38c, IRF4/MUM1, FRDM1 (also known as BLIMP1) and XBP1.
- CD20, CD45 and PAX5 are either negative or weakly positive.
- **CD79a positivity** is demonstrated in 40% of cases.
- **CD10 positivity** is demonstrated in 20% of cases.
- Cytoplasmic IgG is either κ or λ light chain, and CD56 is expressed by tumor cells in 25% of cases. EMA and CD30 are frequently expressed.
- Ki-67 proliferation rate is most often high, i.e. >90% of cases.
- BCL-2, BCL-6 and cyclin D1 expression are negative.

Markers	Expression
CD138	Positive (100%)
CD38	Positive (100%)
VS38c	Positive (100%)
IRF4/MUM1	Positive (100%)
FRDM1	Positive (100%)
XBP1	Positive (100%)
CD79a	Positive (40%)

▪ CD10	▪ Positive (20%)
▪ Cytoplasmic IgG is either κ or λ light chain	▪ Positive (25%)
▪ CD56	▪ Positive (25%)
▪ EMA	▪ Positive (frequently)
▪ CD30	▪ Positive (frequently)
▪ Ki-67	▪ Positive (>90%)
▪ BCL-2	▪ Negative
▪ BCL-6	▪ Negative
▪ Cyclin D1	▪ Negative

- **Prognosis:** Prognosis of plasmablastic lymphoma is generally poor with median survival of 6–12 months. Myc translocation has been associated with worse prognosis.

HAIRY CELL LEUKEMIA

Hairy cell leukemia (HCL) arises from activated small mature B memory cells and most often affects middle and elderly persons. Hairy cell leukemia most often involves peripheral blood, bone marrow and splenic red pulp.

- **Clinical features:** Most patients present with splenomegaly, pancytopenia, weakness, fatigue, pain in left upper quadrant, fever and bleeding. Bone marrow is virtually always involved. Hairy cells are demonstrated in the peripheral **blood** by the presence of cytoplasmic projections, hence named hairy cell leukemia. Hairy cell leukemia is resistant to conventional chemotherapy. Sometimes hairy cells may invade spleen.

Laboratory Diagnosis: Hairy Cell Leukemia

Light Microscopy

- Hairy cells are small- to medium-sized lymphoid cells with bean-shaped nucleus with ground glass chromatin, absent or inconspicuous nucleoli and abundant pale blue cytoplasm with circumferential hairy projections.
- The abundant cytoplasm and prominent cell borders produce '**fried egg appearance**' in bone marrow biopsies.

Cytochemistry

Hairy cells are tartrate-resistant acid phosphatase (TRAP) positive.

Immunophenotyping

Hairy cells express CD20, CD22, CD11c, CD103, CD25, CD123, T-bet, Annexin A1 and DBA 44.

Markers	Expression
▪ CD20	▪ Positive
▪ CD22	▪ Positive
▪ CD11c	▪ Positive
▪ CD103	▪ Positive
▪ CD25	▪ Positive
▪ CD123	▪ Positive
▪ T-bet	▪ Positive
▪ Annexin A1	▪ Positive
▪ DBA 44	▪ Positive

Flow Cytometry

Hairy cells show positivity with pan-B antigens: CD19 and CD20. In addition, these neoplastic cells coexpress CD11c, CD25, CD103 and FMC7.

- **Prognosis of disease:** Complete remissions are achieved in hairy cell leukemia with purine analogs or α-interferon. Overall 10-year survival exceeds 90%. There is an increased risk for secondary cancers such as Hodgkin's disease, NHL and thyroid cancer.

B CELL PROLYMPHOCYTIC LEUKEMIA

B cell prolymphocytic leukemia (B-PLL) arises from B cell prolymphocytes in marginal zone of lymph node, which involves peripheral blood, bone marrow and spleen. Prolymphocytes must constitute >55% of lymphoid cells in peripheral blood. It is difficult to distinguish B cell prolymphocytic leukemia from chronic lymphocytic leukemia and mantle cell lymphoma with leukemic expression.

- **Age group:** B cell prolymphocytic leukemia (B-PLL) accounts for 1% of lymphocytic leukemias. It affects elderly persons of >60 years with a median age of 65–69 years. Men and women are equally affected.
- **Localization of disease:** Leukemic cells of B-PLL are demonstrated in peripheral blood, bone marrow trephine biopsy, spleen and lymph node.
- **Clinical features:** Majority of patients present with massive splenomegaly, rapidly increasing lymphocyte count $>100 \times 10^9/L$ and absent or minimal lymphadenopathy. Anemia and thrombocytopenia are demonstrated in >50% of patients.

Laboratory Diagnosis: B Cell Prolymphocytic Leukemia

Peripheral Blood

B cell prolymphocytic leukemia cells in peripheral blood and bone marrow show >55% and most often >90% of lymphoid cells. These are medium-sized cells with round nucleus, condensed chromatin, a prominent central nucleus and scant basophilic cytoplasm.

Bone Marrow Trephine Biopsy Examination

Bone marrow trephine biopsy examination reveals an interstitial or nodular intertrabecular infiltration of prolymphocytes similar to those demonstrated in peripheral blood.

Light Microscopy (Spleen and Lymph Node)

- Spleen demonstrates expansion of white and red pulp due to infiltration of intermediate to large lymphoid cells with irregular to round nucleus, a central eosinophilic nucleus with abundant cytoplasm.
- Lymph nodes show deposits of similar lymphoid cells in diffuse or vague nodules.

Differential Diagnosis

- B cell prolymphocytic leukemia poses diagnostic difficulty in differentiating from pleomorphic mantle cell lymphoma, marginal zone lymphoma, and chronic lymphocytic leukemia. Diagnosis is established by immunophenotyping and genetic studies.
- Mantle cell lymphoma shows overexpression of cyclin D1 and t(11,14) (q13q32).
- Evaluation of SOX11 amd cyclin D1 expression in pure leukemia cases may require mRNA analysis by quantitative polymerase chain reaction (PCR).

Immunophenotyping

- B cell prolymphocytic leukemia cells express surface IgM/IgD as well as B cell antigens positivity with CD19, CD20, CD22, CD79a, CD79b and FMC7.
- **CD5 positivity** is seen in 20–30% of cases. CD23 positivity is demonstrated in 10–20% of cases.
- **ZAP70** and **CD38** are expressed in 50% of cases. ZAP70 expression does not correlate with IGHV gene mutation status.

Markers	Expression
Surface IgM/IgD	Positive (100%)
CD19	Positive (100%)
CD20	Positive (100%)
CD22	Positive (100%)
CD79a	Positive (100%)
CD79b	Positive (100%)
CD5	Positive (20–30%)
CD23	Positive (10–20%)
ZAP70	Positive (50%)
CD38	Positive (50%)

- **Prognosis of disease:** B cell prolymphocytic leukemia has a poor prognosis with a median survival of 30–50 months. Splenectomy may help in improvement of symptoms. Various chemotherapeutic drugs such as fludarabine and cladribine are administered. A combination of chemotherapy with rituximab may be effective treatment approach. Allogeneic bone marrow transplantation is also considered in these cases.

MULTIPLE MYELOMA

Multiple myeloma is malignant tumor of plasma cells resulting in synthesis of monoclonal light chain and heavy chain immunoglobulins.

- Multiple myeloma or solitary plasmacytoma arises from post-germinal center B cell. There is diverge rearrangements involving IgH, which most often affects elderly adults.
- Proliferation and survival of clonal plasma cells depend on elaboration of IL-6 by plasma cells and marrow stromal cells.
- Patient presents with osteolytic bone lesions, pathologic fractures, hypercalcemia, renal failure, and primary amyloidosis. In decreasing frequency, bones involved in multiple myeloma include vertebral column 66% (especially lumbar regions); ribs 44%; skull 41%; pelvis 28%; femur 24%; clavicle 10%; and scapula 10%.
  - The lesions begin in the medullary cavity, erode cancellous bone and progressively destroy the bony cortex (osteolytic lesions).
  - Patient is prone to pathologic fractures and compressed vertebral fractures.
  - Radiograph study reveals rounded punched-out defects, of 1–4 cm in diameter in an older adult. Such lesions can produce bone pain.
- Hypercalcemia and an elevated serum alkaline phosphatase are common laboratory findings. Most cases of solitary plasmacytoma involving bone evolve into multiple myeloma.
- Molecular genetic alterations include t(4;14), which juxtapose the IgH locus on 14q32 with the fibroblast growth factor receptor 3 (FGFR3) gene on chromosome 4p16. Clonal plasma cells synthesize cytokines such as MIP1a and the receptor activator of NF-κB ligand (RANKL), which serves as an osteoclast-activating factor (OAF).

Laboratory Diagnosis of Multiple Myeloma

Serum Electrophoresis

Serum protein electrophoresis shows monoclonal immunoglobulin (light chain class or single heavy chain) spike of M protein indicates increased level of IgG (50%) or IgM (25%). There is no increase in circulating immunoglobulin is <1% of cases.

Urinary Findings

In 60–70% of cases, increased light chains (either κ or λ), known as Bence Jones protein, are synthesized and excreted in the urine termed Bence Jones proteinuria. The light chains are toxic to renal tubules, and can lead to tubular injury with renal failure.

Bone Marrow Examination

- Bone marrow examination shows >30% of clonal plasma cells, which are well-differentiated with eccentric nuclei and perinuclear halo (clear cytoplasm representing the Golgi apparatus). Clear cytoplasmic droplets contain immunoglobulin.

- Morphology of clonal plasma cells is indistinguishable from normal plasma cells except by their increased numbers. Patient rarely develops plasma cell leukemia.

Bone Marrow Trephine Biopsy Examination

Tumor is composed of sheets of clonal plasma cells that are very similar to normal plasma cells, with eccentric nuclei and abundant pale purple cytoplasm. In some cases, clonal plasma cells may also be poorly differentiated (Fig. 13.35).

- The diminished amount of normal circulating immunoglobulin increases the risk for infections, particularly with bacterial organisms such as *Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus,* and *Escherichia coli.*
- The excessive light chain production may lead to the AL form of amyloidosis, with deposition of amyloid in many organs. Renal failure due to amyloid deposition is most common cause of death. Plasma cell neoplasms and their key features are given in Table 13.31.

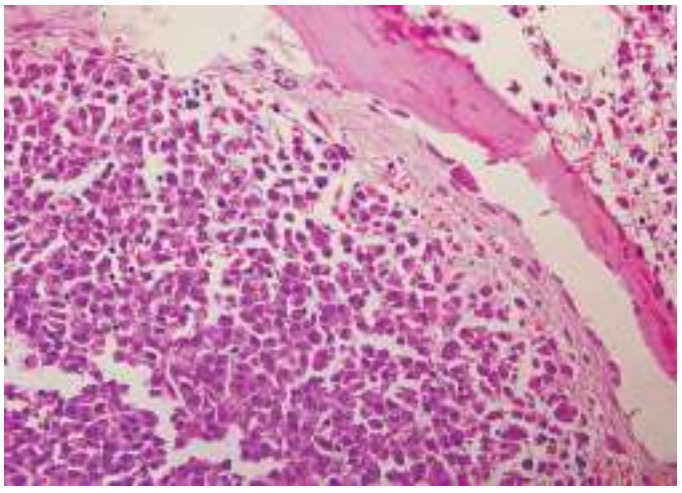


Fig. 13.35: Trephine bone marrow biopsy shows sheets of plasma cells with eccentric nuclei and abundant pale purple cytoplasm (400X).

MATURE T CELL AND NK CELL LYMPHOMAS

T cell and NK cell lymphomas are more common in Asia. Features of non-Hodgkin lymphomas derived from mature T cells and NK cells are given in Table 13.32.

ANGIOIMMUNOBLASTIC T CELL LYMPHOMA

Angioimmunoblastic T cell lymphoma accounts for 15–20% of T cell lymphomas and 1–2% of all non-Hodgkin's lymphoma, which affects 50–70 years of age group with equal sex incidence.

- The eye-catching feature of angioimmunoblastic T cell lymphoma is the presence of a rich small blood vessel meshwork with PAS positive on histologic examination. The tumor cells express CD4, CD10 and other markers of follicular CD4+ helper T cells.
- T cell receptor gene arrangement has been found in 75% of cases and appears to be limited to CD10+ cells.
- Follicular dendritic cells are abundant around the vessels and can be identified by CD21 immunophenotyping. Despite intensive therapy, prognosis is poor.

Clinical Features

Patient presents with generalized lymphadenopathy, hepatosplenomegaly, skin rash with pruritus, ascites, pleural effusion and arthritis. Laboratory investigations demonstrate frequent polyclonal hypergammaglobulinemia, elevated erythrocyte sedimentation rate, and lactic dehydrogenase (LDH), and autoimmune hemolytic anemia (Coombs' positive). Other autoantibodies are demonstrated such as rheumatoid factor, thyroid autoantibodies and anti-smooth muscle antibodies in these cases.

Molecular Genetic Alterations

Southern blotting technique and polymerase chain reaction (PCR) demonstrate the presence of rearrangement of T cell receptor gene in angioimmunoblastic

Table 13.31 Plasma cell neoplasms and their key features

Form	Characteristics
Plasma cell myeloma (multiple myeloma)	▪ Bone marrow or bone marrow trephine biopsy proven plasmacytoma consisting of ≥10% clonal plasma cells ▪ M-spike in serum or urine on electrophoresis ▪ Related organ or tissue damage (CRAB), i.e. hypercalcemia, renal insufficiency, anemia, bone lesions ▪ Myeloma defining events (MDE) must fulfill at least one MDE defining events: (a) serum calcium >11 mg/dl), (b) anemia, (c) bone lesions ≥1 osteolytic lesion on imaging techniques, (d) bone marrow clonal plasma cells ≥60%, (e) involved/uninvolved serum-free light chains ratio ≥100, and (f) presence of ≥1 focal lesion on MRI of spine
Osseous plasmacytoma	Single bone plasmacytoma, which does not produce M protein

Contd...

Table 13.31 Plasma cell neoplasms and their key features (*Contd...*)

Form	Characteristics
Extraosseous plasmacytoma	Nose, paranasal sinuses, nasopharynx, and tonsils
Osteosclerotic myeloma (POEMS syndrome)	▪ Polyneuropathy (chronic inflammatory polyneuropathy) ▪ Organomegaly (hepatomegaly, splenomegaly, or lymphadenopathy) ▪ Endocrinopathy (e.g. gynecomastia, testicular atrophy, M protein)
Nonsecretory myeloma	▪ Monoclonal plasma cells ≥10% in bone marrow ▪ Absence of M protein and serum-free light chain in serum and urine
Asymptomatic (smoldering) myeloma	▪ Monoclonal plasma cells ≥10% in bone marrow ▪ M protein spike in serum or urine >30 g/L ▪ Absence of myeloma-defining events (MDE) or amyloidosis
Covert myeloma	Synthesis of abnormal paraprotein and amyloidosis but without tumor formation

Table 13.32 Features of NHLs derived from mature T cells and NK cells

Disorder	Origin	Age Group	Clinical Features	Genotype
Angioimmunoblastic T cell lymphoma	T cell	Elderly adults	Generalized lymphadenopathy, hepatomegaly, skin rashes, pruritus, autoimmune hemolytic anemia	Rearrangement of T cell receptor gene and immunoglobulin gene
Adult T cell leukemia/lymphoma	Helper T cell	Adults	Cutaneous lesions, bone marrow involvement and hypercalcemia	HTLV-1 provirus demonstrated in neoplastic cells
Peripheral T cell lymphoma	Helper or cytotoxic T cell	Elderly persons	Lymphadenopathy with aggressive behavior	No specific chromosomal abnormalities
Anaplastic large cell lymphoma, ALK positive	Cytotoxic T cell	Children and young adults	Lymphadenopathy and soft tissue involvement with aggressive behavior	Rearrangement of ALK (anaplastic large cell kinase) in a subset
Extranodal NK cell/T cell lymphoma	NK cell (common) or cytotoxic T cell (rare)	Adults	Destructive extranodal masses (most common in sinonasal region) with aggressive course	EBV-associated without specific chromosomal abnormality
Mycosis fungoides	Helper T cell	Adults	Cutaneous lesions, and generalized erythema	No specific chromosomal abnormality
Sézary syndrome	Helper T cell	Adults	Cutaneous lesions, and generalized erythema	No specific chromosomal abnormality
Large granular lymphocytic leukemia	Cytotoxic T cell or NK cell	Adults	Anemia, splenomegaly, neutropenia, anemia accompanied by autoimmune disorder	

lymphoma. However, immunoglobulin gene rearrangement may also be demonstrated in these cases.

Surgical Pathology: Angioimmunoblastic T Cell Lymphoma

Light Microscopy

- Lymph node in angioimmunoblastic T cell lymphoma shows complete or partial effacement, which is composed of polymorphous population of reactive and tumors cells including scattered blasts often Epstein-Barr virus positive. Tumor cells extend into perinodal tissues, often leaving subcapsular sinus patent.
- There is presence of prominent branching of endothelial venules surrounded by PAS positive basement membrane.
- Lymph node also shows expanded follicular cell network and clusters of medium-sized T cells with clear cytoplasm.

Immunophenotyping

- Clonal T cells show positivity for CD3, CD5, CD4, CD10 and PD-1. Residual B cell areas pushed to periphery of lymph node are positive for CD20, CD79a, CD20 and CD79a.
- Clonal T cells may express EBV-encoded RNAs (EBERs) and EBV-latent antigens. Expanded network of dendritic cells shows positivity for CD21, CD23 and CD35.

Cell Type	Markers	Expression
▪ T cells	▪ CD3, CD5, CD4, CD10, PD-1	▪ Positive
▪ Residual B cell areas pushed to periphery of lymph node	▪ CD20, CD79a	▪ Positive
▪ Blast cells	▪ EBV-encoded RNAs (EBERs) and EBV-latent antigens	▪ Positive
▪ Expanded network of dendritic cells	▪ CD21, CD23, CD35	▪ Positive

ADULT T CELL LEUKEMIA/LYMPHOMA

Adult T cell leukemia/lymphoma is derived from a mature CD4+ helper T cell. It is composed of highly pleomorphic lymphoid cells.

- Patient develops widespread disseminated disease, which is caused by human retrovirus T cell leukemia virus 1 (HTLV-1).
- Tumor cells most often show multilobed appearance. Neoplastic T cells express T cell-associated antigens such as CD2, CD3 and CD9 but lack CD7.
- The disease most often occurs in endemic regions for HTLV-1. Males are more affected than females. Adult T cell leukemia/lymphoma is a systemic disease associated with poor prognosis.
- **Localization of disease:** Majority of patients present with widespread involvement of lymph nodes as well as peripheral blood. Circulating tumor cells are recruited from skin, the most commonly extralymphatic site involvement is >50% of cases.
- **Clinical features:** Patient presents with widespread lymphadenopathy. Skin is the most common extra-lymphatic site involved. Several clinical variants of adult T cell leukemia/lymphoma have been described: acute, lymphomatous, chronic and smouldering. Progression from chronic or smouldering variant to the acute variant occurs in 25% of patients most often after a long duration. On clinical examination, lesions of skin have been classified as erythema, papules and nodules. Tumor-like lesion is demonstrated in rare cases.
  - **Acute adult T cell leukemia/lymphoma:** It is most common and characterized by leukemic phase.
  - **Chronic adult T cell leukemia/lymphoma:** It is characterized by exfoliative skin rash.
  - **Lymphomatous adult T cell leukemia/lymphoma:** It is characterized by lymphadenopathy.
  - **Smouldering adult T cell leukemia/lymphoma:** It is characterized by skin or pulmonary lesions with normal TLC and >5% circulating tumor cells.
- **Imaging technique:** In patients with hypercalcemia, imaging studies may reveal osteolytic lesions. Fluorodeoxyglucose (FDG)-positron emission tomography (PET), i.e. FDG-PET is most often positive in 25% of cases after long clinical course in adult T cell leukemia/lymphoma.

Surgical Pathology: Adult T Cell Leukemia/Lymphoma

Light Microscopy

- Lymph node in adult T cell leukemia/lymphoma is characterized by a broad spectrum cytologic features of tumor cells (pleomorphic small, medium and large tubes).
- Rarely, tumor cells may be demonstrated resembling angioimmunoblastic T cell lymphoma. Nuclear chromatin of tumor cells is coarsely clumped with sometimes distinct nucleoli. Multiple indentations of clefts in nuclei are evident. Multinucleated giant cells may be present simulating Reed-Sternberg cells. Mitotic activity is high in the tumor cells.
- In the peripheral blood, tumor cells are multilobed termed flower cells. Giemsa stained blood smear examination reveals multilobed tumor cells with deeply basophilic cytoplasm.
- Tumor cell infiltrating in the bone marrow are most often patchy ranging from sparse to moderate.
- Tumor cells in the skin can produce skin lesions in >50% of patients. Tumor cells in filtratijng into the epidermis form microabscesses. Tumor cells in dermis are present around the blood vessels. Extension of tumor infiltrate may form subcutaneous nodules.
- Histopathology of adult T cell leukemia/lymphoma is shown in Fig. 13.36.

Immunophenotyping

- Tumor cells express E cell associated antigens such as CD2, CD3 and CD5.
- Tumor cells are CD4 positive and CD8 negative but reverse may be seen in most cases.
- CD25 is strongly expressed in nearly all cases.
- CD30 may be positive in transformed cells.
- CCR4 chemokine is expressed by neoplastic cells.

Markers	Expression
▪ CD2	▪ Positive (100%)
▪ CD3	▪ Positive (100%)
▪ CD5	▪ Positive (100%)
▪ CD25	▪ Positive (100%)
▪ CCR4	▪ Positive

- **Prognosis of disease:** The acute and lymphomatous variants of adult T cell leukemia/lymphoma have shorter survival. Death occurs due to infectious agents such as *Pneumocystis pneumoniae*, Cryptococcus and herpes zoster.

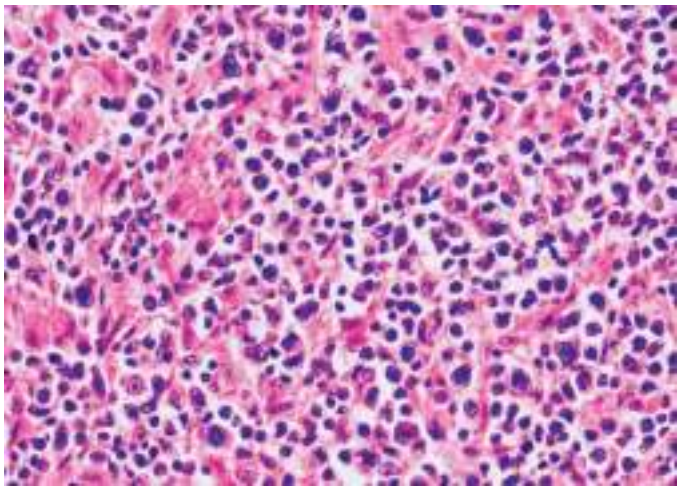


Fig. 13.36: Adult T cell leukemia/lymphoma shows pleomorphic population of neoplastic cells composed of small, medium and large types. Nuclear chromatin is coarsely clumped (400X).

ANAPLASTIC LYMPHOMA KINASE (ALK) POSITIVE ANAPLASTIC LARGE T CELL LYMPHOMA

Anaplastic lymphoma kinase (ALK) positive anaplastic large T cell lymphoma is derived from CD8+ cytotoxic T cells and also known as Ki-1 lymphoma. Primary anaplastic large T cell lymphoma originates *de novo* in lymph node or skin. Secondary anaplastic large T cell lymphoma represents transformation of another lymphomas.

- Tumor is composed of large lymphoid cells of variable size containing horseshoe-shaped nuclei and abundant cytoplasm involving ALK gene and expression of ALK protein and CD30.
- ALK positive anaplastic large T cell lymphoma should be differentiated from primary cutaneous adult anaplastic large cell lymphoma and T cell or B cell lymphoma with anaplastic features and/or CD30 expression.
  - **Epidemiology:** ALK positive anaplastic large T cell lymphoma has bimodal age distribution with one peak in children and another in older patients. It is more frequent in the first three decades of life. Males are more affected than females.
  - **Localization of disease:** ALK positive anaplastic large T cell lymphoma most often involves both lymph nodes and extranodal sites such as soft tissue, skin, bone, liver and lungs. Involvement of central nervous system and gastrointestinal tract is rare. Bone marrow involvement is demonstrated in <30% of cases by using immunohistochemical stains.
  - **Clinical features:** Approximately 70% of patients present with advanced stage III or IV disease with lymphadenopathy in peripheral and/or abdominal regions and often associated with extranodal involvement of bone marrow. About 75% of patients have high fever.
  - **Molecular genetic alterations: Anaplastic lymphoma kinase (ALK)** gene is located on chromosome 2. As a result of chromosomal translocation t(2;5), ALK gene is fused with the NPM (nucleophosmin) gene, resulting in the production of a hybrid NPM–ALK protein. It is demonstrated by ALK-1 (p80) monoclonal a ntibody. Chromosomal translocation and formation of hybrid NPM–ALK gene is seen in majority of children and young adults. These patients have a relatively good prognosis. Anaplastic large T cell lymphoma shows chromosomal translocations involving ALK gene, ALK staining pattern and frequency (Table 13.33).

Surgical Pathology: Anaplastic Large T Cell Lymphoma (ALK Positive)

Light Microscopy

- In ALK positive anaplastic large T cell lymphoma, lymph node exhibits a broad morphologic spectrum and composed of large pleomorphic cells with eccentric horseshoe or doughnut-shaped nuclei with eosinophilic cytoplasm. Morphology of tumor cells may mimic poorly differentiated carcinoma.
- Morphologic of ALK positive anaplastic large T cell lymphoma is shown in Fig. 13.37.

Immunophenotyping

- ALK expression is absent in all normal postnatal human tissues except in rare case in brain.
- Immunophenotyping with specific anti-ALK monoclonal antibodies plays significant role in diagnosing the neoplasm.
- Tumor cells show positivity for epithelial membrane antigen (EMA), CD30 (Ki-1), CD2, CD5, CD4, IL-2 receptor, clustrin, cadherin and galectin in most of the cases. Immunophenotyping in common histologic types of T cell lymphomas is given in Table 13.34.

Markers	Expression
EMA	Positive
CD30 (Ki-1)	Positive
CD2	Positive
CD5	Positive
CD4	Positive
IL-2 receptor	Positive
Clustrin	Positive
Cadherin	Positive
Galectin	Positive

- **Prognosis of disease:** Anaplastic large B cell lymphoma (ALK positive) has good prognosis with 75% cure rate treated with chemotherapy. Relapses most often remain sensitive to chemotherapy. Allogeneic bone marrow transplantation may be

Table 13.33 Categories of anaplastic large T cell lymphoma

Parameters	ALK Positive	ALK Negative
Age group	Children and young persons	Elderly persons
Prognosis with treatment	Good	Poor outcome

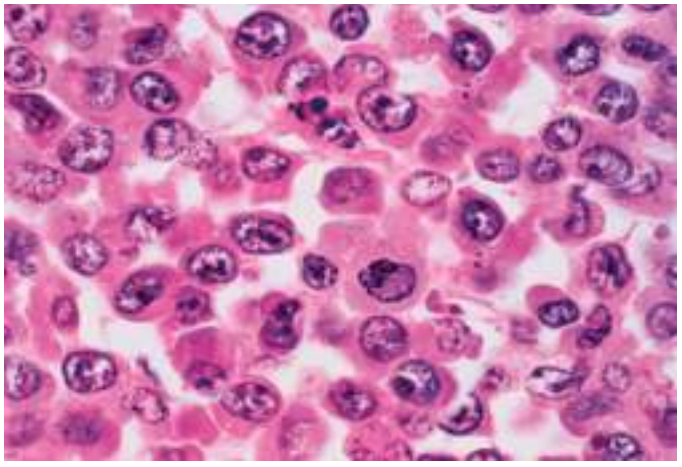


Fig. 13.37: ALK positive anaplastic large T cell lymphoma shows large pleomorphic cells with eccentric horseshoe-shaped nuclei with eosinophilic cytoplasm (400X).

effective in refractory cases. Small molecule inhibitors of ALK kinase and CD-targeting with antibody drug may be administered in relapse cases.

EXTRANODAL NK CELL/T CELL LYMPHOMA

Extranodal NK/T cell lymphoma derived from NK/T cell lineage is characterized by vascular destruction, necrosis and cytotoxic phenotype associated with Epstein-Barr virus.

- **Epidemiology:** Extranodal NK/T cell lymphoma is more prevalent in **Asians** and populations in **Mexico**, **central** and **South America**. It most often affects adults between 44 and 54 years of age. Males are more affected than females.
- **Localization of disease:** Extranodal NK/T cell lymphoma most often involves nasal cavity, nasopharynx, paranasal sinuses and palate. Nasal cavity is most often involved. Other organs may be involved such as skin, soft tissue, gastrointestinal tract. Some cases may be accompanied by involvement of lymph node.
- **Clinical features:** Patient presents with nasopharyngeal mass with obstruction and epistaxis. There are extensive destructive midfacial lesions.
  - Neoplasm may extend to surrounding structures such as nasopharynx, paranasal sinuses, oral cavity, orbit, palate and oropharynx. Bone marrow involvement is uncommon.
  - Disease may disseminate to skin, gastrointestinal tract, central nervous system, tests and cervical lymph nodes during clinical course. Some patients may develop hemophagocytic syndrome.

Surgical Pathology: Extranodal NK Cell/T Cell Lymphoma

Light Microscopy

- Histologic features of extranodal NK cell/T cell lymphoma are similar irrespective of the site of involvement.
- Mucosal sites most often show extensive ulceration.
- Tumor cells infiltrate is diffuse and permeative. There is loss of mucosal glands.
- An angiocentric and angiodestructive growth patterns are most often present. Coagulative necrosis and apoptotic bodies are commonly demonstrated.
- Tumor is composed of small- and medium-sized, large or anaplastic cells with irregularly folded nuclei.
  - Chromatin in granular in small- and medium-sized neoplastic cells.
  - Cytoplasm is moderate in amount either pale or clear in the cells.
  - Mitotic figures are most often demonstrated.

Immunophenotyping

- Most typical immunophenotyping of extranodal NK cell/T cell lymphoma is CD2 positive, CD56 positive, cCD3 epsilon positive, CD3 negative and CD5 negative. CD56 is a specific marker for NK cells but not specific for extranodal NK cell/T cell lymphoma.
- CD43 and CD45RO are most often positive. CD7 is invariably expressed in tumor cells.
- Subset of cases of cytotoxic T cell lineage may express CD5, CD8 and T cell receptor (gamma–delta or alpha–beta). Cytotoxic molecules such as granzyme B, perforin and TIA1 are positive.
- Tumor cells show positivity for HLA-DR, CD25, FAS (CD95) and FASL. CD30 is positive in 30% of cases.
- Nuclear expression of megakaryocyte-associated tyrosine kinase (MATK) is common in these cases.
- EBV encoded RNA (ERER) *in situ* hybridization demonstrates EB virus in neoplastic cells.

Markers	Expression
CD2	Positive (most often)
CD56	Positive (most often)
cCD3 epsilon	Positive
CD56	Positive (not specific marker)
CD43	Positive
CD45RO	Positive
HLA-DR	Positive
CD25	Positive
FAS (CD95)	Positive
FASL	Positive
CD30	Positive (30% of cases)
MATK	Positive (most often)

Table 13.34 Immunophenotyping in common histologic types of T cell lymphomas

Markers	Anaplastic Large T Cell Lymphoma	Peripheral T Cell Lymphomas	T Lymphoblastic Lymphoma
CD3	Positive/negative	Positive	Positive/negative
CD30	Positive (sheets of cells)	Positive (focal cells)	Negative
EMA	Positive	Negative	Negative
ALK1	Positive	Negative	Negative
CD99	Positive/negative	Negative	Positive
TdT	Negative	Negative	Positive

MYCOSIS FUNGOIDES

Mycosis fungoides is an epidermotropic primary T cell lymphoma derived from CD4+ helper T cells composed of small- and medium-sized T cells with cerebriform nuclei. Term mycosis fungoides may be used for classic cases characterized by the presence of cutaneous erythematous patches, plaques, and tumors.

- **Epidemiology:** Mycosis fungoides is the most common type of T cell lymphoma. It constitutes 50% of all cutaneous lymphomas, which most often occurs in adults/elderly. However, it may affect children and adolescents. Males are more affected than females. Disease is more prevalent in crop and vegetable farmers, painters, carpenters and wood workers suggesting environmental etiology of the disease.
- **Localization of disease:** Mycosis fungoides is most often limited to the skin. Extracutaneous dissemination may be demonstrated in advanced stage of disease involving lymph nodes, liver, spleen, blood and lungs. Bone marrow involvement is very rare.
- **Clinical features:** Patient presents with cutaneous patches ranging from plaques or nodules, or generalized erythematous plaques in the early stage. Patient with tumor stage mycosis fungides shows combination of skin lesions with ulceration. Later neoplastic cells may disseminate to lymph nodes, liver, spleen, lungs and blood. Clinical stage of the disease is the most important prognostic factor.

Surgical Pathology: Mycosis Fungoides

Light Microscopy

The histology of the skin lesions varies with the stage of the disease (Fig. 13.38).

- **Early patch stage:** Band-like or lichenoid infiltrates of lymphocytes and histiocytes are demonstrated. Atypical cells are small- and medium-sized with cerebriform nuclei present mainly in the basal layer of epidermis.
- **Plaque stage:** Epidermotropism is prominent with intraepidermal atypical cell collections called Pautrier microabscesses.
- **Tumor stage:** Dermal infiltrates of neoplastic cells are more diffuse with prominent nuclei. Intradermal atypical cell collection is less.

Immunophenotyping

Tumor cells of mycosis fungoides express CD2, CD3, TCR-β, CD5, and CD4 T cell. Cutaneous lymphocyte antigen (CLA) is expressed in most of the cases. Mycosis fungoides; cutaneous plaque shows CD3 positivity (Fig. 13.39).

Markers	Expression
CD2	Positive
CD3	Positive
TCR-β	Positive
CD5	Positive
CD4	Positive
CLA	Positive (in most of the cases)

- **Prognosis of disease:** Most important prognostic factors for mycosis fungoides is the extent of cutaneous and extracutaneous disease as reflected by the clinical stage. Prognosis is poor in advanced

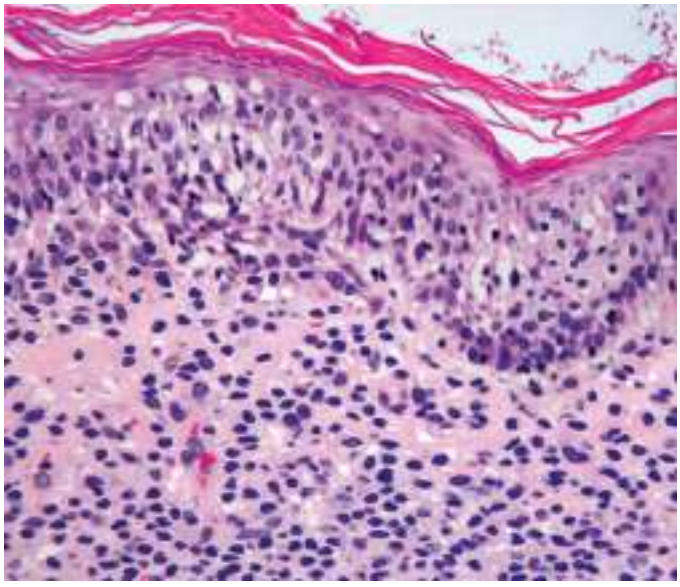


Fig. 13.38: Mycosis fungoides shows dermal infiltrates of neoplastic cells arranged in diffuse pattern with prominent nuclei (400X).

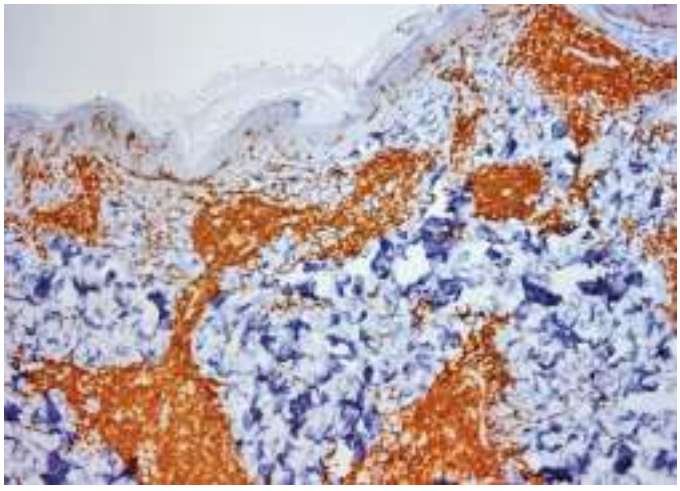


Fig. 13.39: Mycosis fungoides shows positivity for CD3.

disease. Advese prognostic factors for mycosis fungoiders include extracutaneous dissemination, patient age >60 years, elevated lactate dehydrogenase and histologic transformation to blast stage with >25% blast cells.

SÉZARY SYNDROME

Sézary syndrome is defined by the triad of erythroderma, generalized lymphadenopathy and presence of clonal neoplastic T cells with cerebriform nuclei (Sézary-Lutzner cells). Leukemic cells from skin lesion in mycosis fungoides most often involve bone marrow and lymph nodes.

- **Epidemiology:** Sézary syndrome accounts for <5% of all cutaneous T cell lymphoma, which occurs in adults of >60 years of age.
- **Localization of disease:** Sézary syndrome is generalized disease, which can involve any visceral organ in advanced disease especially in oropharynx, lungs and central nervous system. Bone marrow involvement is variable.
- **Clinical features:** Patient presents with erythroderma (intense and widespread reddening of skin), generalized lymphadenopathy, pruritus, plantar or palmar hyperkeratosis, alopecia, ectropion and alteration of nail morphology, i.e. onychodystrophy. Patient with Sézary syndrome develops generalized exfoliative erythroderma associated with cutaneous T cell lymphoma.

Surgical Pathology: Sézary Syndrome

Light Microscopy

- Histologic features in Sézary syndrome may be similar to mycosis fungoides.
- Histologic examination of lymph node shows a dense, monotonous infiltrate of Sézary cells with effacement of normal architecture of lymph node.
- Bone marrow involvement is variable and showing neoplastic T cells (leukemic T cells) which are arranged mainly in interstitial pattern.
- Peripheral blood shows **Sézary-Lutzner cells** with deep-clefted, cerebriform nuclei.

Immunophenotyping

- Neoplastic T cells express CD3, CD4, CD279 (also known as PD1), CD2 and TCR-β in almost all cases.
- Sézary-Lutzner cells express cutaneous lymphocyte antigen (CLS) and skin homing receptor (CR7).
- Flow cytometry analysis of peripheral blood lymphocytes exhibits CD4+ helper T cells in >30% of cases.

Markers	Expression
CD3	Positive
CD4	Positive
CD279 (PD1)	Positive
CD2	Positive
TCR-β	Positive

- **Prognosis of disease:** Sézary syndrome is an aggressive disease with a 5-year survival rate of 10–30%, depending on stage of the disease.
  - Majority of patients die of opportunistic infections involvement of lymph node, and viscera indicates poor prognosis. Degree of peripheral blood involvement at diagnosis affects prognosis.
  - Chromosomal translocations in non-Hodgkin's lymphoma are given in Table 13.35. Interpretation of verious non-Hodgkin's lymphomas is shown in Fig. 13.40.

Table 13.35 Chromosomal translocations in non-Hodgkin's lymphoma (NHL). Adapted from flow cytometry, immunohistochemistry and molecular genetics for hematologic and lymphoid neoplasms

Non-Hodgkin's Lymphoma (Histologic Variants)	Chromosomal Translocations	Genes Involved
Anaplastic large cell lymphoma	t(2;5) (p23;q35)	2ALK; 5NPM
Burkitt's lymphoma	▪ t(8;14) (q24;q32) ▪ t(2;8) (q12;q24) ▪ t(8;22) (q24;q11)	▪ 8-c-Myc, 14 IgH ▪ 2-kappa, 8-c-Myc ▪ 8-c-Myc, 22 lambda IgH

Contd...

Table 13.35 Chromosomal translocations in non-Hodgkin's lymphoma (NHL). Adapted from flow cytometry, immunohistochemistry and molecular genetics for hematologic and lymphoid neoplasms (*Contd...*)

Non-Hodgkin's Lymphoma (Histologic Variants)	Chromosomal Translocations	Genes Involved
Diffuse large B cell lymphoma (DLBCL)	▪ t(3;14) (q27;q32) ▪ t(14;18)	▪ IgH; BCL-6 (30% of cases) ▪ IgH BCL-2 (10–20% of cases)
Cutaneous T cell lymphoma	t(10;14) (q24;q32)	NFK, δ_2 (LYT-10); IgH
Burkitt-like lymphoma	t(14;18) (q32;q21)	IgH; BCL-2
Follicular lymphoma	t(14;18) (q32;q21)	14-IgH; BCL-2
Mantle cell lymphoma	t(11;14) (q13;q32)	BCL-1 (CCND-1); IgH, 11-cyclin D1
Lymphoplasmacytic lymphoma	t(19;14) (p13;q32)	PAX5 (BSAP); IgH
Marginal zone lymphoma (MALT lymphoma)	▪ t(11;18) (q21;q21) ▪ t(1;14) (p22;q32)	▪ AP12-MALT-1 ▪ BCL-10; IgG
Small cell lymphocytic lymphoma/chronic lymphocytic leukemia (CLL)	t(14;19) (q32;q13)	BCL-3, IgH
Plasma cell myeloma	▪ t(4;14) (p16;q32) ▪ t(14;16) (q32;q23) ▪ t(16;32) (q23;q11)	▪ FGFR3; IgH ▪ IgH; c-MAF ▪ c-MAF, Igδ

MALT: Mucosa-associated lymphoid tissue; CLL: Chronic lymphocytic leukemia.

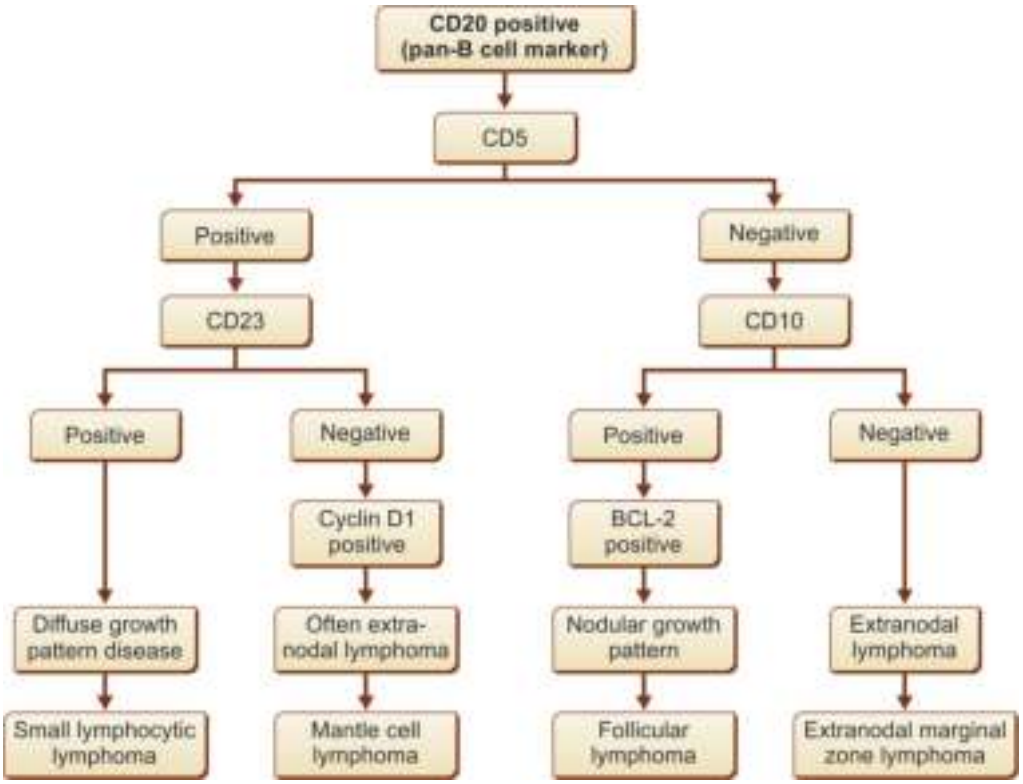


Fig. 13.40: Interpretation of various non-Hodgkin's lymphomas.

DISORDERS OF SPLEEN

SPLEEN: STRUCTURE

Spleen is the largest lymphoid organ. In normal adults, spleen weighs 50–200 g, which contributes in the maturation of red blood cells by pitting function. It has ability to remove solid particles from the cytoplasm of red blood cells without causing injury to the cell membrane.

- Normal red blood cells squeeze through slit pores in the sinusoids, but red blood cells with membrane defect (spherocytosis or elliptocytes) and sickle cells cannot squeeze resulting in red blood cells destruction in spleen termed extravascular hemolysis.
- Spleen is divided into white pulp and red pulp regions, separated by an ill-defined interface known as marginal zone. Organization of lymphoid tissue in the spleen is shown in Fig. 13.41.

WHITE PULP OF SPLEEN

The white pulp of spleen contains periarteriolar lymphoid sheath (PALS), germinal lymphoid follicles, which are surrounded by marginal zone. Marginal zone of spleen contains numerous macrophages, antigen-presenting cells (APCs), slowly recirculating B cells and natural killer cells. Periarteriolar lymph sheath contains T cells. B cells are distributed in the lymphoid follicles. In routine hematoxylin and eosin stained sections, white pulp appears basophilic due to dense heterochromatin in lymphocytes nuclei.

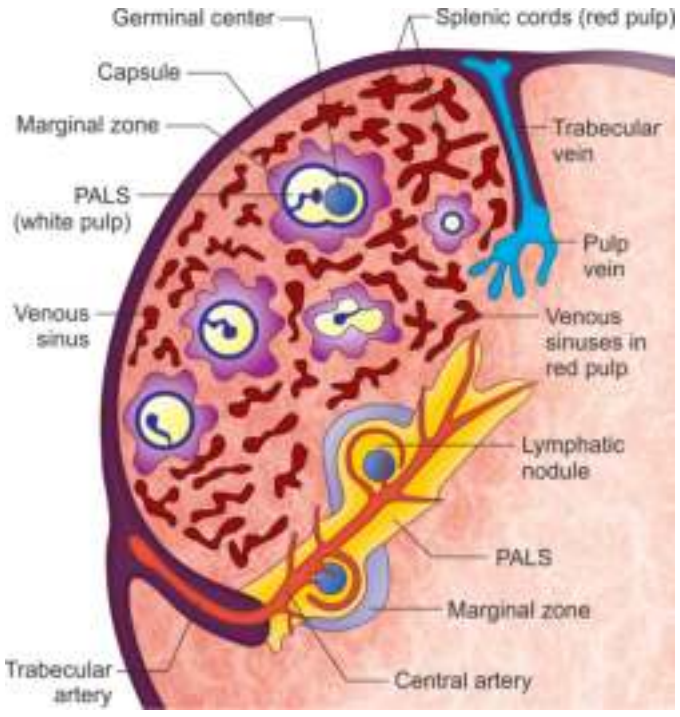


Fig. 13.41: Organization of lymphoid tissue in the spleen. Blood enters the tissue via the trabecular arteries, which give rise to the many-branched central arteries. Some of these end in the white pulp, supplying the germinal centers and mantle zones, but most empty into or near the marginal zones (PALS—periarteriolar lymphoid sheath).

RED PULP OF SPLEEN

The red pulp of spleen contains venous sinuses separated by splenic cords, which participates in the storage of red blood cells, white blood cells and platelets.

- When splenomegaly occurs, excessive pooling of red blood cells may cause fall in the peripheral blood count. Special endothelial cells in red pulp of spleen that express both reticular cells and histiocytic markers are known as **Littoral cells**.
- Blood enters into the spleen via the trabecular artery, which divides into many-branched central arteries. Some of these arteries end in the white pulp, supplying the germinal centers and mantle zones, but most empty into or near the marginal zones. The splenic venous outflow is drained into the portal vein, adding as rich supply of antibodies to the portal vein entering the liver.

FUNCTIONAL DISORDERS OF SPLEEN

THERAPEUTIC SPLENECTOMY

Therapeutic splenectomy is performed in idiopathic thrombocytopenic purpura, chronic myeloproliferative disorders, Hodgkin's disease and non-Hodgkin's lymphoma. Following splenectomy, children under the age of three years are susceptible to infection by pneumococci, streptococci group A, enteric group and *Haemophilus influenzae*, therefore, resulting in septicemia, meningitis and disseminated intravascular coagulation (DIC).

SPLENOMEGALY

Spleen is most often enlarged due to infections and congestive state. Red pulp congestion is most common finding. Massive splenomegaly occurs due to chronic myelogenous leukemia, myelofibrosis, kala-azar, malaria, Gaucher's disease and hepatosplenic T cell lymphoma. Conditions associated with splenomegaly are given in Table 13.36.

FIBROCONGESTIVE SPLENOMEGALY

Fibrocongestive splenomegaly most often occurs in portal hypertension due to cirrhosis and right-sided cardiac failure with cor pulmonale. Decreased portal venous drainage in these disorders leads to fibro-congestive splenomegaly.

Table 13.36 Conditions associated with splenomegaly

Categories	Conditions
Bacterial infections	Infectious endocarditis, tuberculosis, syphilis
Viral infections	Infectious mononucleosis, cytomegalovirus
Parasitic infection	Malaria
Fungal infection	Histoplasmosis
Congestive states	▪ Cirrhosis ▪ Congestive heart failure ▪ Splenic vein thrombosis
Hematologic malignancies	▪ Myeloproliferative disorders ▪ Hodgkin's disease, non-Hodgkin's lymphoma, hepatosplenic T cell lymphoma, splenic marginal B cell lymphoma, mantle cell lymphoma, hairy cell leukemia, multiple myeloma
Immune-related conditions	Systemic lupus erythematosus, rheumatoid arthritis, Gaucher's disease

- The increased portal venous pressure causes dilatation of sinusoids, with slowing of blood flow from the cords to the sinusoids that prolongs the exposure of the blood cells to the cordal macrophages, resulting in excessive trapping and destruction (hypersplenism).
- Perivascular hemorrhages result in organization and formation of Gamna-Gandy bodies (dystrophic calcification).

Surgical Pathology: Fibrocongestive Splenomegaly

Gross Morphology

- Spleen shows irregular tan-white fibrous plaques over the purple capsular surface.
- Cut surface of fibrocongestive splenomegaly shows firm and brown fibrotic nodules termed Gamna-Gandy bodies.

Light Microscopy

Gamna-Gandy body is an organized hemorrhage forming nodule with dystrophic calcification and hemosiderin pigment in spleen (Fig. 13.42).

HYPERSPLENISM

Hypersplenism refers to destruction of one or more blood cell lines by the spleen, which is characterized by splenomegaly, anemia, leukopenia, thrombocytopenia, bone marrow hyperplasia.

- Cytopenias occur due to sequestration of blood elements in the spleen, which can be corrected by splenectomy.
- Diagnostic criteria for hypersplenism include cytopenia of one or more cell lines, bone marrow hyperplasia, splenomegaly and correction of cytopenias following splenectomy. Disorders associated with hypersplenism are given in Table 13.37.

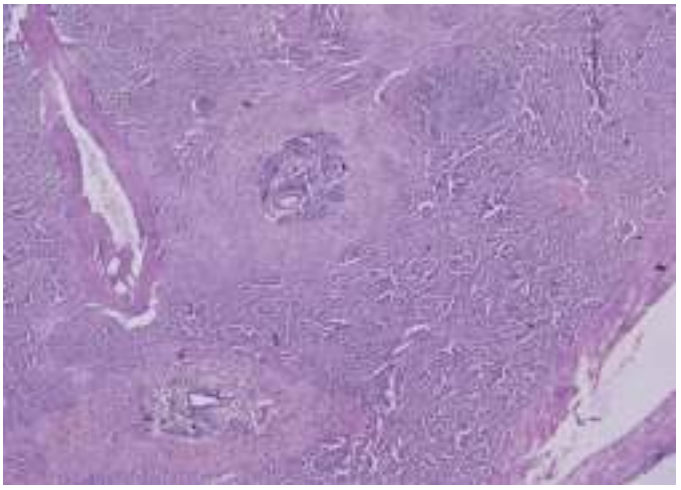


Fig. 13.42: Chronic venous congestion of spleen shows formation of Gamna-Gandy bodies as a result of organization of hemorrhage with dystrophic calcification and hemosiderin pigment in spleen (400X).

AUTOSPLENECTOMY

Sickle cell disease is important cause of autosplenectomy, which leads to susceptibility to disseminated infection with encapsulated bacteria such as pneumococci, meningococci, and *Haemophilus influenzae*.

HYPOSPLENISM

Hyposplenism refers to absence of functioning of spleen following splenectomy. Hematologic findings suggestive of hyposplenism are presence of Howell-Jolly bodies, poikilocytosis, target cells, acanthocytes, and nucleated red blood cells in the peripheral blood smear. Other causes of hyposplenism are Fanconi anemia and sickle cell disease, infiltrative disorders involving spleen and old age.

Table 13.37 Disorders associated with hypersplenism

Mechanism	Disorders
Abnormal sequestration of blood cells with intrinsic defects in normal spleen	Inherited disorders (hereditary spherocytosis and elliptocytosis), malaria, autoimmune hemolytic anemia, autoimmune thrombocytopenia and/or neutropenia
Sequestration of normal blood cells in abnormal spleen	▪ Gaucher's disease ▪ Niemann-Pick disease ▪ Langerhans' cell histiocytosis ▪ Chronic congestive splenomegaly ▪ Malignant infiltrative disorders (CML, AML, splenic marginal B cell lymphoma, mantle cell lymphoma, hairy cell leukemia, hepatosplenic T cell lymphoma, Hodgkin's disease, plasma cell dyscrasias, metastatic carcinoma) ▪ Extramedullary hematopoiesis (severe hemolytic states, chronic idiopathic myelofibrosis) ▪ Chronic infections (tuberculosis and brucellosis) ▪ Vascular tumors ▪ Splenic cysts ▪ Hamartomas
Miscellaneous conditions	▪ Hypogammaglobulinemia ▪ Hypothyroidism ▪ Progressive multifocal leukoencephalopathy

SPLENOSIS

Splenosis refers to splenic implants of splenic tissue over the peritoneal surface, pleural surface, and lung parenchyma following trauma or surgical procedure for splenectomy.

SPLENIC RUPTURE

Splenic rupture most often results from blunt injury to abdominal organs. The hemorrhage can extend into the peritoneal cavity to produce hemoperitoneum. Spontaneous rupture of spleen following minor trauma may occur due to malaria, infectious mononucleosis, subacute bacterial endocarditis, lymphoid neoplasms and typhoid fever.

HEMOSIDEROSIS OF SPLEEN

Thalassemia major is important cause of hemosiderin deposition in spleen, liver, pancreas, myocardium, adrenal glands and pituitary gland. The hemosiderin is deposited as refractile granular golden brown pigment in macrophages lining sinusoids in spleen. Hemosiderin is demonstrated by Pearl's Prussian stain.

REACTIVE SPLENIC DISORDERS

GRANULOMATOUS INFLAMMATION OF SPLEEN

Granuloma in spleen is focal benign process ranging from lipogranulomatous inflammation to caseating and noncaseating inflammation. Caseating granulomas occur due to Mycobacterium tubercle bacilli, fungal infection and X-linked chronic granulomatous disease. Noncaseating granulomas are most often demonstrated in sarcoidosis.

SPLENIC INFARCT

Splenic infarct occurs as a consequence of systemic arterial embolization in a patient with an infective endocarditis involving either aortic or mitral valve.

Wedge-shaped Splenic Infarct

Most splenic infarcts are due to emboli that arise from thrombi in the heart, either cardiac vegetations on valves or mural thrombi.

- Emboli from infective endocarditis lead to septic infarct in spleen, which reach the spleen via splenic artery and obstruct splenic branches resulting in ischemic wedge-shaped infarct with base towards capsule.
- The remaining splenic parenchyma appears dark red. This wedge-shaped infarct gets replaced by granulation tissue resulting into fibrous scar. Patient presents with left upper quadrant pain and splenic enlargement.

Nonwedge-shaped Splenic Infarcts

Nonwedge-shaped splenic infarcts are most often occur in essential thrombocythemia and myelofibrosis. Less common causes are paroxysmal nocturnal hemoglobinuria, sickle cell anemia, aplastic anemia, polyarteritis nodosa and splenic artery aneurysm.

NON-NEOPLASTIC INFILTRATIVE DISORDERS OF SPLEEN

Non-neoplastic disorders involving the spleen are given in Table 13.38.

GAUCHER'S DISEASE INVOLVING SPLEEN

The enlarged Gaucher's spleen is pale and has a firm feel. It is an autosomal recessive disorder error of metabolism due to lack of the enzyme glucocerebrosidase, resulting in accumulation of storage product in cells of the mononuclear phagocyte system.

Table 13.38 Non-neoplastic disorders involving the spleen

Pattern of Involvement of Spleen	Non-neoplastic Disorders
White pulp involvement (reactive lymphoid hyperplasia with or without germinal centers)	▪ Castleman disease ▪ Common variable immunodeficiency state ▪ Autoimmune lymphoproliferative syndrome
Red pulp involvement	▪ Gaucher's disease ▪ Niemann-Pick disease ▪ Extramedullary hematopoiesis
Cystic lesions	▪ *Echinococcus granulosus* ▪ *Histoplasma capsulatum* ▪ Coccidioidomycosis ▪ Miliary tuberculosis ▪ Lipogranuloma
Granulomatous disorders	▪ Infectious mononucleosis (EB virus) ▪ Malarial parasite ▪ *Mycobacterium avium intracellulare* ▪ Cytomegalovirus ▪ Pyogenic bacterial infections

- **Hallmark of Gaucher's disease:** The hallmark of Gaucher's disease is the presence of Gaucher's cells with a distinctive cigarette paper-like cytoplasmic appearance and eccentric nuclei.
  - Gaucher's cells are lipid-laden macrophages present in the red pulp of the spleen, liver sinusoids (Kupffer cells), lymph nodes, lungs (alveolar macrophages), and bone marrow.
  - Gaucher's cells contain specific enzymes such as chitotriosidase and angiotensin-converting enzyme. These Gaucher's disease enzyme markers of macrophage proliferation assay are required to confirm the diagnosis.
- **Clinical variants of Gaucher's disease:** Gaucher's disease has three variants: adult type (normal life span), infantile type (fatal disease) and juvenile type (less severe).

SPLEEN INVOLVEMENT IN AMYLOIDOSIS

Deposition of amyloid in spleen results in splenomegaly. Gross examination shows diffuse lardaceous pattern or the nodular sago pattern due to amyloid deposition (AL or AA) in **white pulp**.

NEOPLASTIC DISORDERS OF SPLEEN

Considering spleen's size and blood flow, the spleen is uncommon site for either primary hematologic malignant disorders (leukemias) or metastatic malignancies (extranodal Hodgkin's disease and NHL) due to immunological role of spleen.

- Spleen involved due to Hodgkin's and non-Hodgkin lymphoma (NHL) shows multiple nodular pale deposits.
- Splenic masses are more likely due to hematologic diseases (leukemias) rather than metastases. Neoplastic disorders of spleen according to recent 2024 World Health Organization are given in Table 13.39.

Table 13.39 Neoplastic disorders of spleen according to recent 2024 World Health Organization

Disorder	Origin	Spleen Morphology	Other Characteristics
Primary splenic lymphoma			
Primary splenic lymphoma	B or T cells	Diffuse large B cell lymphomas (origin germinal center or post-germinal center B cell) with single or multiple nodules of varying size	Diverse chromosomal rearrangement, BCL-6 (30%), BCL-2 (10%) and Myc (5%)
Secondary splenic lymphomas			
Hodgkin's disease (classic variant)	B cells Reed-Sternberg cells are CD30+ (Ki-1) specific marker, CD15+ (Leu M1)	Splenomegaly	Prognosis varies depending on variants and staging

Contd...

Table 13.39 Neoplastic disorders of spleen according to recent 2024 World Health Organization (*Contd...*)

Disorder	Origin	Spleen Morphology	Other Characteristics
Low grade non-Hodgkin's lymphoma	B or T cells	Splenomegaly (single or multiple nodules)	50% of cases show splenomegaly and splenic lymph nodes involvement
Lymphomas presenting with prominent lymphadenopathy			
Splenic marginal B cell lymphoma	B cells	Massive splenomegaly (infiltration in both red and white pulp)	Allelic loss of chromosome 7q31–32 in 40% of cases, trisomy 3 in rare cases, IgH clonally rearranged Peripheral cytopenias
Mantle cell lymphoma	Naïve B cell	Prominent splenomegaly	t(11;14) creating cyclin D1-IgH fusion gene
Hairy cell leukemia	Memory B cell	Prominent splenomegaly	Activating FRAF mutations, rearrangement of IgH
Hepatosplenic T cell lymphoma	T cell	Triad of peripheral cytopenias, sinusoidal tropism and massive splenomegaly (>3000 gm)	Isochrome 7q10 and trisomy 8 strong genetic abnormality
Myeloid neoplasms involving spleen			
Chronic myeloid leukemia with blast crisis	Pluripotent stem cell	▪ Leukemic cells are confined to bone marrow and spleen. Bone marrow and blood contain <10% myeloblasts ▪ Anemia, massive splenomegaly and hepatomegaly. Splenomegaly correlates with magnitude of leukocytosis	Philadelphia chromosome t(9;22)
Acute myeloid leukemia	Myeloid cell	Anemia, neutropenia (recurrent upper respiratory tract infections) and thrombocytopenia (bleeding tendencies), bone tenderness, mild to moderate hepatomegaly and splenomegaly	AML with recurrent genetic alterations
Mast cell disease with systemic mastocytosis	Mast cell	Spleen frequently involved (both red and white pulp involved)	Flow cytometry helpful in establishing diagnosis

SPLENIC MARGINAL ZONE LYMPHOMA

Splenic marginal zone lymphoma (SMZL) is derived from B cell and composed of small lymphocytes replacing splenic white pulp germinal centers and effacing follicle mantle zone. There is presence of transformed blasts, both small and large cells in the red pulp of spleen. SMZL most often involves spleen, splenic hilar lymph nodes, bone marrow and peripheral blood. SMZL cells are frequently demonstrated in the peripheral blood as villous lymphocytes.

- **Epidemiology:** Splenic marginal zone lymphoma constitutes 2% of lymphoid neoplasms, which most often involves adults >50 years of age. Both men and women are equally affected.
- **Localization of disease:** Splenic marginal zone B cell lymphoma involves spleen, splenic hilar lymph nodes, bone marrow and peripheral blood.
- **Clinical features:** Patient most often presents with splenomegaly and sometimes, autoimmune thrombocytopenia and peripheral blood villous lymphocytes. Bone marrow is involved in almost all cases. Peripheral lymphadenopathy is extremely uncommon.

Surgical Pathology: Splenic Marginal Zone Lymphoma

Gross Morphology

Gross examination of the splenic marginal zone lymphoma shows marked expansion of white pulp and infiltration of red pulp by tumor cells.

Light Microscopy

- Normal architecture of spleen is affected by neoplastic cells present in white pulp's mantle zone.
- Tumor cells are small- and medium-sized with dispersed chromatin and pale cytoplasm admixed with transformed cells.

Immunophenotyping

- Tumor cells express surface IgM and IgD. These tumors are positive for CD20 and CD79a.
- Ki-67 staining shows targetoid pattern due to increased growth fraction in both the germinal center as well as marginal zone.
- Tumor cells are negative for CD5, CD10, CD 23, CD43 and annexin-A1.
- SMZL is distinguished by the presence of lymphocytosis and diffuse infiltration of bone marrow by tumor cells.

Markers	Expression
Surface IgM and IgD	Positive
CD20	Positive
CD79a	Positive
Ki-67	Positive
CD5	Negative
CD10	Negative
CD23	Negative
CD43	Negative
Annexin-A1	Negative

- **Molecular genetic alterations:** Antigen receptor genes, e.g. immunoglobulin heavy and light chains, show clonal rearrangements in 50% of splenic marginal zone lymphoma (SMZL) cases. Somatic hypermutation of antigen receptor gene is demonstrated in half of the patients.
  - SMZL lacks recurrent chromosomal translocations.
  - NOTCH2 gene mutation is demonstrated in 10–25% of cases.
  - Few cases of SMZL show recurrent t(2;7) (p12; q21) translocations.
- **Prognosis of disease:** Clinical course of splenic marginal zone lymphoma is indolent with a 10-year survival in >70% of cases. Approximately 10–15% of cases undergo transformation to diffuse large B cell lymphoma (DLBCL) associated with poor prognosis.
- Prognosis is poor in cases with poor general health and large tumor mass.
- NOTCH2, TP53 and KLF2 gene mutations are adverse prognostic factors.

METASTASES IN SPLEEN

Despite its size, the spleen is a rare site for metastases from nonhematological malignancies. Melanomas are aggressive neoplasms that can often be widely metastatic. Most of these masses are tan, but some have brown-black pigmentation from the melanin elaborated by the neoplastic cells.

The most common epithelial malignancies metastasizing to spleen are carcinomas of breast and lung origin. Sarcomas involving the spleen tend to be of dendritic/histiocytic or vascular lineage. Carcinoma metastasizing to spleen is shown in Fig. 13.43.

ANGIOSARCOMA OF SPLEEN

Angiosarcoma is malignant proliferation of primitive mesenchymal cells with vascular differentiation.

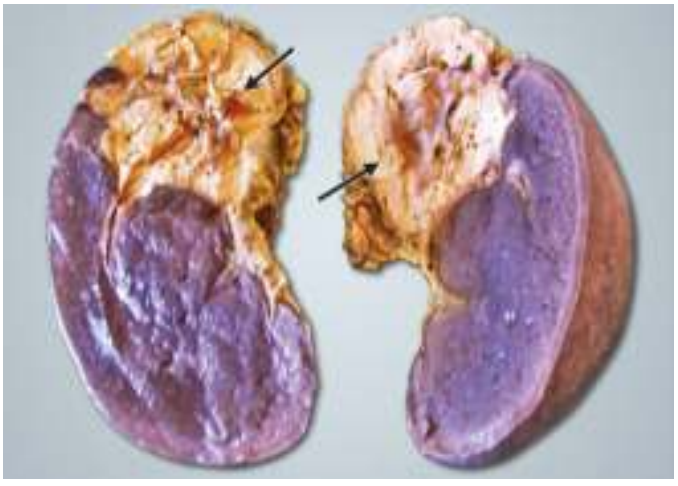


Fig. 13.43: Carcinoma metastasizing to spleen (arrows).

DISORDERS OF THYMUS GLAND

ANATOMY AND HISTOLOGY OF THYMUS GLAND

ANATOMY

Thymus gland situated in the anterior mediastinum of the chest is prominent during third trimester (late fetal life), infancy and childhood. It is populated with T cells. During late fetal life, progenitor cells of bone marrow origin migrate to the thymus and give rise to mature T cells that are exported to the peripheral lymphoid organs. Thymus undergoes atrophy during adulthood. Anterior compartment is most common site for mediastinal masses in >50%. Primary tumors of the mediastinum include thymoma, neurogenic tumors, germ cell tumors and lymphoma. Primary lung cancer can invade mediastinum. Summary of mediastinal masses is given in Table 13.40.

HISTOLOGY

On histologic examination, thymus gland consists of cortex and medulla. Cortex consists of large cells with prominent nucleoli in the superficial zone. In the

Table 13.40 Summary of mediastinal masses

Mediastinum Compartment	Pathological Disorders
Anterior mediastinum	Thymic tumors, retrosternal thyroid mass, germ cell tumors, lymph nodes (lymphomas), cystic hygroma, aortic aneurysm, hernia (Morgagni), pericardial cysts, sternal masses
Middle mediastinum	Lymph nodes (sarcoidosis, tuberculosis, lymphomas, metastases), aortic aneurysm, mediastinal paraganglioma, carcinoma bronchus, fatty mediastinal tumors, mediastinal lipomatosis, hiatal hernia
Posterior mediastinum	Myeloma, metastases, aortic aneurysm, sympathetic ganglion cell tumors, lateral thoracic meningocele, paravertebral abscess, extramedullary hematopoiesis, pancreatic pseudocyst

deeper zone, the cells are small with less prominent nucleoli. Medulla consists of spindle cells with densely stained nuclei and inconspicuous nucleoli. Hassall corpuscles composed of epithelial cells are present in the center of the medullary regions. These become keratinized on enlargement. Thymus also consists of macrophages, dendritic cells, a few B cells; rare neutrophils and eosinophils.

THYMUS HYPERPLASIA

Normally, adult thymus is composed mostly of adipose tissue, with a few clusters of lymphocytes and residual Hassall corpuscles. Approximately 75% of patients diagnosed with myasthenia gravis demonstrate thymic hyperplasia composed of lymphoid cells associated with autoantibody production. These autoantibodies bind to acetylcholine receptor and diminish the receptor function in the skeletal muscle motor end plates, resulting in onset of muscular weakness, particularly with repetitive muscular contraction (Fig. 13.44).

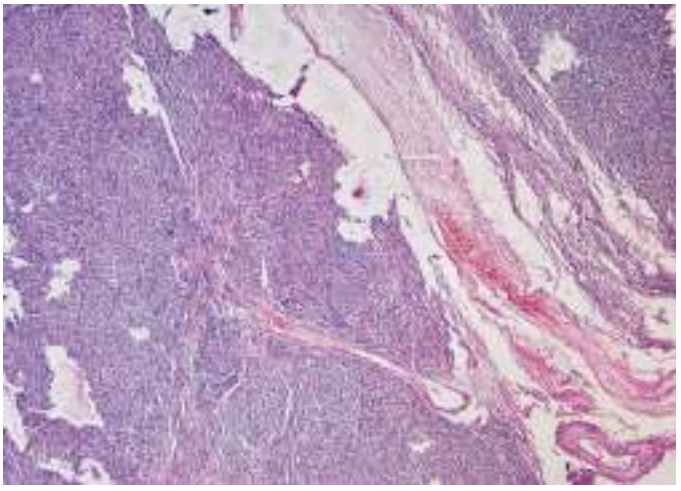


Fig. 13.44: Thymic hyperplasia is composed of lymphoid cells (100X).

Clinical Features

Affected children develop recurrent infections (bacterial, fungal and viral) and tetany from hypoparathyroidism with hypocalcemia.

IMMUNODEFICIENCY DISORDERS OF THYMUS GLAND

DiGEORGE SYNDROME

In DiGeorge syndrome, there is failure of maturation of T cells, but B cells remain unaffected. Thymus gland shows hypoplasia.

- In DiGeorge syndrome, aberrant embryonic development of third and fourth branchial arches, results in hypoplasia of thymus and parathyroid glands as well as anomalies of aortic arch, mandible and ear.
- It can be summed up by **CATH22**, which denotes cardiac defects, abnormal facies, thymic hypoplasia, cleft palate, hypocalcemia and microdeletion of chromosome **22q11**.
- In about 30% of cases, this syndrome is also associated with behavior disorders and psychosis (bipolar disorder and schizophrenia) that develop during adolescence.

WISKOTT-ALDRICH SYNDROME (X-LINKED DISORDER)

Wiskott-Aldrich syndrome is X-linked disorder associated with defects of both B cell and T cell functions (humoral and cellular immunity). It is associated with thymic hypoplasia, eczema, thrombocytopenia, bloody diarrhea, recurrent infections and poor antibody response to polysaccharide antigens. Patient has fatal outcome before six years of age due to bleeding, infection or malignancy (most often lymphoma).

Surgical Pathology: Wiskott-Aldrich Syndrome

Light Microscopy

Thymus gland shows hypoplasia characterized by absence of thymocytes. A few Hassall corpuscles, and only epithelial components may be present.

THYMIC NEOPLASMS

Neoplasms of the thymus gland are thymoma, thymic carcinoma and neuroendocrine tumors including thymic carcinoid. Revised 2024 WHO classification of tumors of thymus gland is given in Table 13.41.

Table 13.41 Revised 2024 WHO classification of tumors of thymus gland

Thymoma	
▪ Thymoma type A	▪ Micronodular thymoma
▪ Thymoma type AB	▪ Microscopic thymoma (rare)
▪ Thymoma type B1	▪ Sclerosing thymoma (rare)
▪ Thymoma type B2	▪ Lipofibroadenoma (rare)
▪ Thymoma type B3	▪ Thymoma C
▪ Metaplastic thymoma	
Thymic Carcinoma	
▪ Squamous cell carcinoma	▪ Lymphoepithelioma-like carcinoma
▪ Basaloid carcinoma	▪ NUT carcinoma
▪ Mucoepidermoid carcinoma	▪ Undifferentiated carcinoma
▪ Sarcomatoid carcinoma	▪ Adenosquamous carcinoma (rare)
▪ Clear cell carcinoma	▪ Hepatoid carcinoma (rare)
▪ Adenocarcinoma (papillary, mucinous, not otherwise specified and adenoid cystic subtypes)	▪ Thymic carcinoma, not otherwise specified
Thymic Neuroendocrine Tumors	
▪ Carcinoid tumor (typical and atypical)	
▪ Large cell neuroendocrine carcinoma (combined large cell neuroendocrine carcinoma)	
▪ Small cell neuroendocrine carcinoma (combined small cell neuroendocrine carcinoma)	
Ectopic Tumors of Thymoma	
▪ Ectopic thyroid tumor	▪ Other rare ectopic tumors
▪ Ectopic parathyroid tumor	

Adapted from 2024 WHO classification of tumors of thymus gland.

THYMOMA

Thymoma is most common primary anterior mediastinal tumor. It is most often located in anterosuperior mediastinum.

- All thymomas, regardless of histology, are associated with invasion and metastases. Reliable indicator of thymoma malignancy is capsular invasion by tumor cells.
- In recent years, histologic classification of thymoma and clinical staging are considered important prognostic factors.
- Poor prognostic factors for thymoma include high stage, B3 or C classification, invasion of capsule and positive tumor margins. Weakly overexpression of podoplanin may predict lymph node metastasis and poor clinical course.
- Thymoma may develop in newborns to elderly persons of >90 years of age.
- There is some correlation between WHO classification of histologic variants of thymoma having aggressive behavior with invasive features. Revised 2024 WHO classification of thymomas (A, B1, B2, B3, AB and C system) is given in Table 13.42.

Clinical Features

Patient presents with clinical manifestations associated with myasthenia gravis such as muscular weakness of the arms and legs, facial drooping of upper eyelid, difficulty in speaking swallowing, double vision and

Table 13.42 WHO classification of thymomas (A, AB, B1, B2, B3 and C system)

WHO Type Categories	Histologic Type	Morphologic Features	Frequency (%)	Invasiveness (%)
A	Medullary type thymoma	▪ Predominantly spindle cells arranged in diffuse or hemangiocytic-like pattern ▪ Lymphocytes absent	8	10
AB	Mixed thymoma	Mixed spindle and epithelial cells	30	40
B1	Predominantly cortical thymoma	▪ Lymphocytes rich without atypical epithelial cells with or without Hassall's corpuscles ▪ Morphology of lymphocytes similar to normal thymus glands	15	45
B2	Cortical thymoma	▪ Higher epithelial cell/lymphocyte ratio with presence of more atypical neoplastic cells ▪ Epithelial cells are polygonal forming lobules and separated by immature T cell	28	70

Contd...

Table 13.42 WHO classification of thymomas (A, AB, B1, B2, B3 and C system) (*Contd...*)

WHO Type Categories	Histologic Type	Morphologic Features	Frequency (%)	Invasiveness (%)
B3	Well differentiated thymic carcinoma	▪ Atypical epithelial cells, polygonal in shape ▪ Atypical epithelial cells are arranged in sheets with minimal lymphocyte components	11	85
C	Nonorganized thymic carcinoma	Nonorganized thymic carcinoma with squamous, lymphoepithelial, clear cell, mucoepidermoid, basaloid, papillary, mucinous or sarcomatoid differentiation	5	5

Adapted from 2024 WHO classification.

fatigue, symptoms related to tumor mass such as chest pain, cough, hoarseness of voice, and pneumonia; and nonmyasthenic paraneoplastic syndrome.

- Thymomas may be associated with pure red cell aplasia, hypogammaglobulinemia, lymphopenia, rheumatoid arthritis, scleroderma, Sjögren's syndrome and systemic lupus erythematosus.
- Significant number of thymoma cases may be asymptomatic, and are diagnosed during routine X-ray chest, CT scan, cardiothoracic surgery and autopsy findings.
- Approximately 65–75% patients are often associated with myasthenia gravis due to autoantibodies against acetylcholine receptors in the motor end plates of skeletal muscles (type II hypersensitivity reaction).
  - The antibodies bind to postsynaptic receptors and block neurotransmission resulting in progressive skeletal muscle weakness involving particularly the external ocular, eyelids and proximal limb muscles. It may cause death due to respiratory muscles paralysis.
  - Thymectomy may reduce the plasma concentrations of autoantibodies and improve clinical picture.

Surgical Pathology: Thymoma

Gross Morphology

- Tumor is well circumscribed, encapsulated and firm.
- Invasive thymoma has poorly-defined edges and tend to ensheath blood vessels and neighboring organs within the mediastinum.
- Average weight of tumor is 150 g. It may weigh up to several kilograms.

Light Microscopy

- Thymoma is composed of neoplastic epithelial cells admixed with non-neoplastic thymic lymphocytes (Fig. 13.45).
- Histologic features of thymoma depend on morphology of cells based on categories defined by revised 2024 WHO classification (A, B1, B2, B3, AB and C). Histologic variant A of thymoma has indolent variant. Other B2 and B3 types are more invasive. Thymic carcinoma or type C is regarded as separate distinctive category of thymoma. Neoplastic epithelial cells may be either spindle on nonspindle shape. Clinicopathological staging of thymoma according to Masaoka is given in Table 13.43.
  - Spindle neoplastic epithelial cells of thymoma are demonstrated in A and AB subtypes. This variant has indolent behavior. It may be associated with hematological malignancies.
  - Nonspindle neoplastic epithelial cell variant of thymoma is also known as cortical thymoma. Epithelioid neoplastic cells are demonstrated in B1, B2 and B3.
- Further subdivision of thymoma depends on the content of neoplastic epithelial cells and non-neoplastic immature T cells.
  - Type A thymoma is composed of densely packed spindle cells admixed with immature T cells.
  - Type B3 thymoma shows sheets of polygonal neoplastic epithelial cells.
  - Types AB, B1 and B2 thymomas show variable and reciprocal content of immature T cells.
- Confirmation of immature T cells is confirmed by CD1a, TdT and CD99.

Immunophenotyping

Neoplastic cells of thymoma show positivity for CK7, CD15 and BCL-2.

Markers	Thymoma According to WHO (ABC System)	Expression
▪ CK7 ▪ CD15 ▪ BCL-2	▪ Type A ▪ Type A ▪ Types A and C	▪ Positive ▪ Positive ▪ Positive

- CK5, CK6 and CK7 are positive in both thymoma and thymic carcinoma.
- CK18, CD5, CD117, MUC1 and GLUT-1 are positive in thymic carcinoma.

- **Laboratory diagnosis:** Most thymomas can be detected by imaging techniques, and confirmed on histologic examination of biopsy from thymoma. Features for laboratory diagnosis of thymoma are given in Table 13.44. Thymoma on imaging technique is shown in Fig. 13.46.

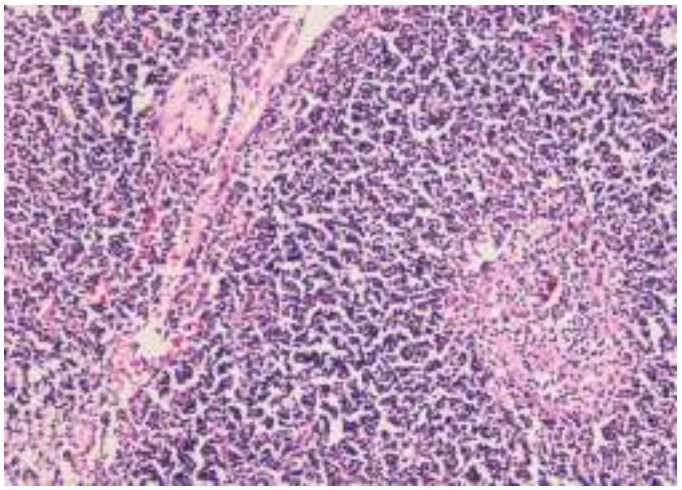


Fig. 13.45: Thymoma is a neoplasm of thymic epithelial cells. Cytologically, bland epithelial cells are admixed with non-neoplastic thymic lymphocytes (100X).

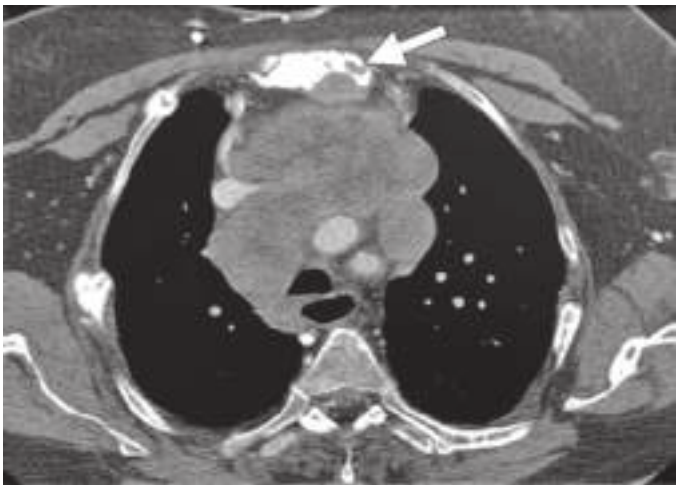


Fig. 13.46: Thymoma on imaging technique. Thymoma presents as sharply demarcated round to oval soft tissue mass in the anterior mediastinum compartment.

Table 13.43 Clinicopathological staging of thymoma according to Masaoka

Stages	Qualifying Features	5-year Survival (%)
I	Completely encapsulated tumor without invasion of capsule	95–100
II	Gross invasion of tumor into surrounding soft tissues or mediastinal pleura and/or microscopic invasion into capsule	80–85
III	Gross invasion of tumor into pericardium, lung and great vessels (intraoperative biopsy obtained for confirmation)	60–70
IVa	Dissemination of tumor to pericardium and/or pleura without contiguous spread as in stage III	40–50
IVb	Distant metastases of tumor to lung, liver, bone and skin	25–30

Table 13.44 Features for laboratory diagnosis of thymoma

Parameters	Diagnostic Features
Terminal deoxynucleotidyl transferase (TdT)	Positive in all stages
T cell markers	Positive for CD1, CD2, CD3, CD4, CD5, CD7, CD8 depend on the stage of thymoma
Common thymocyte stage, positivity for	CD1, CD4, CD8 (most common)
CD117 (KIT)	▪ Positive for thymic carcinoma ▪ Negative for thymoma
Immunophenotyping	▪ Positive for thymic carcinoma ▪ Negative for leukocyte common antigen (LCA)
Electron microscopy	Presence of desmosomes and tonofilaments
TCR gene rearrangement	Germline or deletion

- **Treatment:** Diagnosis and treatment of thymoma are best achieved by complete excision of thymus gland along with tumor.

THYMIC CARCINOMA

Thymic carcinoma is a rare but highly aggressive and easily metastasizing neoplasm derived from thymic epithelial cells. It constitutes 5% of all primary neoplasms of thymus gland. It occurs most frequently in adults between 30 and 60 years of age. It arises *de novo* in the thymus gland.

- Thymic carcinoma presents as multilocular cyst. Unlike thymoma, thymic carcinoma is associated with paraneoplastic syndrome.
- Thymic carcinoma is almost always found at an advanced stage because patients often have atypical symptoms. Gross morphology of thymic carcinoma is shown in Fig. 13.47.

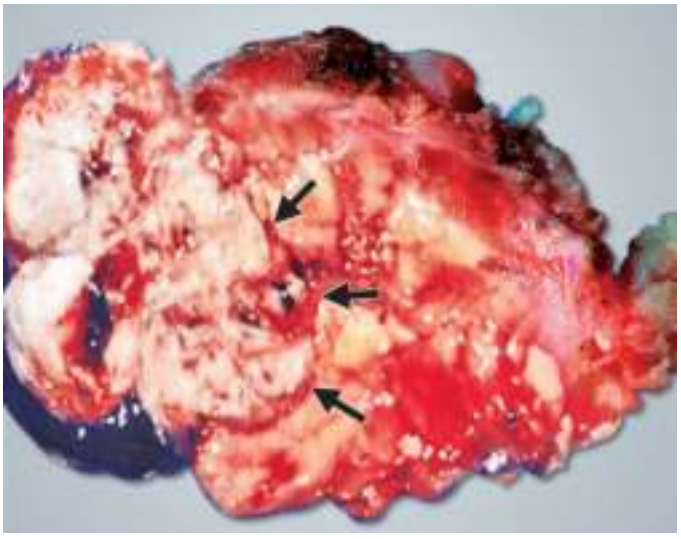


Fig. 13.47: Gross morphology of thymic carcinoma. Cut surface of thymic carcinoma reveals gray-white, firm to hard, gritty with areas of hemorrhage and necrosis (arrows).

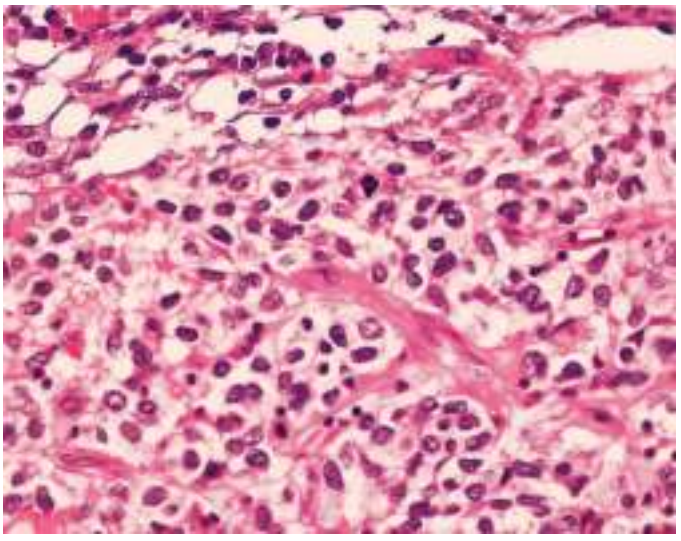


Fig. 13.48: Thymic carcinoma is composed of undifferentiated neoplastic cells (400X).

Surgical Pathology: Thymic Carcinoma

Gross Morphology

Thymic carcinoma is uncapsulated tumor without internal fibrous separation. Cut surface is gray-white, firm to hard, and gritty with areas of hemorrhage and necrosis.

Light Microscopy

- Most common histologic variant of thymic carcinoma is composed of undifferentiated neoplastic cells with or without squamous differentiation.
- There may diverse histopathologic features such as basaloid, adenoid cystic, papillary, clear cell, lymphoepithelial-like or adenocarcinoma type (Fig. 13.48).

Immunophenotyping

Immunophenotyping of thymic carcinoma is shown below. Differential diagnosis of atypical thymoma, thymic carcinoma and lung carcinoma is given in Table 13.45.

Markers	Expression
▪ BCL-2	▪ Positive
▪ CK5	▪ Positive
▪ CK6	▪ Positive
▪ CK7	▪ Positive
▪ CK18	▪ Positive
▪ CD5	▪ Positive
▪ CD117	▪ Positive
▪ MUC1	▪ Positive
▪ GLUT-1	▪ Positive

CK5, CK6 and CK7 are positive in both thymoma and thymic carcinoma.

- Complete resection of the thymic carcinoma is the mainstay of treatment and leads to the best survival rate for patients. However, the complete resection rate is only approximately 50% and the recurrence rate after complete resection is high, up to 40%.

Table 13.45 Differential diagnosis of atypical thymoma, thymic carcinoma and lung carcinoma

Immunophenotyping	Atypical Thymoma	Thymic Carcinoma	Lung Carcinoma
CK7	Positive	Positive	Positive/negative
CD5	Negative	Positive	Negative
CD117	Negative	Positive	Negative
CD1a	Positive in immature thymocytes	Negative	Negative
TTF-1	Negative	Negative	Positive
CD205	Positive	Positive	Positive/negative
FOXN1	Positive (nuclear staining)	Positive (nuclear staining) or negative	Negative

Annexure

Diagnostic workup of Hodgkin disease

- **Clinical history:** Patient (young or >55 years) presents with painless, discrete, and swollen multiple lymph nodes in cervical region, chest pain, cough, shortness of breath and constitutional B symptoms such as intermittent fever >38°C, night sweats, unexplained weight loss (>10% within 6 months), fatigue, recurrent infections, and pruritus. There is family history of development of nodular sclerosis classic variant Hodgkin disease.
- **Physical examination:** Physical examination revealed multiple, painless, and palpable lymph nodes in cervical, axillary, and inguinal regions. Chest tenderness, wheezing and superior vena cava syndrome indicate compression by thoracic mass. Abdominal examination reveals abdominal tenderness, splenomegaly, and hepatomegaly.
- **Imaging techniques:** Ultrasonography and thorax and abdominal computed tomography scan of revealed multiple lymph nodes in the neck, supraclavicular region, and mediastinum. Fluorodeoxyglucose positron emission tomography (FDG-PET) scan revealed marked hypermetabolic lymph nodes, multiple scattered consolidation involving the bones.
- **Laboratory investigations:** The laboratory results showed increased sedimentation rate and C-reactive proteins and neutrophilic leukocytosis.

1. **What is the most likely diagnosis?**

   Answer: Hodgkin disease. Hodgkin disease is follicular center B cell lymphoid malignancy and has two distinct entities: (a) **classic variants of Hodgkin disease** with positive CD30 and CD15 markers (e.g. nodular sclerosis, mixed cellularity, lymphocyte depletion, and lymphocyte-rich); and (b) **nodular lymphocytic-histiocytic predominant variant of Hodgkin disease** with negative CD30 and CD15 markers. Fluorodeoxyglucose positron emission tomography (FDG-PET) radiographic diagnostic tool has emerged in staging. PD-L1 expression and MHC class II positivity on Reed-Sternberg cells are indicative of favorable prognosis after PD-1 blockade. As a rule, patients with early-stage classic variant of Hodgkin disease (stages I–IIa) are treated with combined chemotherapy followed by involved-field radiation therapy (IFRT) in most cases.

2. **What is pathogenesis of Hodgkin disease?**

   Answer: Epstein-Barr virus plays important role in the pathogenesis of Hodgkin disease, which protects Reed-Sternberg cells from apoptosis and CD8+ cytotoxic T cells, which would ordinarily destroy these cells. EBV may cause mutation in the TP53 tumor suppressor gene. Human immunodeficiency virus positive patients are associated with advanced-stage disease at unusual sites and associated with poor outcome after therapy.

3. **What is diagnostic hallmark of Hodgkin disease?**

   Answer: The initial diagnosis of Hodgkin disease can only be confirmed by histologic examination of lymph node biopsy and bone marrow trephine biopsy. The architecture of lymph node is extremely important for an accurate diagnosis. Presence of Reed-Sternberg cells (classic or lacunar, or mononuclear) is the pathologic hallmark of Hodgkin disease.

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- Recent concepts in cellular biology with key points of the disorders are given in boxes in the chapters.
- Immunohistochemistry of surgical specimens and immunophenotyping of hematolymphoid neoplasms have been given in tabulated forms.

Vinay Kamal has more than three-decade of teaching experience at India's premier institution, while serving as Director Professor, Department of Pathology, Maulana Azad Medical College, New Delhi. An alumnus of Post Graduate Institute of Medical Sciences (PGIMS), Rohtak, Haryana, he has also served in the premier institute. He has published articles in national and international journals. Budding medical professionals Dr Anubhav and Dr Vigyat have put sincere efforts in the completion of *Textbook of Pathology*.

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