Molecular Techniques in Radiobiology

Chapter 16 (6th edition)

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Radiobiology for the Radiologist

Introduction

• The birth of molecular biology could be ascribed to the discovery of the structure of DNA by Watson and Francis Crick in 1953
• In the late 1940s and early 1950s, Linus Pauling realized that as amino acids were folded into proteins, they formed three-dimensional structures, and that function was related to the structure
• The whole concept emerged that the sequence of bases, which coded for a protein, ultimately determined function leading the way to breaking the genetic code

The structure of DNA

• DNA - deoxyribonucleic acid is a polymer with the monomer units of nucleotides
• Each nucleotide consists of a 5-carbon sugar (deoxyribose), a nitrogen containing base attached to the sugar, and a phosphate group
• There are four different types of nucleotides found in DNA, differing only in the nitrogenous base:
  - A is for adenine
  - G is for guanine
  - C is for cytosine
  - T is for thymine

RNA and DNA

• RNA – ribonucleic acid, has ribose sugar molecule instead of deoxyribose
• In the cell RNA is usually single-stranded, while DNA is usually double-stranded
• RNA has the base uracil rather than thymine that is present in DNA
• RNA has a much shorter chain of nucleotides
• Unlike DNA, which is located primarily in the nucleus, RNA is found throughout the cell
• There are several types of RNA

Transcription and translation

The flow of genetic information from DNA to protein (gene expression) requires a series of steps:
• In the first step, the DNA code is transcribed in the nucleus into mRNA (messenger RNA); transcription is controlled by other DNA sequences (such as promoters), which show a cell where genes are, and control how often they are copied
• During the second step, the RNA copy made from a gene is then fed through a ribosome, which translates the sequence of nucleotides (with help of ribosomal RNA and transfer RNA) in the mRNA into the correct sequence of amino acids and joins these amino acids together to make a complete protein chain
• The new protein then folds up into its active form
Transcription and translation

- Introns and exons are nucleotide sequences within genes
- Typically introns make up the bulk of the gene
- Introns are removed during the process of splicing
- Remaining exons are joint together
- Only the exons of the DNA are translated

Amino acids and proteins

- Each amino acid is specified by triplet mRNA sequence, codon; four bases give a possibility to have $4 \times 4 \times 4 = 64$ combinations
- There are only 20 amino acids; more than one triplet can code each amino acid
- The vast majority of proteins is composed of a mixture of the same 20 amino acids; chains vary from 5 to > 4000 amino acids

Gene manipulation tools

- The most common form of genetic manipulation (engineering) involves the insertion of new genetic material in the host genome at a random or specific location
- Need to have abilities to recognize specific sequences, cleave DNA, replicate the pieces, insert it into a host

Gene manipulation tools

- Restriction endonucleases: enzymes found in bacteria that can recognize specific DNA sequence and cleave at or near that site
- Recognition sites are short, 4 to 8 nucleotides, and usually read the same in both directions (palindromic sequences)
- There are types I, II, and III, but only type II cut the DNA without modifications, and are used in molecular biology

Gene manipulation tools

- Some endonucleases produce blunt-ended fragments because they cut cleanly through the DNA, cleaving both complementary strands at the same nucleotide position, most often near the middle of the recognition sequence
- Other endonucleases cleave the two strands of DNA at positions two to four nucleotides apart, creating exposed “sticky” ends of single-stranded sequences, very useful for making recombinant molecules

Gene manipulation tools

- A vector is a self-replicating DNA molecule that has the ability to carry a foreign DNA molecule into a host cell
- Vectors are typically used to insert fragments of human DNA, containing a gene of interest, into a bacterium that will self-replicate, producing quantities enough for a study
- There are many types of vectors: plasmids, bacteriophages, bacterial artificial chromosomes (BACs), and viruses
Gene manipulation tools

- BACs (bacterial artificial chromosomes) are vectors based on a type of plasmid with sequences encoding self-replication. BACs can accommodate approximately 300 kilobases (kb) of DNA (plasmids are limited to approximately 10-kb insertions).
- BACs were the primary vector used during the genome-sequencing projects, mainly because a BAC carrying a gene of interest is easily acquired.
- Viruses are highly efficient vectors for introducing foreign genes into mammalian cells.
  - Retroviruses are a type of RNA virus. The RNA genome of retroviruses is transcribed into DNA, which is then integrated into the host genome. Retroviruses can infect virtually every type of mammalian cell, making them very versatile.
  - Adenoviruses are a type of DNA virus.

Gene manipulation tools: hosts

- Mammalian cells do not take up foreign DNA naturally; one of several tricks must be used to bypass natural barriers.
  - Microinjection: DNA can be injected, cell by cell, directly into the nucleus through a fine glass needle.
  - Calcium phosphate precipitation: Cells take up DNA relatively efficiently in the form of a precipitate with calcium phosphate.
  - Cationic lipids: offer some of the highest transfection efficiencies and expression levels to a wide variety of cells, both in suspension and attached.
  - Electroporation: Cells in solution are subjected to a brief electrical pulse that causes holes to open transiently in the membrane, allowing foreign DNA to enter.
  - Viral vectors: The ultimate means of transfection involves the use of a retrovirus. This is analogous to using bacteriophage to get DNA into bacteria. Oncogenes, genes that can cause cancer, and their counterpart, tumor-suppressor genes, can be studied by incorporating them into retro-viral vectors.

Agarose gel electrophoresis

- DNA is negatively charged.
- Can separate pieces of DNA of different sizes.
- After separation is complete ethidium bromide is added, making DNA fluoresce under ultraviolet light to make the position of the DNA visible.
Polymerase chain reaction (PCR)
• DNA polymerase is an enzyme that catalyzes the polymerization of nucleotides into a DNA strand
• Primers (short DNA fragments) contain sequences complementary to the target region
• The polymerase "reads" an intact DNA strand as a template and uses it to synthesize the new strand. This process copies a piece of DNA
• As PCR progresses, the DNA generated is itself used as a template for replication, promoting a chain reaction

Gene analysis: Southern blotting
• Mapping technique, used to detect specific fragments of DNA, named after British biologist Edwin Southern
• Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contains DNA sequence that is complementary to the probe
• Can detect mutations, such as insertions, deletions and sequence differences in DNA

Gene analysis: Chromosome walking
• Used when gene has been mapped to a specific arm of a chromosome, but has not been isolated
• Probes are used to identify an overlapping fragment of DNA from the genomic library

Gene knockouts
• Gene knockout technique involves making one of the genes in a living organism inoperative
• Used to learn about a gene that has been sequenced, but has an unknown or incompletely known function
• Genes in embryonic stem cells are manipulated and inserted into early embryos
• Gene function is inferred from the difference between the knockout organism and normal individuals

Other blotting techniques
• Northern blotting - the technique for separating RNA by gel electrophoresis and is analogous to the Southern blot technique used to study DNA
• Western blotting – used to detect specific proteins in a homogenized tissue sample
  – Proteomics - seeks to define the quantities and interactions of the vast number of proteins in a given cell at a given instant in time

Databases and sequence analysis
• GenBank, a database at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) for the deposition of individually cloned genomic, cDNA and protein sequences
• Related programs, called search engines, were developed to find similar sequences from the ever-growing list of identified genes contained in the databases
• One of the most commonly used programs for finding related sequences from the genome databases is BLAST, found on the NCBI website
Introduction

- Tissue homeostasis depends on the regulated cell division and self-elimination (programmed cell death) of each of its constituent members except its stem cells.
- A tumor arises as a result of uncontrolled cell division and failure for self-elimination.
- Alterations in three groups of genes are responsible for the deregulated control mechanisms that are the hallmarks of cancer cells: proto-oncogenes, tumor-suppressor genes, and DNA stability genes

Proto-oncogenes

- Proto-oncogenes are components of signaling networks that act as positive growth regulators in response to mitogens, cytokines, and cell-to-cell contact.
- A gain-of-function mutation in only one copy of a protooncogene results in a dominantly acting oncogene that often fails to respond to extracellular signals.

Tumor-suppressor genes

- Tumor-suppressor genes are also components of the same signaling networks as proto-oncogenes, except that they act as negative growth regulators.
- They modulate proliferation and survival by antagonizing the biochemical functions of proto-oncogenes or responding to unchecked growth signals.
- In contrast to oncogenes, inactivation of both copies of tumor-suppressor genes is required for loss of function in most cases.

DNA stability genes

- DNA stability genes form a class of genes involved in both monitoring and maintaining the integrity of DNA.
- Loss of these genes results in defective sensing of DNA lesions as well as improper repair of the damaged template.

Mechanisms of carcinogenesis

The malignant progression from normal tissue to tumor to metastasis occurs in a number of discrete "steps" over a period of time.
Oncogenes

- First discovered from studies of retroviruses that cause cancer in animals
- Proto-oncogene has to be activated in order to produce tumor

Mechanisms of oncogene activation

- Transcriptional deregulation by overexpression or abnormal expression of the mRNA of a proto-oncogene is a common theme
- It is a dominant gene, mutation in only one copy leads to its activation
- At least four mechanisms exist:
  - Retroviral integration of proto-oncogene sequences in retroviral genomes through recombination
  - DNA mutation of regulatory sites
  - Gene amplification
  - Chromosome rearrangement

Mechanisms of oncogene activation

Chromosome translocation

- The first real breakthrough in identifying tumor-specific chromosome alterations occurred in the late 1950s when Dr. Peter Nowell found a consistent shortened version of chromosome 22 in individuals afflicted with chronic myelogenous leukemia (CML)

Tumor-suppressor genes

- Recessive gene, both copies of tumor-suppressor gene have to be inactivated in order to suppress malignant transformation
- First discovered through family history studies of patients with hereditary cancers, such as retinoblastoma (Rb gene) or Li-Fraumeni syndrome (p53 gene)

Retinoblastoma studies
Li-Fraumeni syndrome studies

- Extremely rare syndrome, predisposes to development of multiple cancers by young adulthood
- Led to discovery of \( p53 \) gene, its suppression results in a number of tumors

Tumor-suppressor genes

Tumor-suppressor genes

<table>
<thead>
<tr>
<th>Tumor-Suppressor Gene</th>
<th>Syndrome</th>
<th>Tumor</th>
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<tbody>
<tr>
<td>RB</td>
<td>Retinoblasia</td>
<td>Retinoblasia</td>
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<tr>
<td>BRCA2</td>
<td>Familial breast cancer</td>
<td>Breast</td>
</tr>
<tr>
<td>ATM</td>
<td>Neurofibromatosis type 1</td>
<td>Neurofibromatosis</td>
</tr>
<tr>
<td>TP53</td>
<td>Neurofibromatosis type 2</td>
<td>Neurofibromatosis</td>
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<td>APC</td>
<td>Familial adenomatous polyposis</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>p53</td>
<td>Li-Fraumeni Syndrome</td>
<td>Breast, lung, brain tumors, sarcoma</td>
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<tr>
<td>HNF1</td>
<td>von Hippel-Lindau disease</td>
<td>Tumor of kidney, adrenal</td>
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<td>FBN1</td>
<td>Familial hypercholesterolemia</td>
<td>Tumor of colon, breast</td>
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<td>PTEN</td>
<td>Cowden syndrome</td>
<td>Basal cell carcinoma</td>
</tr>
<tr>
<td>BAP1</td>
<td>Multiple endocrine neoplasia</td>
<td>Neuroendocrine</td>
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</tbody>
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The multi-step nature of cancer

- Carcinogenesis is a multi-step process: a number of distinct events that may be separated in time have to occur
- Genetic analysis of cells from solid tumors suggest alterations, mutations, or deletions in multiple signaling genes, either oncogenes or suppressor genes. For example, 6 to 12 mutations have been suggested for the formation of a carcinoma
- The following stages can be identified in tumor development: initiation, promotion, and progression

The multi-step nature of cancer

- Often tumor-suppressor gene is lost through somatic homozygosity: one chromosome of a pair is lost, a deletion occurs in the remaining chromosome, and the chromosome with the deletion replicates
- This process has been documented for a number of tumors

The multi-step nature of cancer

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Functions of oncogenes and tumor-suppressor genes

- Several categories of cell functions are perturbed by mutations in oncogenes and tumor-suppressor genes
- Mostly these are functions related to regulation of proliferation, growth-restriction and apoptosis signals
- Combination in deregulations of these functions lead to tumor initiation, invasion and metastasis

Deregulated proliferation

- Normal cells rely on extracellular growth signals; typically one cell secretes a mitogenic signal to stimulate the proliferation of another cell type
- Signal is initiated at the cell membrane (receptors) and is transduced to the nucleus via a cascade of proteins affecting regulatory functions
- In contrast to untransformed cells, transformed cells become autonomous in regulating their growth by responding to the mitogenic signals they themselves produce

Deregulated proliferation

Failure to respond to growth-restrictive signals

- The oncogenic activation of nuclear oncogenes stimulates the cell into the synthetic phase (S phase), where it duplicates its genetic material before cell division
- Nuclear proto-oncogenes can work as transcription factors by binding to DNA in a sequence-specific manner and forming complexes with themselves or other proteins that will increase mRNA transcription of genes such as cyclin D that promotes cell division

Failure to commit suicide (apoptosis)

- Two major pathways that mediate cell death emanate either from the cell membrane or from the mitochondria
- The signals transmitted by each pathway results in the activation of intracellular proteins, termed caspases, that cleave a diverse number of proteins at specific sites
- Cell lines deficient in Caspases 3 and 9 exhibit substantially reduced levels of apoptosis during development and in response to exogenous stress-inducing stimuli
- Tumor suppressor gene p53 in an important modulator of oncogene-induced apoptosis

Oncogene myc

- Myc codes for a protein that binds to the DNA of other genes and is therefore a transcription factor. When a gene like Myc is altered to cause cancer, the cancerous version of the gene is called an oncogene. The healthy version of the gene that it is derived from is called a proto-oncogene
Escaping senescence

- A telomere is a region of DNA (repeat sequence of TTAGGG) at the end of a chromosome, protecting it from deterioration
- Each time a normal somatic cell divides, the terminal end of the telomere is lost; successive divisions lead to progressive shortening, and after 40 to 60 divisions, vital DNA sequences are lost. At this point, the cell cannot divide further and undergoes senescence
- Cancer cells avoid this process of aging by activating the enzyme telomerase, which is a reverse transcriptase that polymerizes TTAGGG repeats to offset the degradation of chromosome ends that occurs with successive cell divisions; in this way, the cell becomes immortal
- Mutation in tumor-suppressor gene p53 is involved

Angiogenesis

- Angiogenesis, the recruitment of new blood vessels to regions of chronically low blood supply, is essential for the progression of solid tumors to malignancy
- A number of proangiogenic growth factors have been identified. VEGF was the first growth factor isolated that could stimulate proliferation and migration of blood vessel cell lining
- Studies have shown that blocking the binding of VEGF to its receptor inhibits tumor angiogenesis and tumor growth. These findings have led to the development of new antibody approaches for antiangiogenesis therapy for clinical use

Invasion and metastasis

- The genetic circuits that regulate metastasis remain mainly undiscovered
- Decreased expression or impaired function of adhesion molecules, and regulatory alterations in chemical agents for digestion of extracellular matrix (ECM) have been implicated

Gatekeepers and caretakers

- It appears that most tumor-suppressor genes can be broadly divided into two classes that have been called "gatekeepers" and "caretakers."
- Gatekeepers are genes that directly regulate the growth of tumors by inhibiting cell division or promoting cell death, rate limiting for tumor growth. Both alleles (maternal and paternal) must be lost or inactivated for a tumor to develop. The identity of gatekeepers varies with each tissue
- Inactivation of caretaker genes does not directly promote the growth of tumors, but leads instead to genomic instability that only indirectly promotes growth by causing an increase in mutation rate. The targets of the accelerated mutation rate that occurs in cells with defective caretakers are the gatekeeper tumor-suppressor genes, oncogenes, or both

Mismatch repair genes

- Mismatch repair (MR) is responsible for correction of errors of DNA replication and recombination that result in mispaired (but undamaged) nucleotides
- The primary function of mismatch repair genes is to scan the genome as it replicates and to spot errors of mismatch
- Mutations in MR genes were found responsible for the mutator phenotype associated with a predisposition for hereditary nonpolyposis colon cancer (HNPCC) and possibly other familial cancers

Radiation-induced signal transduction

- Ionizing radiation can regulate the expression of early-response genes, resulting in the stimulation of signal transduction pathways and activation of transcription factors
- It may also enhance the response of the cell to radiation in terms of repair and cell-cycle arrest; and provide a mechanism for secondary stimulation of various late-response genes
- Understanding of these defense mechanisms can help exploiting them for treatment of cancer
Gene therapy

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Approaches to gene therapy

- Genes are introduced into tumor cells using viral vectors: retrovirus, adenovirus, and herpesvirus
- There are at least 6 different approaches
  - Suicide-gene therapy
  - Cytotoxic virus targeted to p53-deficient cells
  - Molecular immunology (cancer vaccines)
  - Tumor-suppressor gene therapy
  - Radiation-inducible gene linked to a cytotoxic agent
  - Targeting signal transduction pathways

Suicide-gene therapy

- Suicide-gene therapy is based on transducing cells with a gene that converts a prodrug into a cytotoxic agent
- There is a substantial bystander effect; that is, more cells are killed than transduced initially
- This therapy has produced growth delay and some cures in animal models
- Because of the limited efficiency of gene delivery, suicide-gene therapy needs to be combined with conventional radiotherapy
- Phase I/II clinical trials have shown promise

Targeted p-53 deficient cells

- A cytotoxic virus can be constructed that is engineered to replicate and kill only in cells with mutant p53
- To the extent that mutant p53 is a hallmark of cancer, this treatment differentiates between normal cells and cancer cells
- Growth arrest has been observed in model animal tumors and in early clinical trials by targeting mutant p53

Molecular immunology (cancer vaccines)

- The approach is to provoke a cellular immune response against the cancer by injecting a vaccine genetically engineered to express immune stimulatory molecules or tumor-specific antigens
- Molecular immunology shows some promise in animal models but is generally only effective against small tumor burdens
- Developing strategy is to combine molecular immunology with suicide-gene therapy

Tumor-suppressor gene therapy

- Tumor-suppressor gene therapy is the replacement, with a correct copy, of the mutated gene that initiates or contributes significantly to the malignant phenotype
- The gene p53 has received the most attention of any gene because it is so commonly mutated in human cancers
- Phase I/II clinical trials show some promise in the treatment of non-small-cell lung cancer
- The therapy is limited by a lack of information on the target genes that are essential for maintaining the malignant phenotype and the fact that multiple genetic changes are involved
Radiation-inducible gene linked to a cytotoxic agent

- Combination of the physics of radiation-targeting technology with molecular gene therapy

Targeting signal transduction pathways

A hallmark of the malignant cell is the dysregulation of growth and signal transduction pathways that often result in resistance to radiotherapy. Several potential targets have been identified:

- The epidermal growth factor receptor (EGFR) mediates growth regulation in a wide spectrum of human cancers, and tumors expressing high levels of EGFR appear to be radioresistant
- Raf-1 is a kinase that plays an important role in cell proliferation, differentiation, survival, and angiogenesis and is therefore a prime target for novel cancer therapies
- NF/cB is a cellular transcription factor that plays a central role in the cellular stress response

Summary

- Development of molecular techniques, such as gene identification and manipulation tools greatly advanced identification of specific genes and understanding of genetic pathways responsible for tumor proliferation
- There is a number of approaches to gene therapy; the winning approach will be a synergistic combination of several treatment modalities