PART IV

TOXIC ACTION

CHAPTER 10

Acute Toxicity

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10.1 INTRODUCTION

Acute toxicity of a chemical can be viewed from two perspectives. Acute toxicity may be viewed as a qualitative descriptor of an incident of poisoning. Consider the following statement: "methyl isocyanate gas, accidentally released from a chemical manufacturing facility in 1984, was *acutely toxic* to the residents of Bhopal, India." This statement implies that the residents of Bhopal were exposed to sufficiently high levels of methyl isocyanate over a relatively short time to result in immediate harm. High-level, short-term exposure resulting in immediate toxicity are all characteristics of acute toxicity. Alternatively, acute toxicity may represent a quantifiable characteristic of a material. For example, the statement: "the *acute toxicity* of methyl isocyanate, as measured by its LD_{50} in rats, is 140 mg/kg" defines the acute toxicity of the chemical. Again, the characterization of the quantified effects of methyl isocyanate as being acute toxicity implies that this quantification was derived from a short-term dosing experiment and that the response measured occurred within a short time period following dosing. Considering these qualitative and quantitative aspects, acute toxicity can be defined as *toxicity elicited immediately following short-term exposure to a chemical.* In accordance with this definition, two components comprise acute toxicity: acute exposure and acute effect.

10.2 ACUTE EXPOSURE AND EFFECT

In contrast to acute toxicity, chronic toxicity is characterized by prolonged exposure and sublethal effects elicited through mechanisms that are distinct from those that cause acute toxicity. Typically, acute and chronic toxicity of a chemical are easily distinguished. For example, mortality occurring within 2 days of a single dose of a chemical would be a prime example of acute toxicity (Figure 10.1a). Similarly, reduced litter size following continuous (i.e., daily) dosing of the parental organisms would be indicative of chronic toxicity (Figure 10.1b). However, defining toxicity as being acute or chronic is sometimes challenging. For example, chronic exposure

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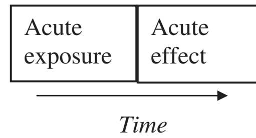
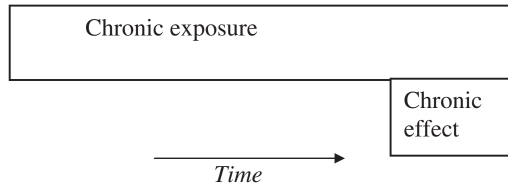
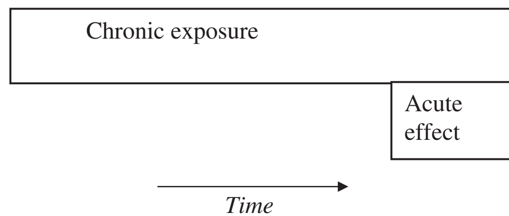
(a) Short-term exposure resulting in immediate effects



(b) Continuous exposure resulting in sublethal effects



(c) Continuous exposure resulting in acute effects



(d) Short-term exposure resulting in later sublethal effects

Time

Figure 10.1 Examples of exposure/effect scenarios that result in either acute toxicity (a), chronic toxicity (b), or mixed acute/chronic toxicity (c, d). Examples for each scenario are provided in the text.

to a persistent, lipophilic chemical may result in sequestration of significant levels of the chemical in adipose tissue of the organism with no resulting overt toxicity. Upon entering the reproductive phase, organisms may mobilize fatty stores, releasing the chemical into the bloodstream, resulting in overt toxicity including death (Figure 10.1c). One could argue under this scenario that chronic exposure ultimately resulted in acute effects. Lastly, acute exposure during a susceptible window of exposure (e.g., embryo development) may result in reproductive abnormalities and reduced fecundity once the organism has attained reproductive maturity (Figure 10.1d). Thus, acute exposure may result in chronic toxicity.

An additional consideration is noteworthy when comparing acute and chronic toxicity. All chemicals elicit acute toxicity at a sufficiently high dose, whereas, all chemicals do not elicit chronic toxicity. Paracelsus' often cited phrase "all things are poison … the dose determines … a poison" is clearly in reference to acute toxicity. Even the most benign substances will elicit acute toxicity if administered at a

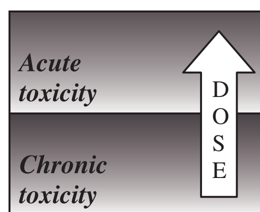


Figure 10.2 Relationships among chemical dose, acute toxicity, and chronic toxicity. All chemicals elicit acute toxicity at a sufficiently high dose. However, chronic toxicity may not occur since dosage elevation may simply lead to acute toxicity.

sufficiently high dose. However, raising the dose of a chemical does not ensure that chronic toxicity will ultimately be attained. Since chronic toxicity typically occurs at dosages below those that elicit acute toxicity, toxicity observed at the higher dosage may simply reflect acute, and not chronic, toxicity (Figure 10.2).

Effects encountered with acute toxicity commonly consist of mortality or morbidity. From a quantitative standpoint, these effects are measured as the lethal dose 50 (LD_{50}), effective dose 50 (ED_{50}), lethal concentration 50 (LC_{50}), or effective concentration 50 (EC_{50}). The LD_{50} and ED_{50} represent the dose of the material that causes mortality (LD_{50}) or some other defined effect (ED_{50}) in 50% of a treated population. The LC_{50} and EC_{50} represent the concentration of the material to which the organisms were exposed that causes mortality (LC_{50}) or some other defined effect (EC_{50}) in 50% of an exposed population. LD_{50} and ED_{50} are typically normalized to the weight of the animal (e.g., milligram chemical/kilogram body weight); whereas, LC_{50} and EC_{50} are typically normalized to the environment in which the organisms were exposed (e.g., milligram chemical/liter water for aquatic organisms). These measures of acute toxicity are used to assign a degree to toxicity to a chemical. For example, the following categories are used by the Organization for Economic Cooperation and Development (OECD):

LD_{50} (mg/kg)	Label
<5 mg/kg	Very toxic
>5 < 50	Toxic
>50 < 500	Harmful
>500	No label required

Results of acute toxicity tests are also used to identify dosages of chemical to be used in sublethal, chronic toxicity evaluations. Finally, results of acute toxicity tests can be used to provide insight to the mode of action of the toxicant as described further below.

10.3 DOSE–RESPONSE RELATIONSHIPS

Acute toxicity of a chemical is quantified by its dose–response curve. This relationship between dose of the chemical administered and the resulting response

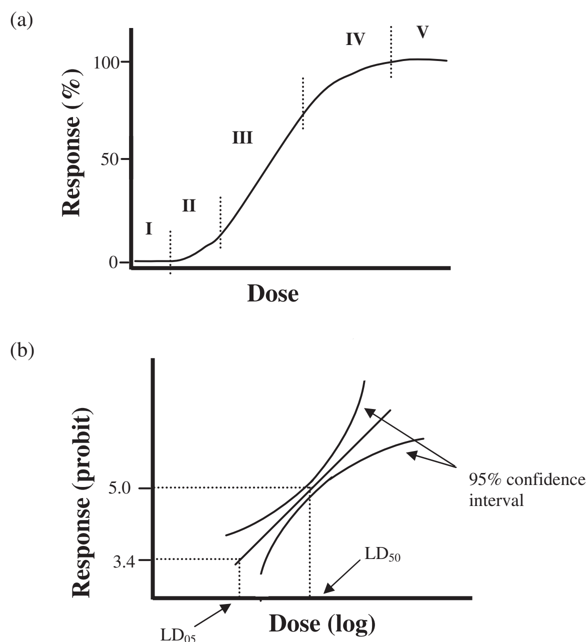


Figure 10.3 The dose–response relationship. (a) Five segments of the sigmoidal dose–response curve as described in the text. (b) Linearized dose–response relationship through log (dose)–probit (response) transformations. Locations of the LD_{50} and LD_{05} are depicted.

is established by treating groups of organisms with various amounts of the chemical. Dosing may be administered orally (e.g., gavage, food, water), through injection, from external application (dermal, ocular), or via environmental exposure (e.g., air). Ideally, doses are selected that will elicit >0% response but <100% response during the course of the experiment. At defined time periods following dosing, effects (e.g., mortality) are recorded. Results are plotted in order to define the dose–response curve (Figure 10.3a). A well-defined dose–response curve generated with a population of organisms whose susceptibility to the chemical is normally distributed will be sigmoidal in shape. The various segments (see Figure 10.3a) of the curve are represented as follows:

Segment I: This portion of the line has no slope and is represented by those doses of the toxicant that elicit no mortality to the treated population of organisms.

Segment II: This segment represents those doses of the toxicant that affects only the most susceptible members of the exposed population. Accordingly, these effects are elicited at low doses and only a small percentage of the dosed organisms are affected.

Segment III: This portion of the line encompasses those doses at which most of the groups of organisms elicit some response to the toxicant. Because most of the groups of exposed organisms respond to the toxicant within this range of dosages, segment III exhibits the steepest slope among the segments.

Segment IV: This portion of the line encompasses those doses of the toxicant that are toxic to even the most tolerant organisms in the populations. Accordingly, high doses of the toxicant are required to affect these organisms.

Segment V: Segment V has no slope and represents those doses at which 100% of the organisms exposed to the toxicant have been affected.

A well-defined dose–response curve can then be used to calculate the LD_{50} for the toxicant. However, in order to provide the best estimate of the LD_{50}, the curve is typically linearized through appropriate transformations of the data. A common transformation involves converting concentrations to logarithms and percentage effect to probit units (Figure 10.3b). Zero percent and 100% responses cannot be converted to probits; therefore, data within segments I and V are not used in the linearization. A 95% confidence interval also can be determined for the linearized dose–response relationship (Figure 10.3b). As depicted in Figure 10.3b, the greatest level of confidence (i.e., the smallest 95% confidence interval) exists at the 50% response level which is why LD_{50} values are favored over some other measure of acute toxicity (e.g., LD_{05}). This high level of confidence in the LD_{50} exists when ample data exist between the 51% and 99% response as well as between the 1% and 49% response.

Additional important information can be derived from a dose–response curve. The slope of the linearized data set provides information on the specificity of the toxicant. Steep slopes to the dose–response line are characteristic of toxicants that elicit toxicity by interacting with a specific target while shallow slopes to the dose–response line are characteristic of toxicant that elicit more nonspecific toxicity such as narcosis (see below). The dose–response line also can be used to estimate the threshold dose. The threshold dose is defined as the lowest dose of the chemical that would be expected to elicit a response under conditions at which the assay was performed. The threshold dose is often empirically estimated as being a dose less than the lowest dose at which an effect was measured but higher than the greatest dose at which no effect was detected. Conceptually, the threshold dose is defined as the intercept of segments I and II of the dose–response curve (Figure 10.3a). Statistically, the threshold dose can be estimated from the linearized dose–response curve as the LC_{05}. This value will closely approximate the threshold dose and can be statistically derived from the entire data set (i.e., the dose–response line). However, confidence in this value is greatly compromised since it is derived from one end of the line (Figure 10.3b).

10.4 NONCONVENTIONAL DOSE–RESPONSE RELATIONSHIPS

The low-level effects of chemicals have received attention among pharmacologists for over 100 years. A current resurgence in interest among pharmacologists in low-level effects stems from use of homeopathic approaches to treating disease. Proponents of homeopathy maintain that low levels of toxic materials stimulate physiological responses that can target disease without eliciting adverse effects in the individual undergoing treatment. Homeopathic principles may have application in toxicology based upon the premise that exposure to some chemicals at subthreshold levels, as defined by standard acute toxicity evaluations, can elicit toxicological

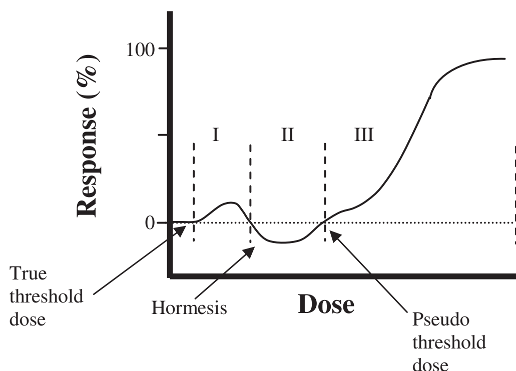


Figure 10.4 Nonconventional dose–response relationship involving low-dose effects and compensation. I: True initiation of the response followed by a compensatory response that returns the effects to the 0% level. II: A negative response due to overcompensation (hormesis) followed by recovery to the 0% response level. III: The standard sigmoidal dose–response relationship.

as well as pharmacological effects. Both pharmacological and toxicological homeopathy may be the consequence of hormesis.

Hormesis is defined as an overcompensatory response to some disruption in homeostasis resulting in a U or inverted U-shaped deflection at the low end of the dose–response curve. Accordingly, hormesis typically presents as an effect opposite to that elicited at higher doses of the chemical. For example, a chemical that stimulates corticosteroid secretion at high doses resulting in hyperadrenocorticism might elicit a hormetic response at low doses resulting in corticosteroid deficiency. A hypothetical nonconventional dose–response relationship resulting from such interactions is depicted in Figure 10.4. At the true threshold dose, the organisms begin to exhibit increased stimulation in corticosteroid secretion. However, at slightly higher doses, a compensatory response occurs whereby corticosteroid secretion is decreased in order to maintain homeostasis within the organism. Overcompensation may actually result in a decrease in corticosteroid secretion at certain toxicant dosages. Finally, the compensatory abilities of the organism are overcome by the high doses of the toxicant at the "pseudo" threshold dose, above which the standard dose–response relationship occurs. Nonconventional dose–response relationships have been observed with respect to both acute and chronic toxicity and are particularly relevant to the risk assessment process when establishing levels of exposure that are anticipated to pose no harm.

10.5 ALTERNATIVE METHODS

Conventional acute toxicity assessments performed with rodents can involve the use of a significant number of animals (e.g., 10 doses with10 animals per dose performed with males and females = 200 animals). Various approaches have been proposed as a means of reducing animal usage in the performance of acute toxicity tests.

10.5.1 Up-Down Method

Various derivations of the up-down method have been reported, but essentially, this method involves dosing a single animal with a starting dose of the chemical. This starting dose can be judged based upon toxicity tests performed with other species or with similar compounds. If the animal dies, then a second animal is dosed at a lower concentration. This progression of single dose levels administered to a single animal continues until several dosages are identified that do not elicit mortality. Similarly, if the starting dose is not lethal, then subsequent doses are administered at progressively higher levels until lethal dosages are identified. This method has proven valuable in bracketing the LD_{50} often with the use of less than 10 animals.

10.5.2 Fixed-Dose Method

The fixed-dose method has proven utility in assigning a chemical into a toxicity category. For example, fixed-doses of 5, 50, and 500 mg/kg might each be administered to five animals and responses used to classify a chemical accordingly the scheme present in Section 10.2. The acute toxicity of the chemical would be classified based upon the lowest dose that elicited no significant response among the treated animals.

10.5.3 *In Vitro* Methods

Cytotoxicity tests performed with human cell lines have been shown to be good predictors of blood concentrations of chemicals that are lethal to humans. The strength of the predictive value of cytotoxicity tests seems to be in the fact that for many chemicals, the cellular targets through which the chemicals cause toxicity are common to all cells (e.g., membranes, mitochondrial respiratory enzymes, etc.). The predictive value of cytotoxicity tests can be improved when used in conjunction with toxicokinetic modeling to consider factors related to adsorption, distribution, metabolism, and elimination that are relevant to *in vivo* toxicity but are inadequately represented in cultured cells.

10.6 MECHANISMS OF ACUTE TOXICITY

An exhaustive review of the mechanisms by which chemicals cause acute toxicity is beyond the scope of this chapter. However, certain mechanisms of toxicity are relevant since they are common to many important classes of toxicants. Some of these mechanisms of acute toxicity are discussed.

10.6.1 Narcosis

Narcosis in toxicology is defined as toxicity resulting from chemicals associating with and disrupting the lipid bilayer of membranes. Narcotics are classified as either nonpolar (Class 1) or polar (Class 2) compounds. Members of both classes of compounds are lipid soluble. However, Class 2 compounds possess constituents

that confer some charge distribution to the compound (e.g., aliphatic and aromatic amines, nitroaromatics, alcohols). The aliphatic hydrocarbon (C5 through C8) are examples of powerful Class 1 narcotics, whereas ethanol is an example of a Class 2 narcotic. The affinity of narcotics to partition into the nonpolar core of membranes (Class 1 narcotics) or to distribute in both the polar and nonpolar components of membranes (Class 2 narcotics) alters the fluidity of the membrane. This effect compromises the ability of proteins and other constituents of the membranes to function properly, leading to various manifestation of narcosis. The central nervous system is the prime target of chemical narcosis and symptoms initially include disorientation, euphoria, giddiness, and progress to unconsciousness, convulsion, and death.

10.6.2 Acetylcholinesterase Inhibition

Acetylcholine is a neurotransmitter that functions in conveying nerve impulses across synaptic clefts within the central and autonomic nervous systems and at junctures of nerves and muscles. Following transmission of an impulse across the synapse by the release of acetylcholine, acetylcholinesterase is released into the synaptic cleft. This enzyme hydrolyzes acetylcholine to choline and acetate and transmission of the nerve impulse is terminated. The inhibition of acetylcholineasterase results in prolonged, uncoordinated nerve or muscle stimulation. Organophosphorus and carbamate pesticides (Chapter 4) along with some nerve gases (e.g., sarin) elicit toxicity via this mechanism.

Inhibitors of acetylcholinesterase function by binding to the substrate-binding site of the enzyme (Figure 10.5). Typically, the inhibitor or a biotransformation derivative of the inhibitor (e.g., the phosphodiester component of organophosphorus compounds) covalently binds to the enzyme resulting in its inhibition. Inhibition persists until the bound inhibitor is hydrolytically cleaved from the enzyme. This inhibition may be range from minutes in duration to permanent. Toxic effects of cholinesterase inhibition typically are evident when the enzyme activity is inhibited by ~50%. Symptoms include nausea and vomiting, increased salivation and sweating, blurred vision, weakness, chest pains. Convulsions typically occur between 50% and 80% enzyme inhibition with death at 80–90% inhibition. Death is most commonly due to respiratory failure.

10.6.3 Ion Channel Modulators

Ion transport is central to nerve impulse transmission both along the axon and at the synapse, and many neurotoxicants elicit effects by interfering with the normal transport of these ions (Figure 10.6). The action potential of an axon is maintained by the high concentration of sodium on the outside of the cell as compared to the low concentration inside. Active transporters of sodium (Na^+K^+ ATPases) that actively transport sodium out of the cell establish this action potential. One action of the insecticide dichlorodiphenyltrichloroethane (DDT) resulting in its acute toxicity is the inhibition of these Na^+K^+ ATPases resulting in the inability of the nerve to establish an action potential. Pyrethroid insecticides also elicit neurotoxicity through this mechanism. DDT also inhibits $Ca^{2+}Mg^{2+}$ ATPases that are important to neuronal repolarization and the cessation of impulse transmission across synapses.

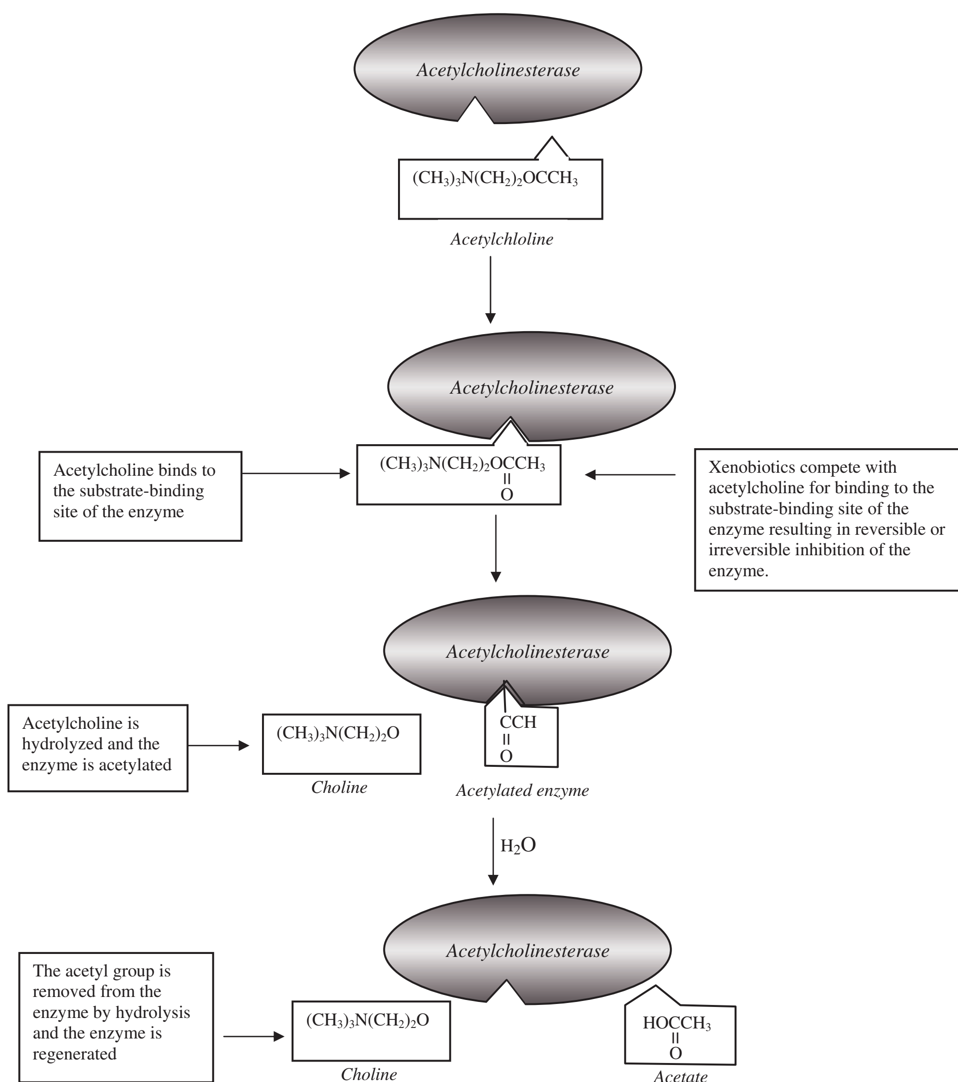


Figure 10.5 Hydrolysis of acetylcholine by the enzyme acetylcholinesterase and its inhibition by toxicants such as organophosphorus and carbamate insecticides.

The $GABA_A$ receptor is associated with chloride channels on the postsynaptic region of the neuron, and binding of gamma-aminobutyric acid (GABA) to the receptor causes opening of the chloride channel. This occurs after transmission of the nerve impulse across the synaptic cleft and postsynaptic depolarization. Thus, activation of $GABA_A$ serves to prevent excessive excitation of the postsynaptic neuron. Many neurotoxicants function by inhibiting the $GABA_A$ receptor resulting in prolonged closure of the chloride channel and excess nerve excitation. Cyclodiene insecticides (e.g., dieldrin), the organochlorine insecticide lindane, and some pyrethroid insecticides all elicit acute neurotoxicity, at least in part, through this mechanism. Symptoms of $GABA_A$ inhibition include dizziness, headache, nausea,

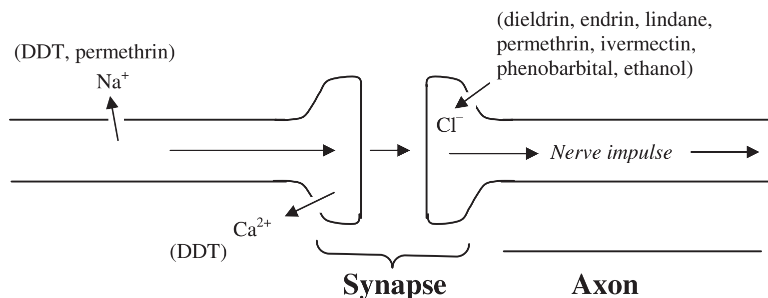


Figure 10.6 Ion channels that facilitate nerve impulse transmission and that are susceptible to perturbation by various toxicants and drugs. Ion transport inhibitors are indicated in parentheses.

vomiting, fatigue, tremors, convulsions, and death. Avermectins constitute a class of pesticides that are used extensively in veterinary medicine to treat a variety of parasitic conditions. While the mode of toxicity of these compounds is not precisely known, they appear to bind a distinct subset of chloride channels (GABA-insensitive chloride channels) resulting in disruptions in normal chloride transport across nerve cell membranes. Barbituates (e.g., phenobarbital) and ethanol elicit central nervous system effects, at least in part, by binding to $GABA_A$ receptors. However, unlike the previously discussed chemicals, these compounds enhance the ability of gamma-aminobutyric acid to bind the receptor and open the chloride channel. Accordingly, these compounds suppress nerve transmission which contributes to the sedative action of the chemicals.

10.6.4 Inhibitors of Cellular Respiration

Cellular respiration is the process whereby energy, in the form of ATP, is generated in the cell while molecular oxygen is consumed. The process occurs along respiratory assemblies that are located in the inner mitochondrial membrane. Electrons derived from NADH or $FADH_2$ are transferred along a chain of electron carrier proteins. This step-by-step transfer leads to the pumping of protons out of the mitochrondrial matrix resulting in the generation of a membrane potential across the inner mitochrondrial membrane (Figure 10.7). Protons are pumped out of the mitochrondrial matrix at three locations along the respiratory chain. Site 1 consists of the NADH-Q reductase complex; site 2 consists of the QH_2-cytochrome c reductase complex; and, site 3 is the cytochrome c-oxidase complex. Adenosine triphosphate (ATP) is generated from adenosine diphosphate (ADP) when protons flow back across the membrane through an ATP synthetase complex to the mitochrondrial matrix. The transfer of electrons culminates with the reduction of molecular oxygen to water.

Many chemicals can interfere with cellular respiration by binding to the cytochromes that constitute the electron transport chain and inhibiting the flow of electrons along this protein complex. The pesticide rotenone specifically inhibits electron transfer early in the chain with inhibition of proton transport beginning at site 1. Actimycin A inhibits electron transfer and proton pumping at site 2. Cyanide, hydrogen sulfide, and azide inhibit electron flow between the cytochrome oxidase

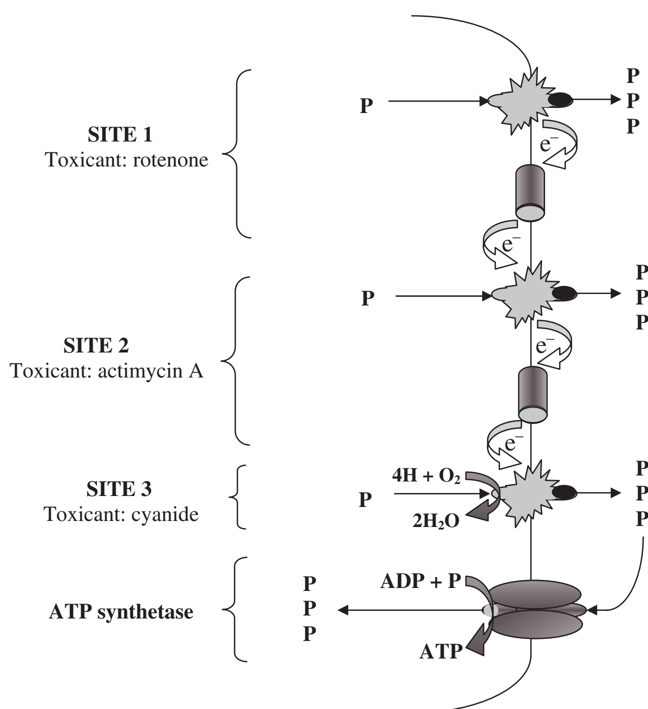


Figure 10.7 Electron (e^-) transport along the inner mitochondrial membrane resulting in the pumping of protons (P) out of the mitochondrial matrix. Protons are shuttled back into the matrix through the ATP synthetase complex where ATP is generated. Sites of toxicant action are indicated.

complex and O_2 preventing the generation of a proton gradient at site 3. Symptoms of toxicity from the inhibition of respiratory chain include excess salivation, giddiness, headache, palpitations, respiratory distress, and loss of consciousness. Potent inhibitors such as cyanide can cause death due to respiratory arrest immediately following poisoning.

Some chemicals do not interfere with electron transport leading to the consumption of molecular oxygen, but rather, interfere with the conversion of ADP to ATP. These uncouplers of oxidative phosphorylation function by leaking protons across the inner membrane back to the mitochrondrial matrix. As a result, a membrane potential is not generated, and energy required for the phosphorylation of ADP to ATP is lost. The uncoupling of oxidative phosphorylation results in increased electron transport, increased oxygen consumption, and heat production. The controlled uncoupling of oxidative phosphorylation is a physiologically relevant means of maintaining body temperature by hibernating animals, some newborn animals, and in some animals that inhabit cold environments. Chemicals known to cause uncoupling of oxidative phosphorylation include 2,4-dinitrophenol, pentachlorophenol, and dicumarol. Symptoms of intoxication include accelerated respiration and pulse, flushed skin, elevated temperature, sweating, nausea, coma, and death.

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SAMPLE QUESTIONS

1. The term "acute toxicity" can be used as a qualitative descriptor of an incidence of toxicity or a quantitative measure of toxicity. Describe how the term "acute toxicity" functions in both capacities.

2. To paraphrase Paracelsus: "all things are poison … the dose determines … a poison." Why is this old adage relevant primarily to acute (as opposed to chronic) toxicity?

3. Why is the LD_{50} value used as a measure of acute toxicity as opposed to the LD_{10}?

4. Which segment of a dose–response curve (I through V) is most important in defining the acute toxicity of a chemical? Why?

CHAPTER 11

Chemical Carcinogenesis and Mutagenesis

ROBERT C. SMART

11.1 DNA DAMAGE AND MUTAGENESIS

DNA is chemically reactive and is subject to chemical modifications that can alter the coding properties of the bases and also cause single or double strand breaks in the DNA backbone. DNA can be modified by endogenous processes such as a base deamination (i.e., cytosine is spontaneously deaminated to form uracil), oxidative stress, lipid peroxidation, and spontaneous hydrolysis to produce apurinic/apyriminidinic base sites (AP site) in the DNA. DNA can also be modified by exogenous or environmental agents including ionizing radiation, UV radiation, chemotherapeutics, and chemical carcinogens (Figure 11.1). If there is an error in the repair of the DNA damage or if the damage is not repaired, an error could occur in the newly synthesized DNA resulting in a mutation in the daughter cell. A mutation is a permanent heritable alteration in the DNA which alters the base sequence. A germinal mutation occurs in ova or sperm cells and can be passed to future generations while a somatic mutation occurs in nongerm cells and cannot be passed to future generations. DNA damaging agents produce three general types of genetic alterations: (1) gene mutations which include point mutations involving single base pair substitutions that can result in amino acid substitutions in the encoded protein and frame shift mutations involving the loss or gain of one or two base pairs resulting in an altered reading frame and gross alterations in the encoded protein; (2) chromosome aberrations including gross chromosomal rearrangement such as deletions, duplications, inversions, and translocations; and (3) aneuploidy which involves the gain or loss of one or more chromosomes. Point mutations are further classified as missense or nonsense mutations. A missense mutation produces an altered protein in which an incorrect amino acid has been substituted for the correct amino acid. A nonsense mutation is an alteration that produces a stop codon and results in a truncated protein. A point mutation can also be characterized based on the mutagen-induced substitution of one base for another within the DNA. When a

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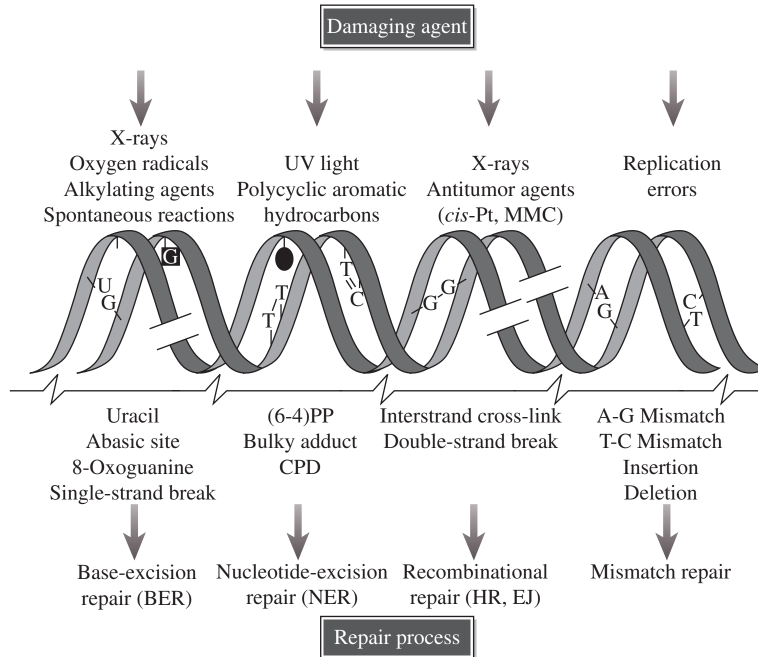


Figure 11.1 DNA damage and repair. DNA damaging agent produce specific type of DNA damage, and these are repaired by specific type DNA repair mechanisms. UVB produces 6-4 photoproducts (6-4)PP and cyclobutane pyrimidine dimers (CPD). *cis*-PT, cicplatinum; MMC, mitomycin C; HR, homologus recombinations; EJ, end joining. Adapted from Hoeijmakers, J. J. *Nature* **411**:366, 2001.

point mutation produces a substitution of a purine for another purine (i.e., guanine for adenine) or a pyrimidine for another pyrimidine (i.e., thymine for cytosine) the mutation is referred as a transition. If a purine is substituted for a pyrimidine or vice versa (i.e., thymine for adenine or guanine for cytosine), the mutation is referred to as a transversion.

Fortunately, cells of higher eukaryotes have four efficient repair systems that can repair specific types of DNA damage (Figure 11.1). Base excision repair (BER) repairs deaminated DNA, AP sites, alkylated DNA, oxidized bases, and single strand breaks. Nucleotide excision repair (NER) repairs DNA that contains large bulky adducts (i.e., polycyclic aromatic hydrocarbons [PAHs]) as well as UV-induced bulky cyclobutane pyrimidine dimers and 6-4 photoproducts. Recombinational repair includes homologous recombination repair and end joining repair, and these repair double strand breaks in DNA. The fourth system is mismatch repair, and it repairs base mismatches between bases on opposing strands of DNA. Cell of higher eukaryotes respond to DNA damage by engaging cell cycle checkpoints which pause the cell in the cell cycle to allow time for DNA repair, or if the damage is too extensive, damaged cells commit to apoptosis (programmed cell death).

11.2 GENERAL ASPECTS OF CANCER

Carcinogenesis is the process through which cancer develops. Chemical carcinogenesis is the study of the mechanisms through which chemical carcinogens induce cancer and also involves the development/utilization of experimental systems aimed at determining whether a substance is a potential human carcinogen. An important aspect of toxicology is the identification of potential human carcinogens.

Cancer is not a single disease but a large group of diseases, all of which can be characterized by the uncontrolled growth of an abnormal cell to produce a population of cells that have acquired the ability to multiply and invade surrounding and distant tissues. It is this invasive characteristic that imparts its lethality on the host.

Epidemiology studies have revealed that the incidence of most cancers increase exponentially with age (Figure 11.2). Epidemiologists have interpreted this exponential increase in cancer incidence to denote that three to seven critical mutations or "hits" within a single cell are required for cancer development. Molecular analyses of human tumors have confirmed the accumulation of mutations in critical genes in the development of cancer. Most cancers are monoclonal in origin (derived from a single cell) and do not arise from a single critical mutation but from the accumulation of multiple critical mutations in relevant target genes within a single cell (Figure 11.3). Initially, a somatic mutation occurs in a critical gene, and this mutation provides a proliferative advantage to the cell and results in the expansion or proliferation of the mutant clone. Over time, an additional mutation in a critical gene that occurs within this clone provides a further selective growth advantage. This process of mutation and selection is repeated over time and eventually results in clone of cells with mutations in multiple critical genes. It often requires decades

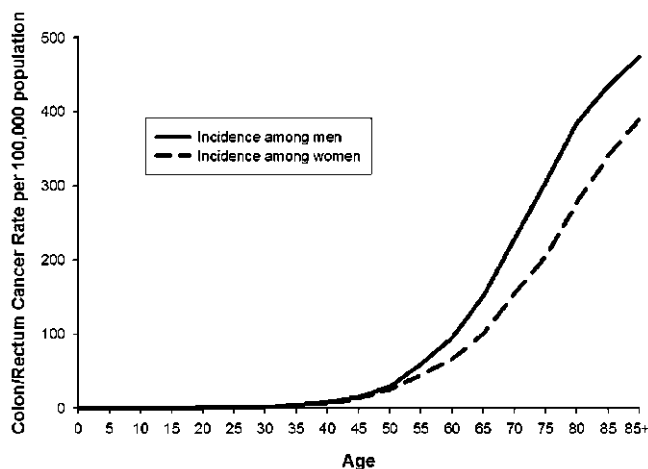


Figure 11.2 Age-Related Colon/Rectum Cancer Incidence in the United States (2000–2003). Epidemiological studies have revealed the incidence of most human cancers increase exponentially with age. NCI SEER. *Cancer Statistics Review, 1975–2003*. http://seer.cancer.gov.

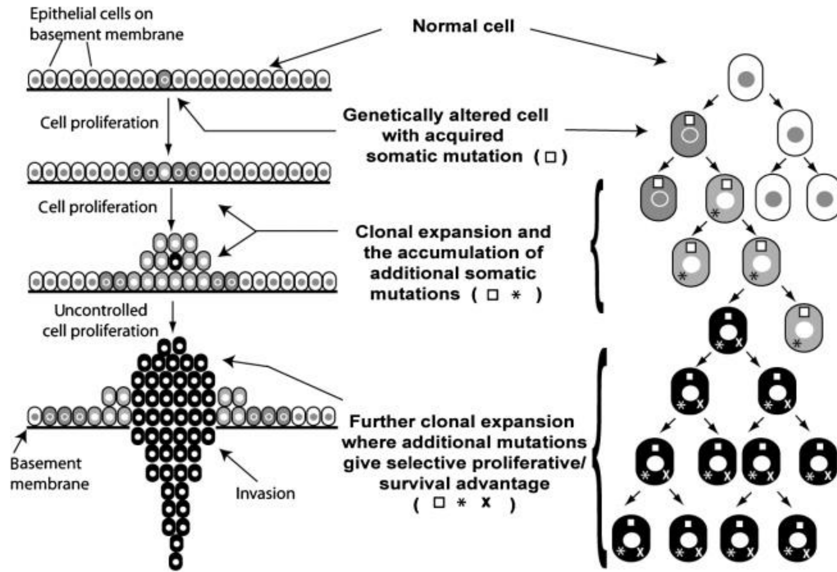


Figure 11.3 Monoclonal nature of cancer. Most cancers are monoclonal in origin and are derived from the accumulation of sequential mutations in an individual cell. Each additional mutation within the cell provides a further proliferative/survival advantage, and this process drives clonal expansion and tumor development.

for a cell clone to accumulate multiple critical mutations and for the progeny of this cell to clonally expand to produce a clinically detectable cancer. Thus, the time required for accumulation of mutations in critical genes within a cell is related to the observation that cancer incidence increases exponentially with age.

Specific genes found in normal cells, termed proto-oncogenes, are involved in the positive regulation of cell proliferation and cell survival and are frequently mutated in cancer. Mutational activation of these proto-oncogenes results in a gain of function in which the altered gene product continually stimulates cell proliferation or increases cell survival (block apoptosis). Such proto-oncogenes with gain-of-function mutations are now referred to as oncogenes. Another family of genes, known as tumor suppressor genes, can be mutationally inactivated during carcinogenesis, resulting in a loss of function. Tumor suppressor genes and the proteins they encode often function as negative regulators of cell proliferation or positive regulators of apoptosis. Tumor suppressor genes containing loss-of-function mutations encode proteins that are inactive so they are unable to inhibit cell proliferation or induce apoptosis in response to DNA damage or activated oncogenes. DNA stability genes responsible for genome maintenance are also mutated in some cancers, and their reduced activity contributes to genomic instability and the accumulation of mutations in oncogenes and tumor suppressor genes. The activation of oncogenes and inactivation of tumor suppressor genes within a cell as well as the alteration in genes responsible for genomic maintenance (repair, checkpoints, etc.) are important mutational events in carcinogenesis (Figure 11.4). A simple analogy can be made to the automobile; tumor suppressor genes are analogous to the brakes on the car while

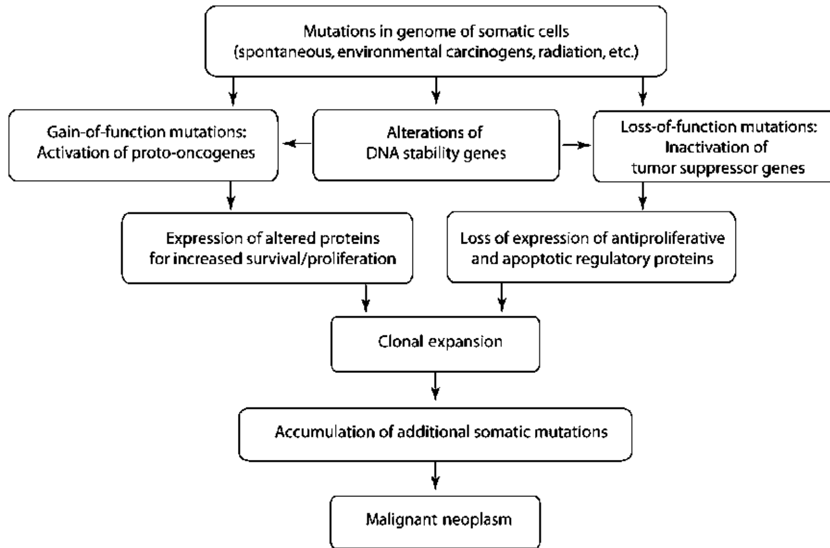


Figure 11.4 General aspects of the cancer process. Somatic mutations involving gain-of-function mutations in proto-oncogenes and loss-of-function mutations in tumor suppressor genes provide for a selective proliferative/survival advantage and are critical events in tumorigenesis. Inactivating mutations in genes involved in genomic maintenance can result in genomic instability.

the proto-oncogenes are analogous to the accelerator pedal, and DNA stability genes are analogous to the automobile mechanic. Mutations within tumor suppressor genes inactivate the braking system while mutations in proto-oncogenes activate the acceleration system. Altering both the cellular brakes and cellular accelerator results in uncontrolled cell proliferation while mutations in genes responsible for DNA stability and genomic maintenance is akin to having an inept mechanic. Mutations in oncogenes, tumor suppressor genes, and DNA stability genes provide a selective growth advantage to the cancer cell through enhanced cell proliferation, decreased apoptosis, and increased genomic instability (Figure 11.4).

Cancer is a type of a neoplasm or tumor. While technically a tumor is defined as only a tissue swelling, it is now used as a synonym for a neoplasm. A neoplasm or tumor is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with the normal tissue, and persists after cessation of the stimuli that evoked it. There are two basic types of neoplasms, termed benign and malignant. The general characteristics of these tumors are defined in Table 11.1. Cancer is the general name for a malignant neoplasm. In terms of cancer nomenclature, most adult cancers are derived from epithelial cells (colon, lung, breast, skin, etc.) and are termed carcinomas. Sarcomas are derived from mesenchymal tissues, while leukemias and lymphomas are derived from blood-forming cells and lymphocytes, respectively. Melanoma is derived from melanocytes, and retinoblastoma, glioblastoma, and neuroblastoma are derived from the stem cells of the retina, glia, and neurons, respectively. According to the American Cancer Society, (1) the lifetime risk for developing cancer in the United States is one in three for women and one

TABLE 11.1 Some General Characteristics of Malignant and Benign Neoplasms

Benign	Malignant
Generally slow growing	May be slow to rapid growing
Few mitotic figures	Numerous mitotic figures
Well differentiated and architecture resembles that of parent tissue	Some lack differentiation, disorganized; loss of parent tissue architecture
Sharply demarcated mass that does not invade surrounding tissue	Locally invasive, infiltrating into surrounding normal tissue
No metastases	Metastases

in two for men; (2) in 2009 about 1.4 million new cancer cases are expected to be diagnosed, not including carcinoma *in situ* or basal or squamous cell skin cancer; and (3) cancer is a leading cause of death in the United States and approximately 23% of all deaths are due to cancer.

11.3 HUMAN CANCER

Although cancer is known to occur in many groups of animals, the primary interest of most cancer research is focused on human cancer. Nevertheless, much of the mechanistic research and carcinogen assessment is carried out in experimental models, usually rodent models. To begin to appreciate the complexity of this subject, it is important to first have some understanding of human cancer and its etiologies.

11.3.1 Causes, Incidence, and Mortality Rates of Human Cancer

Cancer cases and cancer deaths by sites and sex for the United States are shown in Figure 11.5. Breast, lung, and colon and rectum cancers are the major cancers in females while prostate, lung, and colon and rectum are the major cancer sites in males. A comparison of cancer deaths versus incidence for a given site reveals that prognosis for lung cancer cases is poor while that for breast or prostate cancer cases is much better. Age-adjusted cancer mortality rates (1930–2005) for selected sites in males are shown in Figure 11.6 and for females is shown in Figure 11.7. The increase in the mortality rate associated with lung cancer in both females and males is striking and is due to cigarette smoking. It is estimated that 87% of lung cancers are due to smoking. Lung cancer death rates in males and females began to increase in the mid 1930s and mid 1960s, respectively. These time differences are due to the fact that cigarette smoking among females did not become popular until the 1940s while smoking among males was popular in the early 1900s. Taking into account these differences along with a 20–25-year lag period for the cancer to develop explains the differences in the temporal increase in lung cancer death rates in males and female. Another disturbing statistic is that lung cancer, a theoretically preventable cancer, has recently surpassed breast cancer as the cancer responsible for the greatest number of cancer deaths in women. In addition to lung cancer, smoking also plays a significant role in cancer of the mouth, esophagus, pancreas, pharynx,

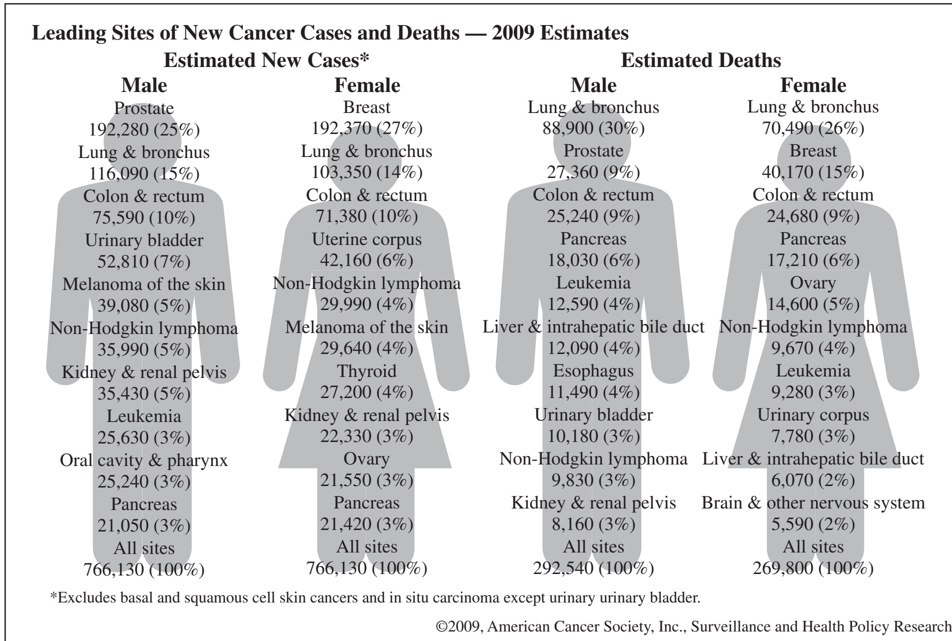


Figure 11.5 Cancer Cases and Cancer Deaths by Sites and Sex—2009 Estimates. Reprinted with permission of the American Cancer Society. *Cancer Facts and Figures 2007*. Atlanta: American Cancer Society.

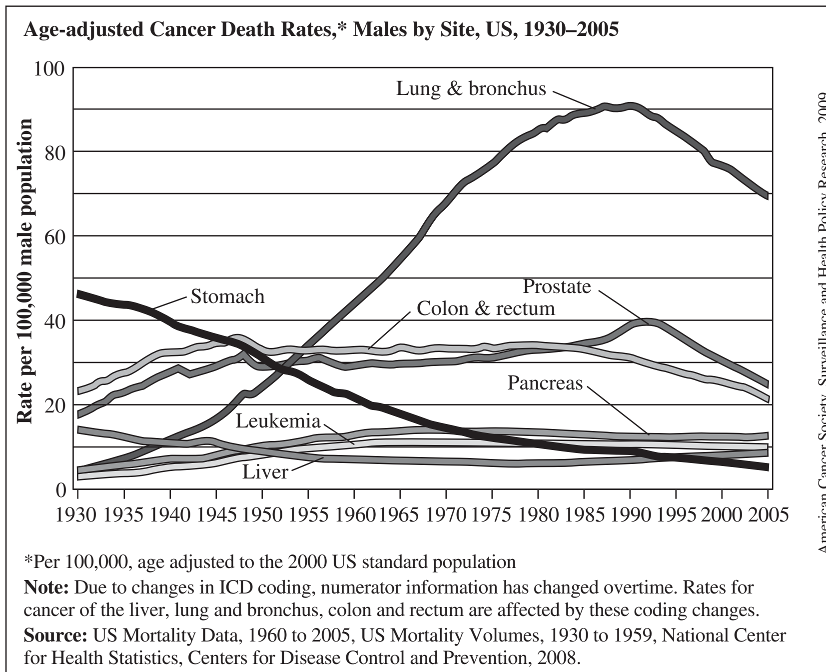


Figure 11.6 Age-Adjusted Cancer Death Rates in Males from 1930–2005. Reprinted with permission of the American Cancer Society. *Cancer Facts and Figures 2009*. Atlanta: American Cancer Society.

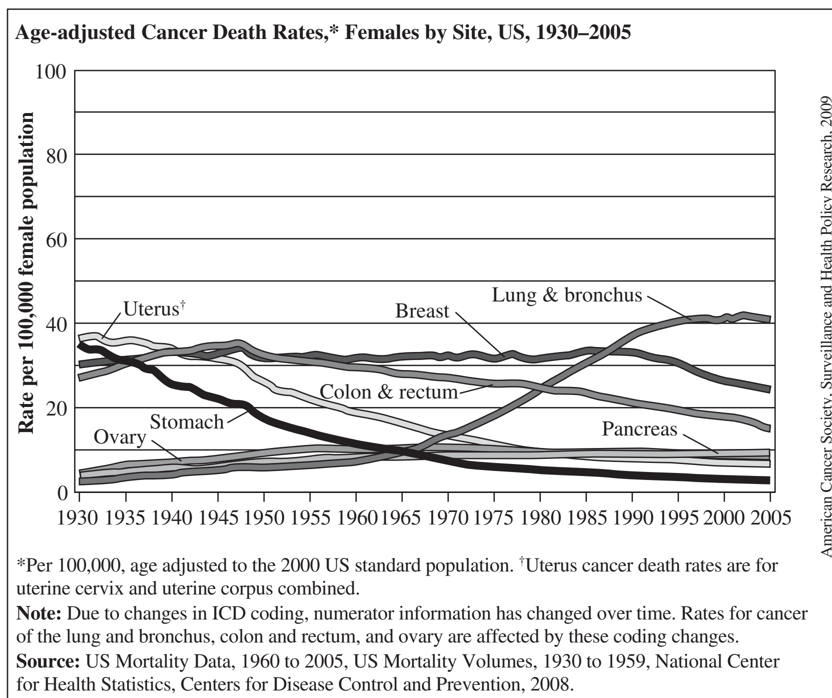


Figure 11.7 Age-Adjusted Cancer Death Rates in Females from 1930–2005. Reprinted with permission of the American Cancer Society. *Cancer Facts and Figures 2009.* Atlanta: American Cancer Society.

larynx, bladder, kidney, and uterine cervix. In general, it is important to realize that death and incidence rates for some types of cancers are increasing while the rates for others are decreasing or remaining constant.

Major insights into the etiologies of cancer have been attained through epidemiological studies that relate the role of hereditary, environmental, and cultural influences on cancer incidence as well as through laboratory studies using model rodent/cellular systems. Cancer susceptibility is determined by complex interactions between age, environment, and an individual's genetic makeup. It is estimated from epidemiological studies that 35–80% of all cancers are associated with the environment in which we live and work. The geographic migration of immigrant populations and differences in cancer incidence between communities has provided an important information regarding the role of the environment and specific cancer incidences. For example, Japanese immigrants and the sons of Japanese immigrants living in California begin to assume a cancer death rate similar to the California white population (Figure 11.8). These results implicate a role for the environment in the etiology of cancer. It should be noted that the term environment is not restricted to exposure to man-made chemicals in the environment but applies to all aspects of our lifestyle, including smoking, diet, cultural and sexual behavior, occupation, natural and medical radiation, and exposure to substances in air, water, and

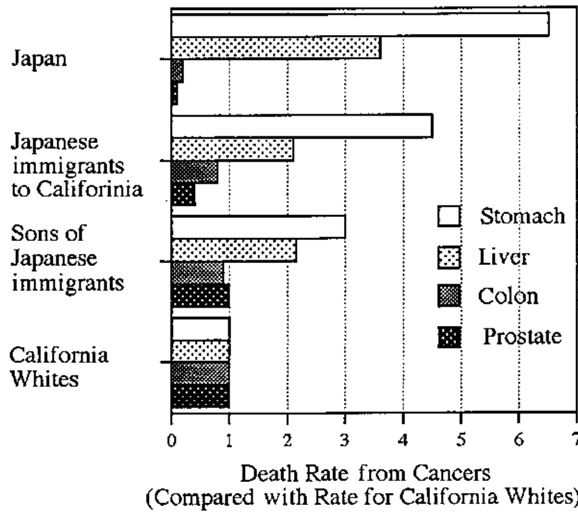


Figure 11.8 Cancer death rates in Japanese immigrants in the United States. The cancer death rate for each type of cancer is normalized to one for California White males and then compared to Japanese immigrants of a similar age. Adapted from Cairns, J. *Readings in Scientific American—Cancer Biology*, p. 13, 1986.

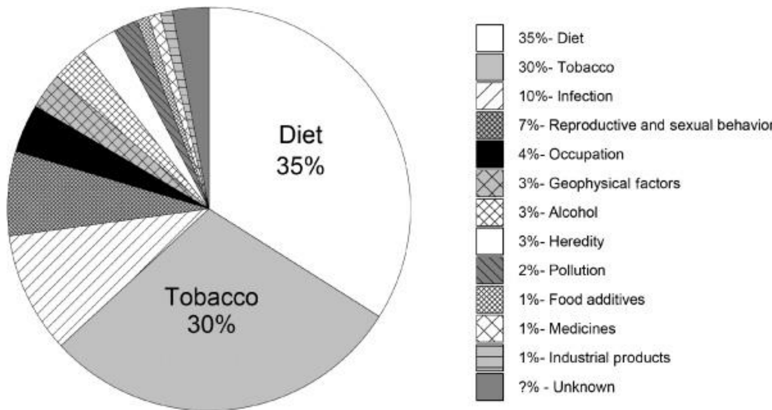


Figure 11.9 Proportion of cancer deaths attributed to various different factors. Values are a best estimate as determined by epidemiological studies. Adapted from Doll, R. and R. Peto. *The Causes of Cancer: Quantitative Estimates of Avoidable Risks of Cancer in the United States Today*. Oxford Medical Publications, 1981.

soil. The major factors associated with cancer and their estimated contributions to human cancer incidence are listed in Figure 11.9. Only a small percentage of total cancer occurs in individuals with a hereditary mutation/hereditary cancer syndrome (~5%). However, an individual's genetic background is the "stage" in which the cancer develops and susceptibility genes have been identified in humans. For

example, genetic polymorphisms in enzymes responsible for the activation of chemical carcinogens may represent a risk factor as is the case for polymorphisms in the *N*-acetyltransferase gene and the risk of bladder cancer. These types of genetic risk factors are of low penetrance (low to moderate increased risk); however, increased risk is usually associated with environmental exposure. While the values presented in Figure 11.9 are a best estimate, it is clear that tobacco use and diet constitute the major factors associated with human cancer incidence. If one considers all of the categories that pertain to man-made chemicals, it is estimated that their contribution to human cancer incidence is approximately 10%. However, the factors listed in Figure 11.9 are not mutually exclusive since there is likely to be interaction between these factors in the multistep process of carcinogenesis.

11.3.2 Known Human Carcinogens

Two of the earliest observations that exposure of humans to certain chemicals or substances is related to an increased incidence of cancer were made independently by two English physicians, John Hill in 1771 and Sir Percival Pott in 1776. Hill observed an increased incidence of nasal cancer among snuff users while Pott observed that chimney sweeps had an increased incidence of skin cancer of the scrotum. Pott attributed this to topical exposure to soot and coal tar. It was not until a century and a half later in 1915 when two Japanese scientists, Yamagiwa and Ichikawa, substantiated Pott's observation by demonstrating that multiple topical applications of coal tar to rabbit skin produced skin carcinomas. This experiment is important for two major reasons: (1) it was the first demonstration that a chemical or substance could produce cancer in animals, and (2) it confirmed Pott's initial observation and established a relationship between human epidemiology studies and animal carcinogenicity. Because of these important findings, Yamagiwa and Ichikawa are considered the fathers of experimental chemical carcinogenesis. In the 1930s, Kennaway and coworkers isolated active carcinogenic chemicals from coal tar and identified one as benzo[a]pyrene, a PAH that results from the incomplete combustion of organic molecules. Benzo[a]pyrene has also been identified as one of the carcinogens in cigarette smoke. The p53 tumor suppressor gene can be mutationally inactivated by numerous carcinogens, including the carcinogenic metabolite of benzo[a]pyrene.

Epidemiological studies have provided sufficient evidence that exposure to a variety of chemicals, agents, or processes are associated with human cancer. For example, the following causal associations have emerged between exposure and the development of specific cancers; vinyl chloride and hepatic cancer, amine dyes and bladder cancer, benzene and leukemia, diethylstilbestrol and clear cell carcinoma of the vagina and cigarette smoking and lung cancer. Naturally occurring chemicals or agents such as asbestos, aflatoxins, nickel, and certain arsenic compounds are also associated with an increased incidence of certain human cancers. Both epidemiological studies and rodent carcinogenicity studies are important in the identification and classification of potential human carcinogens. The strongest evidence for establishing whether exposure to a given chemical is carcinogenic in humans comes from epidemiological studies. However, these studies are complicated by the fact that it often takes 20–30 years after carcinogen exposure for a clinically detectable cancer to develop. This delay is problematic and can result in inaccurate historical

exposure information and additional complexity due to the interference of a large number of confounding variables. Most importantly, this lag period can also prevent the timely identification of a putative carcinogen and result in unnecessary human exposure. Therefore, methods to identify potential human carcinogens have been developed. The long-term rodent bioassay, also known as the 2-year rodent carcinogenesis bioassay (see Chapter 20), is currently used in an attempt to identify potential human carcinogens. It is clear that almost all, if not all, human carcinogens identified to date are rodent carcinogens; however, it is not known if all rodent carcinogens are human carcinogens. Indeed, identification of possible human carcinogens based on rodent carcinogenicity is complicated (see below). Table 11.2 contains the list of the known human carcinogens as reported by the National Toxicology Program's 11th Report on Carcinogens. The entire 11th Report on Carcinogens which succinctly summarizes the information for the classification of each listed agent (246 agents) is available at http://ntp.niehs.nih.gov. In addition, the

TABLE 11.2 Known Human Carcinogens Listed in 11th Report on Carcinogens (National Toxicology Program)

Aflatoxins
Alcoholic Beverage Consumption
4-Aminobiphenyl
Analgesic Mixtures Containing Phenacetin
Arsenic Compounds, Inorganic
Asbestos
Azathioprine
Benzene
Benzidine
Beryllium and Beryllium Compounds
1,3-Butadiene
1,4-Butanediol Dimethanesulfonate (Myleran)
Cadmium and Cadmium Compounds
Chorambucil
1-(2-Choroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU)
Bis(Chloromethyl) Ether and Technical Grade Chloromethyl Methyl Ether
Chromium Hexavalent Compounds
Coal Tar Pitches
Coal Tars
Coke Oven Emissions
Cyclophosphamide
Cyclosporin A
Diethylstilbestrol
Dyes Metabolized to Benzidine
Environmental Tobacco Smoke
Erionite
Estrogens, Steroidal
Ethylene Oxide
Hepatitis B Virus
Hepatitis C Virus
Human Papilloma Viruses: Some Genital-Mucosal Types
Melphalan

TABLE 11.2 ***Continued***

Methoxsalen with Ultraviolet A Therapy (PUVA)
Mineral Oils (Untreated and Mildly Treated)
Mustard Gas
2-Naphthylamine
Neutrons
Nickel Compounds
Radon
Silica, Crystalline (Respirable Size)
Smokeless Tobacco
Solar Radiation
Soots
Strong Inorganic Acid Mists Containing Sulfuric Acid
Sunlamps/sunbeds
Tamoxifen
2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)
Thiotepa
Thorium Dioxide
Tobacco Smoking
Vinyl Chloride
Ultraviolet Radiation, Broad Spectrum
Wood Dust
X-Radiation and Gamma Radiation

International Agency for Research on Cancer (IARC) maintains their own complete list of human carcinogens as well as carcinogenic complex mixtures and occupations associated with increased cancer incidence (http://monographs.iarc.fr/ENG/Classification/index.php). *In vitro* mutagenicity assays are also used to identify mutagenic agents that may have carcinogenic activity (see Section 11.3.4).

11.3.3 Classification of Human Carcinogens

Identification and classification of potential human carcinogens through the 2-year rodent carcinogenesis bioassay is complicated by species differences, use of high doses (MTD, the maximum tolerated dose), the short life span of the rodents, high background tumor incidence in some organs, sample size, and the need to extrapolate from high to low doses for human risk assessment. MTD is the highest dose used in the rodent bioassay and is operationally defined in toxicology as the highest daily dose of a chemical that does not cause overt toxicity in a 90-day study in laboratory mice or rats. Although these problems are by no means trivial, the rodent 2-year bioassay is still considered the "gold standard" assay for the identification of potential human carcinogens. The criteria used for the classification of carcinogens by the National Toxicology Program, 11th Report on Carcinogens 2005 is as follows: *known human carcinogen category* which is reserved for those substances for which sufficient evidence of carcinogenicity from studies in humans exists, indicating a cause and effect relationship between exposure to the substance and human cancer. The *reasonable anticipated to be a human carcinogen category* includes those substances for which there is limited evidence of carcinogenicity in humans and/or

sufficient evidence of carcinogenicity in experimental animal, indicating a cause and effect relationship between exposure to the substance and cancer. Conclusions regarding carcinogenicity in humans or experimental animals are based on expert, scientific judgment with consideration given to all relevant information. The 11th Edition of the Report on Carcinogens contains 246 entries, 58 of which are listed as known to be human carcinogens and the remaining 188 listed as reasonably anticipated to be human carcinogens. The complexity of classifying agents as to their human carcinogenic potential is complex, and this is best demonstrated by examining the criteria and the classification system used by the IARC (Table 11.3). Carcinogens are generally classified by the weight of evidence for carcinogenicity referred to as sufficient, limited, or inadequate based on both epidemiological studies and animal data. In 2005, Environmental Protection Agency (EPA) revised their guidelines for carcinogen risk assessment and their carcinogen classification scheme. New guidelines emphasize the incorporation of biological mechanistic data in the analysis and do not rely solely on rodent tumor data. The six alphanumeric categories listed in Table 11.3 have been replaced by five descriptors for classifying

TABLE 11.3 International Agency for Research on Cancer (IARC) Classification of Carcinogens

Group 1: The agent is *carcinogenic to humans*.
This category is used when there is *sufficient evidence of carcinogenicity* in humans. Exceptionally, an agent may be placed in this category when evidence of carcinogenicity in humans is less than *sufficient* but there is *sufficient evidence of carcinogenicity* in experimental animals and strong evidence in exposed humans that the agent acts through a relevant mechanism of carcinogenicity.
Group 2
This category includes agents for which, at one extreme, the degree of evidence of carcinogenicity in humans is almost *sufficient*, as well as those for which, at the other extreme, there are no human data but for which there is evidence of carcinogenicity in experimental animals. Agents are assigned to either Group 2A (*probably carcinogenic to humans*) or Group 2B (*possibly carcinogenic to humans*) on the basis of epidemiological and experimental evidence of carcinogenicity and mechanistic and other relevant data. The terms *probably carcinogenic* and *possibly carcinogenic* have no quantitative significance and are used simply as descriptors of different levels of evidence of human carcinogenicity, with *probably carcinogenic* signifying a higher level of evidence than *possibly carcinogenic*.
Group 2A: The agent is *probably carcinogenic to humans*.
This category is used when there is *limited evidence of carcinogenicity* in humans and *sufficient evidence of carcinogenicity* in experimental animals. In some cases, an agent may be classified in this category when there is *inadequate evidence of carcinogenicity* in humans and *sufficient evidence of carcinogenicity* in experimental animals and strong evidence that the carcinogenesis is mediated by a mechanism that also operates in humans. Exceptionally, an agent may be classified in this category solely on the basis of *limited evidence of carcinogenicity* in humans. An agent may be assigned to this category if it clearly belongs, based on mechanistic considerations, to a class of agents for which one or more members have been classified in Group 1 or Group 2A.

TABLE 11.3 *Continued*

Group 2B: The agent is *possibly carcinogenic to humans*.
This category is used for agents for which there is *limited evidence of carcinogenicity* in humans and less than *sufficient evidence of carcinogenicity* in experimental animals. It may also be used when there is *inadequate evidence of carcinogenicity* in humans but there is *sufficient evidence of carcinogenicity* in experimental animals. In some instances, an agent for which there is *inadequate evidence of carcinogenicity* in humans and less than *sufficient evidence of carcinogenicity* in experimental animals together with supporting evidence from mechanistic and other relevant data may be placed in this group. An agent may be classified in this category solely on the basis of strong evidence from mechanistic and other relevant data.
Group 3: The agent is *not classifiable as to its carcinogenicity to humans*.
This category is used most commonly for agents for which the evidence of carcinogenicity is *inadequate* in humans and *inadequate* or *limited* in experimental animals. Exceptionally, agents for which the evidence of carcinogenicity is *inadequate* in humans but *sufficient* in experimental animals may be placed in this category when there is strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans. Agents that do not fall into any other group are also placed in this category. An evaluation in Group 3 is not a determination of noncarcinogenicity or overall safety. It often means that further research is needed, especially when exposures are widespread or the cancer data are consistent with differing interpretations.
Group 4: The agent is probably not carcinogenic to humans.
This category is used for agents for which there is *evidence suggesting lack of carcinogenicity* in humans and in experimental animals. In some instances, agents for which there is *inadequate evidence of carcinogenicity* in humans but *evidence suggesting lack of carcinogenicity* in experimental animals, consistently and strongly supported by a broad range of mechanistic and other relevant data, may be classified in this group.

Source: IARC. http://monographs.iarc.fr/ENG/Preamble/currentb6evalrationale0706.php.

human carcinogenic potential. Carcinogens are classified by the EPA as: (1) carcinogenic to humans, (2) likely to be carcinogenic in humans, (3) suggestive evidence of carcinogenic potential, (4) inadequate information to assess carcinogenic potential, and (5) not likely to be a human carcinogen.

11.3.4 Usefulness and Limitations of Mutagenicity Assays for the Identification of Carcinogens

Identification and classification of potential human carcinogens through the 2-year rodent carcinogenesis bioassay is complicated by species differences, use of high doses (MTD), the short life span of the rodents, sample size, and the need to extrapolate from high to low doses for human risk assessment. In addition, the 2-year rodent bioassay to is costly to conduct (>2–4 million dollars) and can take 2–4 years before complete results can be obtained. Since many carcinogens are mutagens, short-term test systems to evaluate the mutagenicity or genetic toxicity of compounds were developed with the idea that these tests could be used to quickly and inexpensively detect/identify chemical carcinogens. Short-term

genotoxicity/mutagenicity assays were developed in a variety of organisms including bacteria, yeast, *Drosophila*, and human and rodent cells. These mutagenic assays or short-term genotoxicity tests directly or indirectly measure point mutations, frame-shift mutations, chromosomal damage, DNA damage and repair, and cell transformation.

In the 1970s, it was reported that mutagenicity could predict rodent carcinogenicity 90% of the time; however, after extensive evaluation, it is now considered that mutagenicity can predict rodent carcinogenicity approximately 60% of the time. For certain classes of carcinogens, such as the PAHs, short-term mutagenicity tests are generally highly accurate at predicting rodent carcinogenicity. However, for other classes of carcinogens such as the halogenated hydrocarbons, short-term genotoxicity tests often fail to detect these rodent carcinogens. Many of these halogenated hydrocarbons probably function through an epigenetic mechanism/tumor promoting mechanism.

In a seminal study published in 1987 by Tennant et al., 73 chemicals previously tested in the rodent 2-year carcinogenesis bioassay were examined in four widely used short-term tests for genetic toxicity. The short-term assays measured mutagenesis/genotoxicity using the *Salmonella* assay (Ames Assay) and mouse lymphoma assay, and chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells. The concordance (% agreement between short-term genotoxicity test and rodent bioassay results) of each assay with the rodent bioassay data was approximately 60%. Within the limits of the study, there was no evidence of complementarity among the four tests, and no battery of tests constructed from these assays improved substantially on the overall performance of the *Salmonella* assay. When interpreting the results of short-term test for genetic toxicity assays, it is important to consider (1) the structure and physical properties of the test compound; (2) there is only 60% concordance between short-term test for genetic toxicity and rodent carcinogenicity; (3) epigenetic versus genetic mechanisms of carcinogenesis; (4) the existence of noncarcinogenic mutagens; (5) a positive result in the *Salmonella* assay is a good predictor (83%) of rodent carcinogenicity; and (6) a negative result in the *Salmonella* assay only predicts a negative rodent bioassay 51% of the time. It is this latter statistic that lowers the concordance of the assay to 60%, which is largely due to agents that function through an epigenetic mechanism. It is also important to keep in mind that there is accumulating evidence that some compounds that are negative in short-term tests for mutagenicity can induce oxidative DNA damage *in vivo* through the direct or indirect production of reactive oxygen species. These compounds are *in vivo* mutagens but are negative in the short-term test of genetic toxicity. Several bacterial and mammalian short-term tests for mutagenicity and genotoxicity as well as their biochemical and genetic rationale are described in Chapter 20 on toxicity testing.

11.4 CLASSES OF AGENTS THAT ARE ASSOCIATED WITH CARCINOGENESIS

Chemical agents that influence cancer development can be divided into two major categories based on whether or not they are mutagenic in *in vitro* mutagenicity assay. DNA damaging agents (genotoxic) are mutagenic in *in vitro* mutagenicity

assays and are considered to produce permanent alterations in the genetic material of the host *in vivo*, and epigenetic agents (nongenotoxic) are not mutagenic in *in vitro* assays. These agents are not believed to alter the primary sequence of DNA but are considered to alter the expression or repression of certain genes and/or produce perturbations in signal transduction pathways that influence cellular events related to proliferation, differentiation, or apoptosis. Epigenetic/nongenotoxic agents contribute to the clonal expansion of cells containing an altered genotype (DNA alterations) to form tumors; however, in the absence of such DNA alterations, these epigenetic agents have no effect on tumor formation.

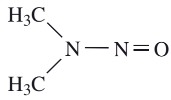
11.4.1 DNA Damaging Agents

DNA damaging agents can be divided into four major categories: (1) Direct-acting carcinogens are intrinsically reactive compounds that do not require metabolic activation by cellular enzymes to covalently interact with DNA. Examples include *N*-methyl-*N*-nitrosourea and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; the alkyl alkanesulfonates such as methyl methanesulfonate; the lactones such as beta propiolactone and the nitrogen and sulfur mustards. (2) Indirect-acting carcinogens require metabolic activation by cellular enzymes to form the ultimate carcinogenic species that covalently binds to DNA. Examples include dimethylnitrosamine, benzo[a]pyrene, 7,12-dimethylbenz[a]anthracene, aflatoxin B1 and 2-acetylaminofluorene (Figure 11.10). (3) Radiation and oxidative DNA damage can occur directly or indirectly. Ionizing radiation produces DNA damage through direct ionization of DNA to produce DNA strand breaks or indirectly via the ionization of water to reactive oxygen species that damage DNA bases. Ultraviolet radiation (UVR) from the sun is responsible for approximately 1 million new cases of human (nonmelanoma) skin cancer each year. Reactive oxygen species can also be produced by various chemicals and cellular processes including respiration and lipid peroxidation. (4) Inorganic agents such as arsenic, chromium, and nickel are considered DNA damaging agents, although in many cases, the definitive mechanism is unknown.

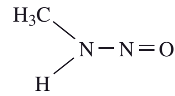
11.4.2 Epigenetic Agents

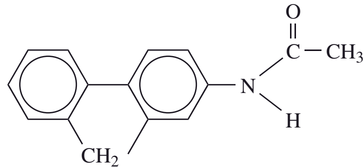
Epigenetic agents that influence carcinogenesis are not thought to alter the primary sequence of DNA, but rather they are considered to alter the expression or repression of certain genes and/or produce perturbations in signal transduction pathways that influence cellular events related to proliferation, differentiation, or apoptosis. Some mechanisms through which epigenetic agents produce their effects include: (1) alterations posttranslational modification of transcription factors, (2) activation of specific cellular kinases, (3) receptor–ligand interactions, (4) chromatin modifications involving promoter region methylation and histone modifications; and (5) immunosuppression. Many epigenetic agents favor the proliferation of cells with an altered genotype (cells containing a mutated oncogene(s) and/or tumor suppressor gene(s)) and allow the clonal expansion of these altered or "initiated" cells. Epigenetic agents can be divided into four major categories: (1) hormones such as conjugated estrogens and diethylstilbestrol, (2) immunosuppressive xenobiotics such as azathioprine and cyclosporin A; (3) solid state agents that include plastic implants and asbestos, and (4) tumor promoters in rodent models that include

Procarcinogen | Proximate carcinogen | Putative ultimate carcinogen

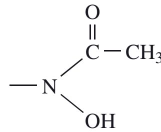


Dimethylnitrosamine

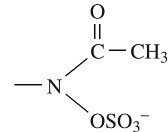




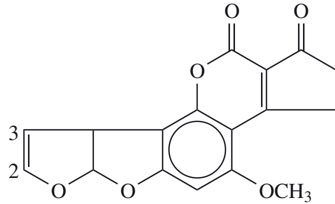
2-Acetylaminofluorene (AAF)



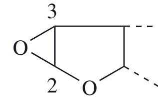
N-Hydroxy-AAF



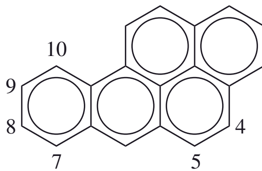
Sulfate ester Of *N*-hydroxy-AAF



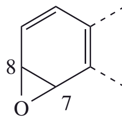
Aflatoxin B_1



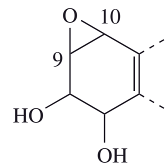
Aflatoxin B_1 2, 3-epoxide



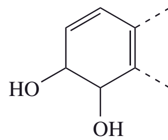
Benzo(a)pyrene (BP)



BP-7, 8-epoxide



BP-7, 8-dihydro-7, 8-diol-9, 10-epoxide



BP-7, 8-dihydro-7, 8-diol

Figure 11.10 Examples of DNA-damaging carcinogens.

12-*O*-tetradecanoylphorbol-13-acetate, peroxisome proliferators, TCDD, and phenobarbital (Figure 11.11). In humans, diet (including caloric, fat, and protein intake), excess alcohol, late age of pregnancy are considered to function through a promotion mechanism. While smoking and UVR have initiating activity, both are also considered to have tumor-promoting activity. By definition, tumor promoters are not classified as carcinogens since they are considered inactive in the absence of initiated cells. However, an altered genotype or an initiated cell can arise from spontaneous mutations resulting from imperfect DNA replication/repair, oxidative

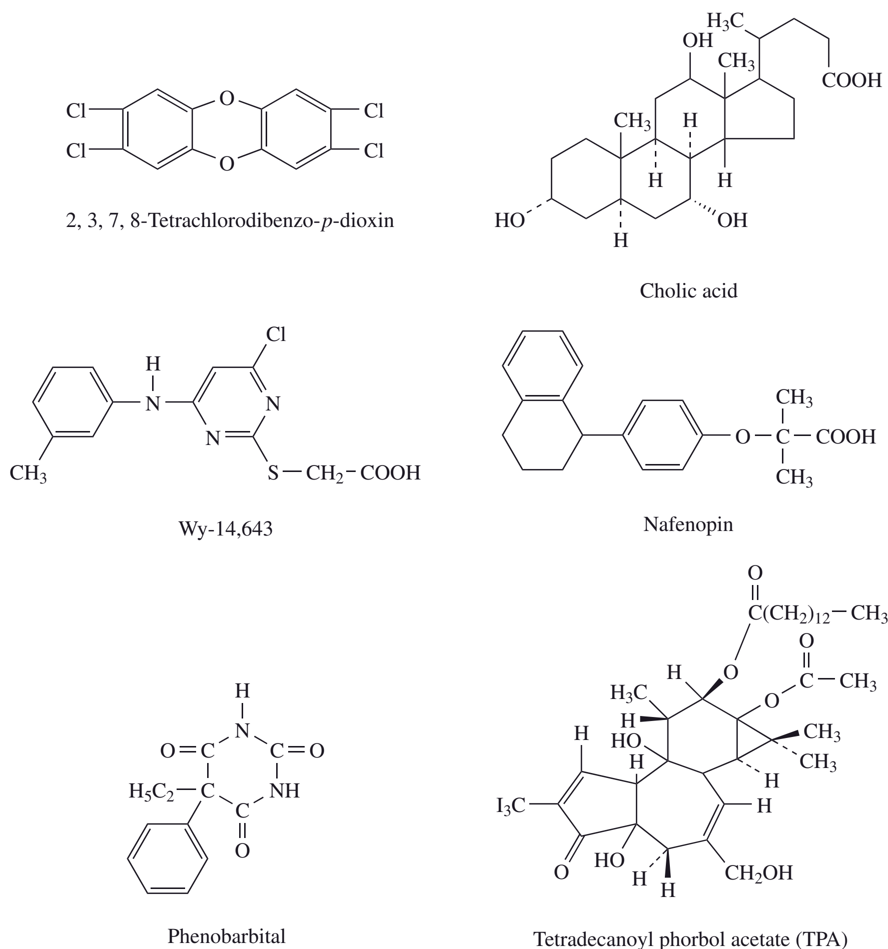


Figure 11.11 Examples of tumor promoters.

DNA damage, or can result from environmental carcinogens. Theoretically, in the presence of a tumor promoter, these mutant cells would clonally expand to form a tumor. Therefore, the nomenclature becomes somewhat a matter of semantics as to whether the tumor promoter should or should not be classified as a carcinogen. Certain hormones and immunosuppressive agents are classified as human carcinogens although it is generally considered that these agents are not carcinogenic in the absence of initiated cells, but rather, like tumor promoters, may only allow for the clonal expansion of cells with an altered genotype.

11.5 GENERAL ASPECTS OF CHEMICAL CARCINOGENESIS

There is indisputable evidence to support the somatic mutation theory of carcinogenesis which states that cancer is caused by mutations within somatic cells. As stated earlier, cancer development (carcinogenesis) involves the accumulation of

mutations in multiple critical genes, and these mutations can result from endogenous processes or from environmental carcinogens. Many chemical carcinogens can alter DNA through covalent interaction (DNA adducts or alkylation) or direct and/or indirect oxidative DNA damage. Some chemical carcinogens are intrinsically reactive and can directly covalently bind to DNA while others require metabolic activation via cytochrome P450 to produce reactive electrophilic intermediates capable of covalently binding to DNA (Figure 11.12). In the 1950s, Elizabeth and James Miller observed that a diverse array of chemicals with divergent structures could produce cancer in rodents. In an attempt to explain this, they hypothesized that these diverse chemicals are metabolically activated to common electrophilic metabolites that are capable of interacting with nucleophilic sites in the DNA. The Millers termed this the electrophilic theory of chemical carcinogenesis. From this concept of metabolic activation of carcinogens, the terms parent, proximate, and ultimate carcinogen were developed (Figure 11.12). A parent carcinogen is a compound that must be metabolized in order to have carcinogenic activity; a proximate carcinogen is an intermediate metabolite requiring further metabolism resulting in the ultimate carcinogen which is the actual metabolite that covalently binds to the DNA and is responsible for producing mutations. The cell has many defense systems to detoxify the carcinogenic species, including cellular antitioxidants (i.e., vitamin C

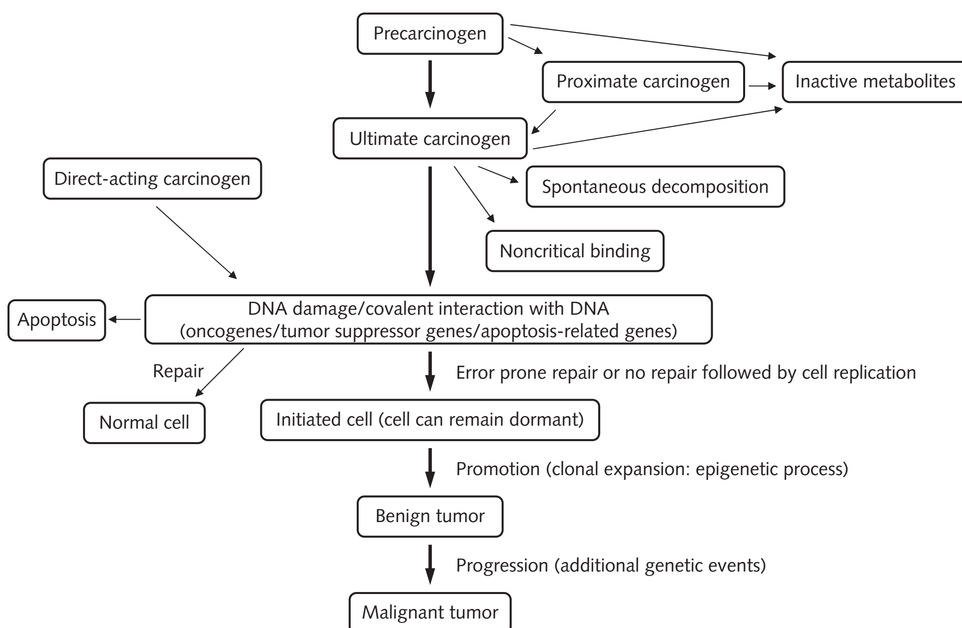


Figure 11.12 General aspects of multistage chemical carcinogenesis. Carcinogens can form reactive species that damage DNA. If damage occurs in a critical gene and either error prone repair or no repair is followed by cell replication, a mutation can result. This mutated cell is now referred to as an "initiated cell." Tumor promoters allow for the clonal expansion of an initiated cell to produce a benign tumor. This benign tumor can progress to a malignant tumor, and this involves additional genetic changes in critical genes.

and E) and nucleophiles (i.e., glutathione [GSH]) as well as a whole host of Phase I and Phase II enzymes. In addition, reactive carcinogenic species may bind to non-critical sites in the cell resulting in detoxification or they can undergo spontaneous decomposition. If the carcinogenic species binds to DNA, the adducted DNA can be repaired, producing a normal cell. If there is error in the repair of the DNA or the DNA adduct is not repaired before the cell replicates, an error in the newly synthesized DNA could occur and, if so, a mutation would occur in the DNA of the daughter cell. If this change has occurred in a critical gene, for example, in a proto-oncogene or tumor suppressor gene, it would represent an important mutagenic event(s) in carcinogenesis.

The mutationally altered cell or "initiated cell" has an altered genotype and may remain dormant (not undergo clonal expansion) for the lifetime of the animal; however, additional mutations or "hits" in critical genes followed by clonal expansion could lead to tumor development as described earlier in this chapter. In addition to this mechanism, chemical carcinogenesis in experimental models can be divided into at least three stages termed initiation, promotion, and progression (Figure 11.12); this model is often referred to as the initiation/promotion model of chemical carcinogenesis. As mentioned above, the "initiated cell" may remain dormant (not undergo clonal expansion) for the lifetime of the animal. However, if the animal is repeatedly exposed to a tumor promoter, it will provide a selective growth advantage to the "initiated cell" which will clonally expand to produce a benign tumor. This process is termed tumor promotion and is an epigenetic process favoring the growth of cells with an altered genotype. The development of a malignant tumor from a benign tumor encompasses a third step, termed progression, and involves additional genetic changes.

Higher doses of carcinogen or multiple doses of carcinogen can produce tumors without tumor promoter treatment; under these circumstances, the chemical agent is often referred to as a complete carcinogen and the model as the complete carcinogenesis model.

11.5.1 Initiation-Promotion Model

Experimentally, the initiation-promotion process has been demonstrated in several organs/tissues including skin, liver, lung, colon, mammary gland, prostate and bladder, as well as in variety of cells in culture. While tumor promoters have different mechanisms of action and many are organ specific, all have common operational features (Figure 11.13). These features include: (1) following a subthreshold dose of initiating carcinogen, chronic treatment with a tumor promoter will produce many tumors; (2) initiation at a subthreshold dose alone will produce very few if any tumors; (3) chronic treatment with a tumor promoter in the absence of initiation will produce very few if any tumors; (4) the order of treatment is critical; that is, you must first initiate and then promote; (5) initiation produces an irreversible change; and (6) promotion is reversible in the early stages; for example, if an equal number of promoting doses are administered but the doses are spaced further apart in time, tumors would not develop or would be greatly diminished in number. Many tumor promoters are organ specific. For example, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), also known as phorbol 12-myristate 13-acetate (PMA), belongs to a family of compounds known as phorbol esters. Phorbol esters are isolated from

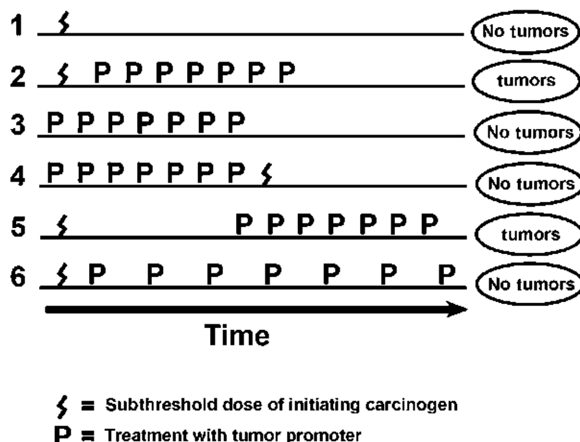


Figure 11.13 Diagrammatic scheme of the initiation promotion model. Topical application of a subthreshold dose of an initiating carcinogen to mouse skin results in no tumor formation; however, if this dose is followed by repetitive treatment with a tumor promoter, then tumors develop. Initiation is an irreversible genetic event leading to the development of an "initiated cell" that can remain dormant until exposed to a tumor promoter. Tumor promoters produce the clonal expansion of the initiated cell to form a tumor.

croton oil (derived from the seeds of the croton plant) and are almost exclusively active in skin. Phenobarbital, DDT, chlordane, TCDD, and peroxisome proliferators Wy 24,643, clofibrate, and nafenopin are hepatic tumor promoters. TCDD is also a promoter in lung and skin. Some bile acids are colonic tumor promoters while various estrogens are tumor promoters in the mammary gland and liver. There are multiple mechanisms of tumor promotion, and this may explain the organ-specific nature of the many promoters.

It is generally accepted that many tumor promoters allow for the clonal expansion of initiated cells by interfering with signal transduction pathways and/or altering expression of genes that are involved in the regulation of cell proliferation, differentiation, and/or apoptosis. While the precise mechanisms of many tumor promoters are not completely understood at the molecular/biochemical level, current research is providing new and promising mechanistic insights into how tumor promoters allow for the selective growth of initiated cells.

11.5.2 Metabolic Activation of Chemical Carcinogens and DNA Adduct Formation

Having described the general aspects of chemical carcinogenesis including the initiation-promotion model, we will now examine some aspects of chemical carcinogenesis in more detail. Metabolic activation of chemical carcinogens by cytochrome P450 is well documented. The metabolism of benzo[a]pyrene has been extensively studied, and at least 15 major Phase I metabolites have been identified. Many of these metabolites are further metabolized by Phase II enzymes to produce numerous different metabolites. Extensive research has elucidated which of these

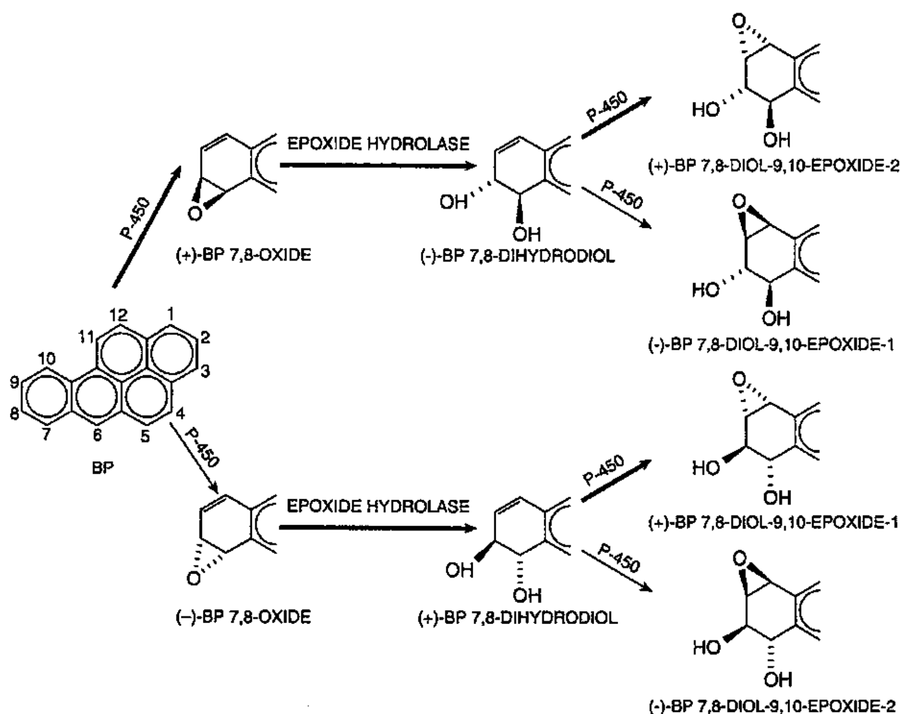


Figure 11.14 Metabolic activation of benzo[a]pyrene to the ultimate carcinogenic species. Benzo[a]pyrene is metabolized by cytochrome P450 and epoxide hydrolase to form the ultimate carcinogen, (+)benzo[a]pyrene 7,8 diol-9,10 epoxide-2. Adapted from Conney, A. H. *Cancer Res.* **42**:4875, 1982.

metabolites and pathways are important in the carcinogenic process. As shown in Figure 11.14, benzo[a]pyrene is metabolized by cytochrome P450 to benzo[a]pyrene-7,8 epoxide which is then hydrated by epoxide hydrolase to form benzo[a]pyrene-7,8-diol. Benzo[a]pyrene-7,8-diol is considered the proximate carcinogen since it must be further metabolized by cytochrome P450 to form the ultimate carcinogen, the bay-region diol epoxide, (+)-benzo[a]pyrene-7,8-diol-9,10-epoxide-2. It is this reactive intermediate that binds covalently to DNA-forming DNA adducts. (+)-Benzo[a]pyrene-7,8-diol-9,10-epoxide-2 binds preferentially to deoxyguanine residues, forming N-2 adduct. (+)-Benzo[a]pyrene-7,8-diol-9,10-epoxide-2 is highly mutagenic in eukaroytic and prokaryotic cells and is carcinogenic in rodents. It is important to note that not only is the chemical configuration of the metabolites of many PAHs important for their carcinogenic activity, but so is their chemical conformation/stereospecificity (Figure 11.11). For example, four different stereoisomers of benzo[a]pyrene-7,8-diol-9,10 epoxide are formed, each one only differs with respect to whether the epoxide or hydroxyl groups are above or below the plane of the flat benzo[a]pyrene molecule and yet only one, (+)-benzo[a]pyrene-7,8-diol-9,10-epoxide-2, has significant carcinogenic potential. Many PAHs are metabolized to bay-region diol epoxides. The bay region theory suggests that the bay-region diol epoxides are the ultimate carcinogenic metabolites of PAHs.

DNA can be altered by strand breakage, oxidative damage, large bulky adducts, and alkylation. Carcinogens such as *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine and methyl methanesulfonate alkylate DNA to produce N-alkylated and O-alkylated purines and pyrimidines. Ionizing radiation and reactive oxygen species commonly oxidize guanine to produce 8-oxoguanine. Formation of DNA adducts may involve any of the bases, although the N7 position of guanine is one the most nucleophilic sites in DNA. Of importance is how long the adduct is retained in the DNA. (+)-Benzo[a]pyrene-7,8-diol-9,10-epoxide-2 forms adducts mainly at guanine N-2 while aflatoxin B1 epoxide, another well-studied rodent and human carcinogen, binds preferentially to the N7 position of guanine. For some carcinogens, there is a strong correlation between the formation of very specific DNA adducts and tumorigenicity. Quantitation and identification of specific carcinogen adducts may be useful as biomarkers of exposure. Importantly, the identification of specific DNA adducts has allowed for the prediction of specific point mutations that would likely occur in the daughter cell providing there was no repair of the DNA adduct in the parent cell. As will be discussed in a later section, some of these expected mutations have been identified in specific oncogenes and tumor suppressor genes in chemically induced rodent tumors, providing support that the covalent carcinogen binding produced the observed mutation. In several cases, specific base pair changes in p53 tumor suppressor gene in human tumors are associated with a mutational spectrum that is consistent with exposure of the individual to a specific carcinogen. For example, the mutation spectrum identified in p53 in human tumors thought to result from the exposure of the individual to UVR, aflatoxin, and benzo[a]pyrene (from cigarette smoke) are consistent with the observed specific mutational damage in p53 induced by these agents in experimental cellular systems.

11.6 ONCOGENES

If the interaction of a chemical carcinogen with DNA leading to a permanent alteration in the DNA is a critical event in chemical carcinogenesis, then the identification of these altered genes and the function of their protein products is essential to our understanding of chemical carcinogenesis. It was not until the early to mid-1980s that the identification of specific genes that were mutationally altered by chemical carcinogens became known. Certain normal cellular genes, termed proto-oncogenes, can be mutated by chemical carcinogens providing a selective growth advantage to the cell. The mutational activation of proto-oncogenes is strongly associated with tumor formation, carcinogenesis, and cell transformation. Proto-oncogenes are highly conserved in evolution and their expression is tightly regulated. Their protein products function in the control of normal cellular proliferation, differentiation, and survival. However, when these genes are altered by a mutation, chromosome translocation, gene amplification, or promoter insertion, an abnormal protein product or an abnormal amount of product is produced. Under these circumstances these genes have the ability to transform cells *in vitro* and are termed oncogenes. Over 200 oncogenes have been identified with approximately 30 oncogenes having a major role in human cancer.

Most oncogene protein products appear to function in one way or another in cellular signal transduction pathways that are involved in regulating cell growth,

differentiation, or apoptosis. Signal transduction pathways are used by the cells to receive and process information to ultimately produce a biological cellular response. These pathways are the cellular circuitry conveying specific information from the outside of the cell to the nucleus (Figure 11.15). In the nucleus, specific genes are expressed, and their encoded proteins produce the evoked biological response. Oncogenes encode proteins that are components of this cellular circuitry and can be classified with respect to their biological function (Table 11.4). If a component of the circuit is altered, then the entire cellular circuit of which the component is a part is altered. It is not difficult to imagine how an alteration in a pathway that regulates cellular proliferation could have very profound effects on cellular homeo-

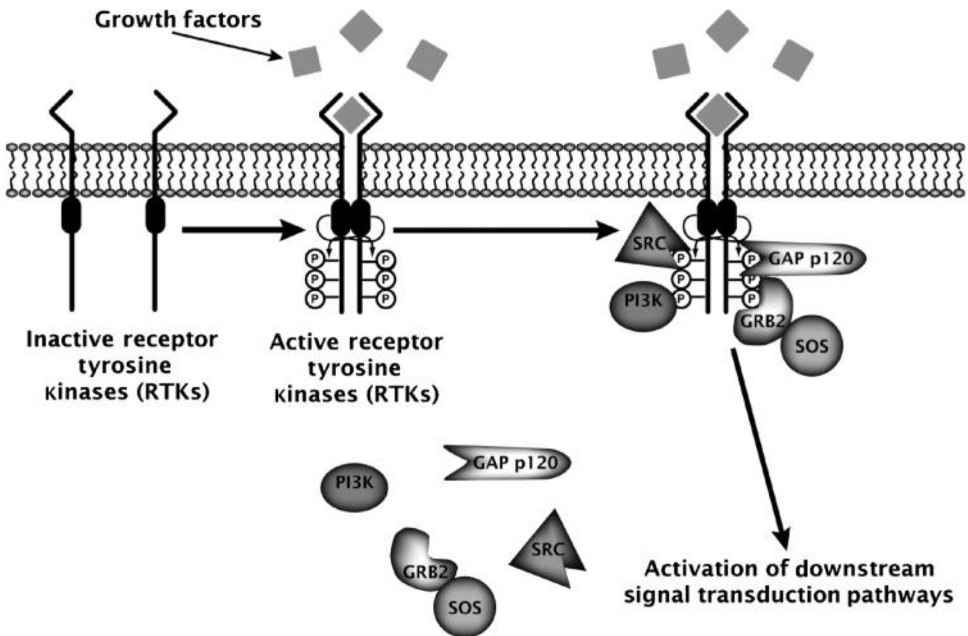


Figure 11.15 Generic signal transduction pathway involving receptor tyrosine kinase (RTK). An extracellular growth factor signal is conveyed via receptors, GTPases (Ras), kinases and, ultimately, to transcription factors that alter gene expression and produce a cellular response.

TABLE 11.4 Human Oncogene Classification

Oncoprotein Families	Oncogenes
Growth factors	PDGF, HGF, TGFα, VEGF, WNT-1, IGF-2
Receptor tyrosine kinases (RTKs)	ERBB1, ERBB2, KIT, RET, MET
Nonreceptor tyrosine kinase	SRC, ABL, YES, LCK
Guanosine triphosphatases (GTPases)	H-RAS, K-RAS, N-RAS
Serine/threonine kinases	RAF-1, B-RAF, AKT, PIM-1, BCR
Transcription factors	MYC, FOS, JUN, ETS, REL, MYB, GLI
Survival proteins	BCL-2, AKT, E2F1, MDM2

ERBB1, EGF receptor (EGF-R); ERBB2, HER2 or NEU receptor; MET, HGF receptor (HGF-R).

stasis. Indeed, the alteration of pathways by oncogenes is the molecular basis through which oncogenes contribute to the cancer process.

11.6.1 Ras Oncogene

Ras genes are frequently mutated in chemically induced animal tumors and are among the most frequently detected mutated oncogenes in human tumors. Approximately 20–30% of all human tumors contain mutated *RAS*. The Ras subfamily includes H-Ras, K-Ras, N-Ras, and all have been found to be mutationally activated in numerous types of tumors from a large variety of species, including humans.

Activated Ras oncogenes have been detected in a large number of animal tumors induced by diverse agents including physical agents, such as radiation and a large number of chemical carcinogens. Some chemical carcinogens bind covalently to DNA, forming specific adducts which, upon DNA replication, yield characteristic alterations in the primary sequence of the H-Ras proto-oncogene. The study of the Ras oncogene as a target for chemical carcinogens has revealed a correlation between specific carcinogen-DNA adducts and specific activating mutations of Ras in chemically induced tumors. For example, 7,12-dimethylbenz[a]anthracene, a PAH carcinogen, is metabolically activated to a bay-region diol epoxide which binds preferentially to adenine residues in DNA. Skin tumors isolated from mice treated with 7,12 dimethylbenz[a]anthracene (DMBA) contain an activated H-Ras oncogene with an A to T transversion of the middle base in the 61st codon of H-ras. Therefore, the identified mutation in Ras is consistent with the expected mutation produced by the DMBA-DNA adduct. Likewise, rat mammary carcinomas induced by nitrosomethylurea contain a G to A transition in the 12th codon of H-Ras, and this mutation is consistent with the modification of guanine residues by this carcinogen. Based on these events, the alteration of Ras by specific chemical carcinogens appears to be an early event in carcinogenesis.

Ras proteins function as membrane-associated molecular switches operating downstream of a variety of membrane receptors. Ras is in the off position when it is bound to guanosine diphosphate (GDP); however, when a growth factor receptor is activated by the binding of its ligand, the activated receptor stimulates the guanine nucleotide exchange factor, SOS (son of sevenless), which causes Ras to exchange guanosine triphosphate (GTP) for GDP, and now Ras is bound to GTP and is in the "on" position. Ras communicates this "on" signal downstream to the other proteins in the signaling circuitry. The best-characterized pathways involve the activation of a kinase cascade that results in the activation of various transcription factors. Theses transcription factors regulate the expression of genes involved in cell proliferation, and the cell is instructed to proliferate. As mentioned, Ras is a molecular switch and once Ras has conveyed the "on" signal, Ras must turn itself "off." Ras has intrinisic GTPase activity which hydrolyzes GTP to form GDP and Ras is now "off" position. Another protein, termed GAPp120 (GTPase activating protein) aids Ras in GTP hydrolysis. When Ras is mutated by a gain of function mutation in certain codons, including the 12th, 13th, or 61st codon, the intrinsic GTPase activity of Ras is greatly diminished as is its ability to interact with GAP. The net effect is that mutated Ras is now an oncogene and is essentially stuck in the "on" position, continually sending a proliferative signal to the downstream circuitry.

11.7 TUMOR SUPPRESSOR GENES

Activation of oncogenes results in a gain of function while inactivation of tumor suppressor genes results in a loss of function. Tumor suppressor genes encode proteins that generally function as negative regulators of cell proliferation or positive regulators of apoptosis. The majority of tumor suppressor genes were first identified in rare familial cancer syndromes and later found be mutated in sporadic cancers through somatic mutation. Major tumor suppressor genes, their proposed function, as well as the cancer syndrome they are associated with, are shown in Table 11.5. When tumor suppressor genes that negatively regulate cell proliferation are inactivated by allelic loss, point mutation, or chromosome deletion, the result is uncontrolled cell proliferation. Generally, if one allele of a tumor suppressor gene is inactivated, the cell is normal (this gene is referred to as haplosufficient). However, when both alleles are inactivated, the ability to control cell proliferation is lost.

11.7.1 p53 Tumor Suppressor Gene

p53 aka TP53 encodes a 53 kDa protein. p53 is mutated in 50% of all human cancer and is the most frequently known mutated gene in human cancer. The majority (~80%) of p53 mutations are missense mutations and p53 is mutated in approximately 70% of colon cancers, 50% of breast and lung cancers, and 97% of primary melanomas. In addition to point mutations, allelic loss, rearrangements, and deletions of p53 occur in human tumors. p53 is a transcription factor and participates in many cellular functions including cell cycle regulation, DNA repair,

TABLE 11.5 Human Tumor Suppressor Genes

Gene Name	Familial Cancer Syndrome	Protein Function	Sites/Types of Commonly Associated Neoplasms
TP53	Li–Fraumeni syndrome	Transcription factor	Most human cancers
RB1	Hereditary retinoblastoma	Transcriptional modifier	Retinoblastoma, osteosarcoma
APC	Familial adenomatous Polyposis	B-catenin degradation	Colon, stomach, intestine
CDKN2A ($p16^{INK4A}$)	Familial malignant melanoma	Cyclin-dependent kinase inhibitor	Melanoma, pancreas
CDKN2A ($p14^{ARF}$)		p53 stabilizer	Melanoma
PTCH	Gorlin syndrome	Transmembrane receptor	Basal cell skin carcinoma, ovary, heart
PTEN	Cowden syndrome	PIP3 phosphatase	Hamartoma, glioma, uterus
TGFBR2		Transmembrane receptor	Colon, stomach, ovary

and apoptosis. The p53 protein is composed of 393 amino acids and single missense mutations can inactivate the p53. Unlike Ras genes which have a few mutational codons that result in its activation, the p53 protein can be inactivated by hundreds of different single point mutations in p53. It has been proposed that the mutation spectrum of p53 in human cancer can aid in the identification of the specific carcinogen that is responsible for the genetic damage; that is to say that different carcinogens cause different characteristic mutations in p53. Some of the mutations in p53 reflect endogenous oxidative damage, while others such as the mutational spectrum in p53 in hepatocellular carcinomas from individuals exposed to aflatoxin demonstrate a mutation spectrum characteristic aflatoxin. In sun-exposed areas where skin tumors develop, the mutations found in p53 in these tumors are characteristic of UV light-induced cyclobutane pyrimidine dimers and, finally, the mutation spectrum induced by (+)-benzo[a]pyrene-7,8-diol-9,10-epoxide-2 in cells in culture is similar to the mutational spectrum in p53 in lung tumors from cigarette smokers. Thus, certain carcinogens produce a molecular signature which may provide important information in understanding the etiology of tumor development.

p53 has been termed the "guardian of genome" because it controls a G1 checkpoint, regulates DNA repair and apoptosis. DNA damage results in the accumulation of p53 and the activation of p53 function. p53 prevents cells with damaged DNA from entering the S-phase of the cell cycle until the DNA damage is repaired. If the DNA damage is severe, p53 can cause the cell to undergo apoptosis (Figure 11.16). Mutation of p53 disrupts these functions, leading to the accumulation of mutations as cells enter S phase with damaged DNA (mutator phenotype; genetic instability) and further development of malignant clones.

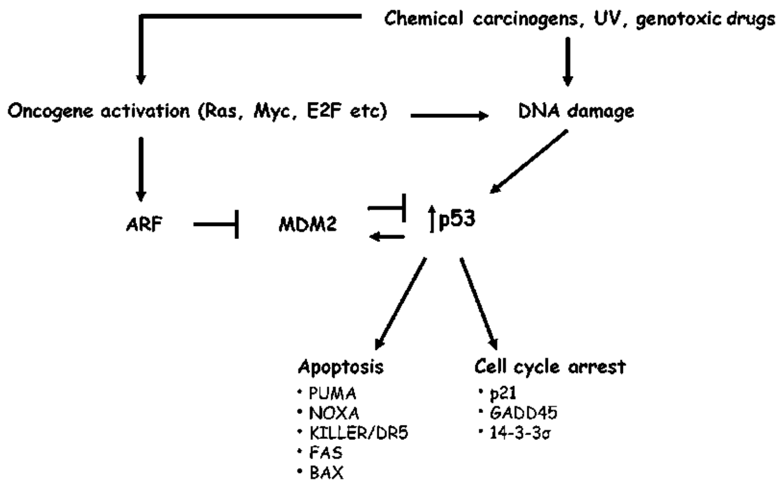


Figure 11.16 *p53* regulates apoptosis and cell cycle progression. In response to DNA damage or oncogene activation, the *p53* protein undergoes posttranslational modifications that increase its stability and activity. *p53* accumulates in the cell and can regulate the expression of genes involved in apoptosis and cell cycle arrest. Oncogene activation is also believed to activate components of the DNA damage response pathway to further increase *p53*. MDM2 is a feedback inhibitor of *p53* and targets *p53* for proteasomal degradation.

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SAMPLE QUESTIONS

1. What does the somatic mutation theory state?
2. What are the three major categories of genes involved in cancer development?
3. Cancer susceptibility is determined by complex interactions between______________, __________and ______________.
4. What is the major reason for the 60% concordance between rodent carcinogenicity and mutagenicity in short term tests?
5. Briefly describe how the study of cancer rates of groups of people that emigrate from one country to another has provided important information on the causes of cancer.
6. Describe the role of Ras in cancer. Be sure to describe its normal function and how this function is altered in carcinogenesis.
7. Describe the normal function of p53 protein in the cell and how a mutation in this gene contributes to the development of cancer.

CHAPTER 12

Teratogenesis

JILL A. BARNES and IDA M. WASHINGTON

12.1 INTRODUCTION

Teratology is the study of abnormal development, and teratogenesis is the production of an abnormal organism. The term teratology is derived from the Greek word *teratos*, which means "monster." An agent is considered to be a teratogen if it increases the occurrence of structural or functional abnormalities in offspring when given to either parent before conception, to the mother during pregnancy, or to the developing embryo or fetus. Teratogens affect the developing embryo or fetus without significant toxicity in the mother; these agents may include chemicals, environmental factors, viruses, radiation, toxic plants, and metabolite deficiencies or excesses. The mechanisms by which teratogens disrupt development are still largely unknown. However, a number of general principles have emerged regarding the interaction of teratogens with the developing embryo.

The field of teratology had its origins in the early twentieth century with the observation in the 1920s that pregnant women exposed to ionizing radiation produced children with neural and skeletal defects. In the 1940s, a connection between maternal rubella infection and neonatal death and abnormalities was recognized. Experiments by Warkany and colleagues in the 1940s demonstrated abnormal growth and development of mammalian embryos after maternal exposure to dietary deficiency or irradiation. Interest in the field of teratology increased significantly in the 1950s and 1960s when human infants with severe limb defects were born to mothers dosed with the sedative thalidomide during pregnancy.

In order to understand the principles and mechanisms of teratogenesis, one must first understand how the embryo develops normally. Thus, this chapter will begin with an overview of normal embryonic development, followed by a review of basic principles of teratogenesis. Mechanisms of teratogenesis will be illustrated by describing specific teratogenic agents and current knowledge about how these factors disrupt normal embryogenesis. The chapter concludes with a discussion of future considerations in the field of teratology. Significant progress has been made in this field of study during the past half-century, but there is much yet to understand about molecular aspects of embryonic development and the mechanisms of teratogenesis.

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12.2 OVERVIEW OF EMBRYONIC DEVELOPMENT

12.2.1 Fertilization

Fertilization typically occurs in the ampulla of the uterine tube and represents the union of male and female germ cells to form a single-cell embryo, the zygote. Maternal and paternal chromosomes arrange on the mitotic spindle for the first mitosis, followed by a series of rapid mitotic divisions. The genetic sex of the mammalian embryo is determined at fertilization, when a spermatozoon carrying an X or Y sex chromosome combines with an oocyte carrying an X sex chromosome, to produce a female (XX) or male (XY) offspring.

12.2.2 Cleavage Stages

Morula The single-cell zygote undergoes a series of rapid mitotic divisions to produce a solid ball of cells, the morula, which is surrounded by an acellular layer, the zona pellucida. A group of large cells (inner cell mass) located centrally within the morula will form the embryo, while the smaller peripheral cells (outer cell mass) will form the extraembryonic membranes and placenta.

Blastula A fluid-filled cavity, the blastocele, begins to form between the cells of the morula as the embryo transitions to the blastula stage. During this stage, the blastocele enlarges to form a large central fluid-filled cavity. The cells of the embryoblast (future embryo) move to one pole of the blastula and form two layers, the epiblast and hypoblast. The outer cell mass becomes the trophoblast, which forms the wall of the blastula. The zona pellucida degenerates and disappears as the embryo "hatches" and then implants in the uterine mucosa. Implantation occurs in most species at approximately 5–8 days post fertilization (Table 12.1).

12.2.3 Determination

The zygote is capable of forming all cells of the body, a quality called totipotency. This capability persists through several cell divisions. As development proceeds, the potential of each cell becomes narrowed as its fate is progressively fixed. This process is called determination and is necessary for subsequent cellular differentiation.

TABLE 12.1 Comparison of Gestation in Several Species

Species	Number of Days after Conception		
	Implantation	Embryonic Period[a]	Fetal Period
Human	6–7	20–56	56–280
Rabbit	6–8	8–16	17–34
Rat	6–8	9–17	18–22
Mouse	5–7	7–16	17–20

[a] Period of organogenesis and greatest teratogenic risk.

12.2.4 Gastrulation

Gastrulation is the stage of development during which the three primary germ layers (ectoderm, mesoderm, and endoderm) are formed. Gastrulation begins with the appearance of the primitive streak on the surface of the embryonic disc. Cells on the surface of the embryo migrate to the primitive streak and invaginate to form two new layers, the endoderm and mesoderm. This process occurs in a cranial to caudal direction. When gastrulation is complete, the primitive streak disappears and the remaining surface layer forms the ectoderm.

12.2.5 Differentiation

After the three germ layers of the embryo are established, cells in different regions of these layers begin to differentiate into components of developing organs to serve specific functions. During differentiation, cells pass through several stages of increasing complexity to achieve a fully functional state. They develop characteristics specific to their cell type, which involves the proliferation or disappearance of certain organelles and the synthesis of certain intracellular or secreted proteins.

12.2.6 Organogenesis

Organogenesis is the stage during which organ systems are formed from the three primary germ layers (ectoderm, mesoderm, endoderm) that were established during gastrulation.

Ectoderm The original ectoderm layer is composed of neural ectoderm, nonneural ectoderm, and neural crest. Neural ectoderm forms the central nervous system, retina and olfactory epithelium, pineal gland, and posterior pituitary gland. Nonneural ectoderm forms surface structures and their derivatives, such as epidermis and associated hair, nails, and glands. The neural crest originates between the neural and nonneural ectoderm and migrates to form numerous derivatives, including most of the peripheral nervous system.

Mesoderm The original mesoderm layer becomes subdivided into paraxial, intermediate, and lateral plate regions. The paraxial mesoderm forms somitomeres in the head region and somites in the body region of the embryo. These temporary structures will further subdivide to form dermis, voluntary muscles, cartilage, bone, and connective tissue of the trunk and limbs, as well as voluntary muscles of the head and a few bones of the skull. Intermediate mesoderm forms the kidneys, gonads, ducts, and accessory glands of the urogenital system, as well as the adrenal cortex. Lateral plate mesoderm splits into somatic and splanchnic layers, which form the body wall of the embryo and wall of the gut tube, respectively.

Endoderm The endoderm layer forms the lining of the gut tube and derivatives of the embryonic gut, including respiratory tract and pancreas, liver, thyroid, parathyroid, tonsils, and thymus. Endoderm also lines the urinary bladder and urethra, and the auditory tube, middle ear, and tonsillar fossa.

12.2.7 Fetal Period

There is no distinct demarcation between the end of the embryonic period and the beginning of the fetal period. In general, organ primordia are established during the embryonic period, and rapid growth and differentiation of these organs occurs during the fetal period. In the fetus, organs and organ systems undergo structural and functional maturation. Species-dependent features start to become apparent in the early fetal period.

12.3 PRINCIPLES OF TERATOGENESIS

12.3.1 Wilson's Principles

In 1959, James Wilson proposed six basic principles of teratology. Fifty years later, these principles remain important basic tenets in the field of teratology. These principles include the following:

1. Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which it interacts with environmental factors.
2. Susceptibility to teratogens varies with the developmental stage at the time of exposure.
3. Teratogenic agents act in specific ways on developing cells and tissues to initiate abnormal developmental processes.
4. The access of adverse environmental influences to developing tissues depends on the nature of the influences.
5. The final manifestations of altered development are death, malformation, growth retardation, and functional disorder.
6. Manifestations of altered development increase in frequency and in degree as dosage increases from no effect to 100% lethality.

12.3.2 Critical Period

The result of exposing an embryo to a teratogenic compound or condition depends on its developmental stage at the time of exposure (Principle #2 above). During the zygote to blastula stages, teratogens may affect numerous cells and cause embryonic death. Alternatively, few cells of the early embryo may be affected by a teratogenic compound, resulting in embryonic compensation and recovery. Malformations are most likely to result from teratogenic exposure during the stage of organogenesis, when organ systems are formed. Each organ system has a different critical period, during which it is most susceptible to the effects of teratogenic agents. In general, there is a decline in teratogenic susceptibility during the fetal period, which is the stage of organ growth. Exposure to teratogens during the fetal period may result in growth retardation or functional impairment (Figure 12.1).

12.4 MECHANISMS OF TERATOGENESIS

In general, factors that cause congenital abnormalities can be either genetic or environmental. In humans, it is estimated that approximately 20% of malformations

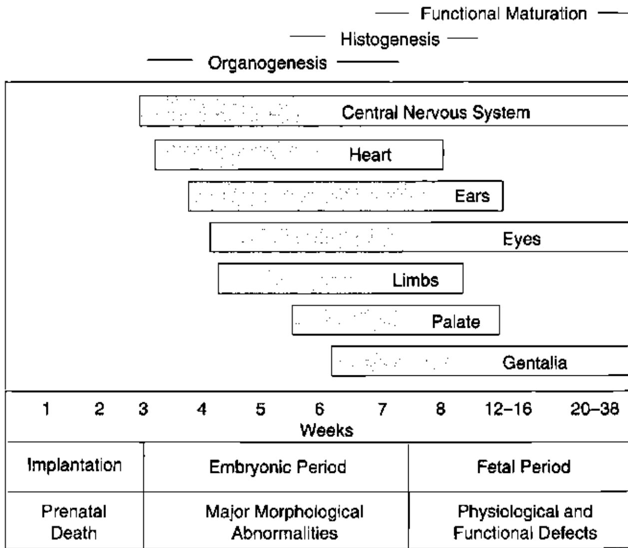

Figure 12.1 Sensitivity to teratogenic exposure at different stages of embryonic and fetal development.

are due to genetic factors and approximately 10% are due to environmental exposure to teratogens such as drugs, chemicals, or infectious agents. This leaves the vast majority of malformations, approximately 70%, for which the etiology is unknown.

12.4.1 Genetic Factors

Mutations Mutations are alterations in the DNA sequence of an organism. In general, mutations can be classified as spontaneous or induced and will cause a structural change to the DNA, which may then lead to altered function of a gene. Spontaneous mutations typically occur at rates of one per million. Induced mutations are generally a result of exposure to chemical or physical agents (e.g., radiation), which alter DNA.

Some examples of known mutations include X-linked muscular dystrophy (in cats and dogs) which leads to an abnormal dystrophin gene or gangliosidosis, resulting in a deficiency of β-galactosidase. In humans, Marfan syndrome is an example of a mutation where a defective glycoprotein product of the fibrillin gene (FBN1) antagonizes the product of the normal allele.

Chromosomal Abnormalities Large-scale alterations to DNA segments can lead to chromosomal abnormalities. When the chromosome number of a cell is altered by either the addition or loss of a chromosome, the condition is called aneuploidy. Monosomy and trisomy refer to the condition where a pair of chromosomes either loses or adds to its pair, respectively. Examples in humans include Down syndrome, which is trisomy of chromosome 13, and Klinefleter syndrome, which is characterized by the addition of an X chromosome.

12.4.2 Teratogens

According to the principles of teratogenesis, a teratogen must cause a specific malformation through a specific mechanism during a period in which the conceptus is susceptible to that mechanism (Karnofsky, 1965). Clearly, there are multiple mechanisms known to cause malformations that are in agreement with these principles. It is difficult, if not impossible, to discuss all of the known or potential mechanisms responsible for inducing malformations. These include DNA attack, enzyme inhibition, interference with hormonal action, alterations of gene signaling pathways, reactive oxygen species, and insult to membranes, proteins, and mitochondria. Examples of agents and/or mechanisms known to cause malformations are described below.

Drugs and Other Xenobiotics

Ethyl Alcohol Fetal alcohol syndrome occurs in infants of women with severe alcoholism during pregnancy. Since ethyl alcohol can readily cross the placenta, this agent is exceptionally dangerous to the developing embryo and fetus. Children who are affected are developmentally and mentally retarded. Studies in mice show that ethyl alcohol interferes with neural crest cell migration, causes apoptosis (cell death) of neurons in the developing forebrain, and detrimentally alters the activity of cell adhesion molecules.

Dioxin Dioxins are halogenated hydrocarbons which are used in many industrial processes and have been linked to congenital defects in humans who have been exposed to the compound as an herbicide. Exposure of pregnant mice to dioxin leads to cleft palate as well as kidney, brain, and other defects in the offspring. *In vitro* studies of palate cells demonstrated that exposure to dioxins altered cell proliferation and differentiation of the palate epithelial cells which have high-affinity receptors for the compound.

Diethylstilbestrol (DES) DES is a synthetic estrogen that was used for nearly 30 years in the prevention of miscarriage or other complications of pregnancy. Unfortunately, female offspring of women treated with DES in the early stages of pregnancy showed an increased risk of reproductive tract abnormalities. After decades of experiments, the complex genetic messenger mechanisms responsible for DES-induced defects are better understood. Studies have shown that pregnant mice exposed to DES have repressed expression of HOX-a-10 gene in the paramesonephric duct. DES, acting primarily through the estrogen receptor, represses Wnt 7a gene expression, which in turn prevents Hox expression. Lack of Hox expression prevents activation of the gene Wnt 5a which codes for a protein required for cellular division of the developing uterus.

Thalidomide Thalidomide was chiefly sold and prescribed during the late 1950s and early 1960s to pregnant women as an antiemetic and as an aid to help them sleep. However, this drug turned out to be a potent teratogen in rabbits and primates, including humans. Thalidomide has severe teratogenic effects from 20 to 36 days of gestation in humans. Lack of long bone development in the limbs, defects

of the gastrointestinal (GI) tract, heart, eye, ear, and renal defects have all been documented as a result of thalidomide exposure. The teratogenic effects of thalidomide have been attributed to its ability to detrimentally affect the production of angiogenesis factors in the developing limb buds and other target tissues by causing the downregulation of specific genes.

Plants

Numerous poisonous plants have been identified to cause congenital defects in animals with considerable species variations.

***Veratrum Californicum* (Skunk Cabbage/False Hellebore)** Ewes that consume this particular plant on the fourteenth day of gestation produce offspring with congenital cyclopean deformities of the head, cleft palate, limb deformities, and tracheal stenosis. Teratogeneic compounds present in this plant include cyclopamine, cycloposine, and jervine. These toxic alkaloids have been shown to interfere with Sonic Hedgehog signaling pathways.

Lupinus Species There are more than 100 species of lupins and some of these have been shown to be teratogenic. Pregnant cows that ingest these particular plants produce calves with malformations of the forelimbs. This condition is often referred to as "crooked calf disease." Limb abnormalities consist of contracture of the flexure muscles, arthrogryposis associated with disproportionate growth of joints, and shortening and rotation of bones. A quinolizidine alkaloid is considered to be the teratogenic agent.

Infectious Agents

Several infectious agents that can cross the placenta and infect the developing fetus are significant causes of defects in humans as well as domestic animals. These can include bacteria, protozoa, fungi, or viruses.

Rubella Virus (German Measles) Infants born to women infected with the rubella virus during the first 3 months of pregnancy are at a significant risk of developing congenital defects. Abnormalities include cardiac malformations, microcephaly, deafness, ocular defects, and mental retardation. As the fetus matures, the risk of defects is reduced, and defects are infrequently seen after the twentieth week of gestation in humans. Maternal immunity, either from immunization or following infection, will prevent congenital infection.

Feline Panleukopenia Virus Transplacental infection with this particular parvovirus in cats can have significant effects on fetal development which relate directly to the stage of gestation at the time of infection. Early infection may result in fetal resorption or death. Cerebellar hypoplasia and retinal dysplasia occurs in cats infected during late pregnancy. If the dam is infected during the last 2 weeks of pregnancy, kittens will have severe cerebellar hypoplasia, which is characterized by ataxia, tremors, and hypermetria.

12.5 FUTURE CONSIDERATIONS

The discovery of environmental agents that cause congenital malformations is extremely important to the health of human as well as animal populations. The question remains: how do we determine which agents are teratogens? Recent reports show that experimental data from 11 groups of known human teratogens across 12 species showed huge amounts of variation in positive predictability (Bailey et al., 2005). Thus, it appears that animal studies are reasonably predictive for animals but, to date, the best human data may, in fact, be epidemiological. This is not particularly surprising, given the amount of variables one must consider. In summary: susceptibility to teratogenesis varies between different species, different strains, and among individuals; affected individuals frequently show different phenotypes, and all these aspects are influenced by genetic makeup, environmental factors, and metabolic and placental differences. Results are further affected by anatomical differences, differences in routes of administration, dose levels, and strategies, differences in absorption, distribution, metabolic activation, sensitivity and excretion, and by typically stressful laboratory handling and housing conditions which can impair health (Bailey et al., 2005). The future dictates that we should employ any and all experimental strategies, including *in vitro* embryonic stem cell tests and whole embryo culture as well as animal studies, to best determine which, if any, of the thousands of chemicals that humans and animals are continuously exposed to may be dangerous to the developing offspring.

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SAMPLE QUESTIONS

1. An agent that specifically disrupts cells in the developing endoderm layer could produce congenital defects in the

- **a.** Adrenal cortex
- **b.** Retina
- **c.** Vertebrae
- **d.** Pancreas
- **e.** Skin

2. Exposure to a teratogenic agent during organogenesis would most likely cause

a. Fetal growth retardation but no congenital defects
b. Structural or functional congenital defects
c. Embryonic death
d. Maternal toxicity
e. Delayed implantation

3. Which of the following best describes the critical period?

a. The critical period is the same for all organs.
b. The critical period always occurs prior to implantation.
c. The critical period usually occurs during organogenesis.
d. The critical period is the stage at which teratogenic agents cause embryonic death.
e. The critical period is the primary stage of organ growth and functional maturation.