CHAPTER 13

Teratogenesis

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13.1 INTRODUCTION

Developmental toxicity is any morphological or functional alteration caused by chemical or physical insult that interferes with normal growth, homeostasis, development, differentiation, and/or behavior. Teratology is a specialized area of embryology. It is the study of the etiology of abnormal development (the study of birth defects). Teratogens therefore are xenobiotics and other factors that cause malformations in the developing conceptus. Examples of teratogens may include (Figure 13.1): pharmaceutic compounds, substances of abuse, hormones found in contraceptive agents, cigarette components, and heavy metals. Also included in this category are viral agents, altered metabolic states induced by stress, and nutrient deficiencies (e.g., folic acid deficiency).

13.2 PRINCIPLES OF TERATOLOGY

James Wilson (in 1959) proposed six principles of teratology. A simplified version of these is as follows:

1. Susceptibility to teratogenesis depends on the embryo's genotype that interacts with adverse environmental factors (G × E interaction).
2. The developmental stage of exposure to the conceptus determines the outcome.
3. Teratogenic agents have specific mechanisms through which they exert there pathogenic effects.
4. The nature of the teratogenic compound or factor determines its access to the developing conceptus/tissue.
5. The four major categories of manifestations of altered development are death, malformation, growth retardation, and functional deficits.
6. The manifestations of the altered development increase with increasing dose (i.e., no effect to lethality).

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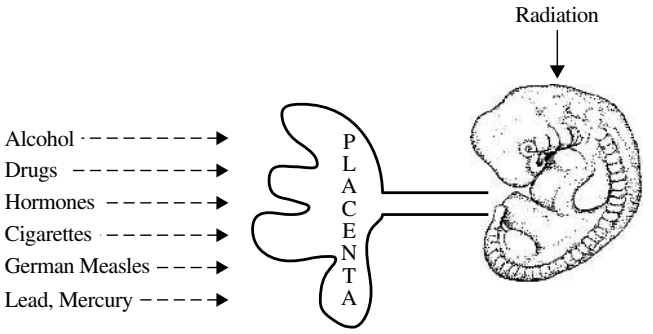


Figure 13.1 Placenta and susceptibility to teratogens.

When describing teratogens, one may think of three basic characteristics of teratogens:

1. A given teratogen may be organ specific.
2. It may be species specific.
3. It can be dose specific.

Further discussion detailing these characteristics will be encountered later in the chapter when historical examples are addressed.

13.3 MAMMALIAN EMBRYOLOGY OVERVIEW

Prior to specific discussion of teratogenicity, an embryology review is provided to facilitate the understanding of the principles and descriptions associated with the effects of teratogen exposure. This section will address the development from the zygote state to the attainment of the three germ layers (gastrula). Figure 13.2 diagrams the events leading to the development of the three-layered embryo, the gastrula. Formation of the zygote marks the beginning of early embryonic development. The embryo proceeds from morula to the blastocyst while still within the zona pellucida. The aforementioned morula will give rise to the structure that attaches the early embryo to the uterus and feeds the embryo (trophoblast). Mammalian development is characterized by the formation of the blastocele-bearing embryo, the blastula (Figure 13.3). The blastula contains the mass of cells that will give rise to the actual embryo (conceptus). These cells, termed the inner cell mass (ICM), differentiate into ectoderm and endoderm prior to implantation. The ectoderm will eventually give rise to the epidermis and associated structures, the brain, and nervous system. The endoderm will give rise to glandular tissue such as the liver and pancreas and the linings of the gastrointestinal and respiratory tracts.

The inner cell mass gives rise to the epiblast (develops into ectoderm) and the hypoblast (develops into endoderm). Cells of the epiblast migrate toward the midline of the early embryo (Figures 13.4 and 13.5). The primitive streak is active proliferation of the cells with a loss of the basement membrane separating the epiblast and endoderm. The epiblast cells migrate and intermingle with the endoderm cells. The anterior end of

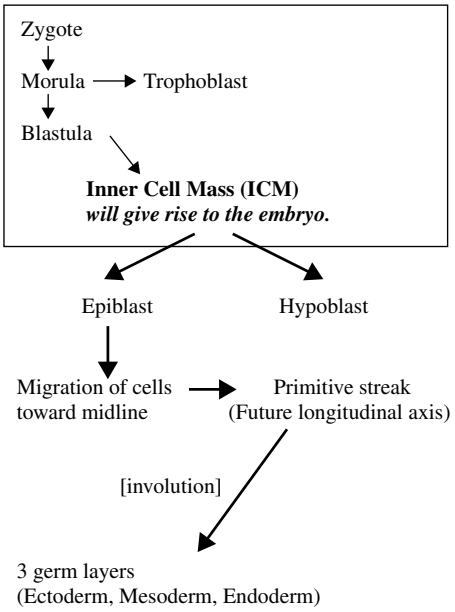
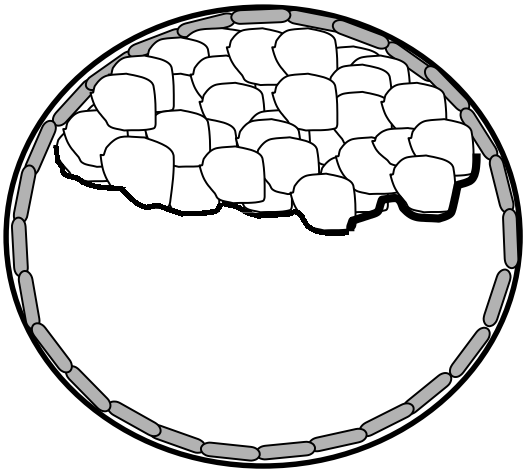


Figure 13.2 Development of the zygote to a three germ cell layered embryo.



In the blastocyst stage (zona-intact) there is a
distinct inner cell mass and an outer layer of trophectoderm cells.

Figure 13.3 Blastula containing the inner cell mass that gives rise to the embryo proper.

the streak is defined by the Hensen's node. This node (also termed the primitive node) is associated with the organization of the developing embryo. The cellular migration (involution) leads to the creation of the third germ layer (the mesoderm). Somites are derived from the mesoderm. Figure 13.6 demonstrates the early stage embryo with visible somites and the succeeding embryonic to fetal stages. Somites are blocklike

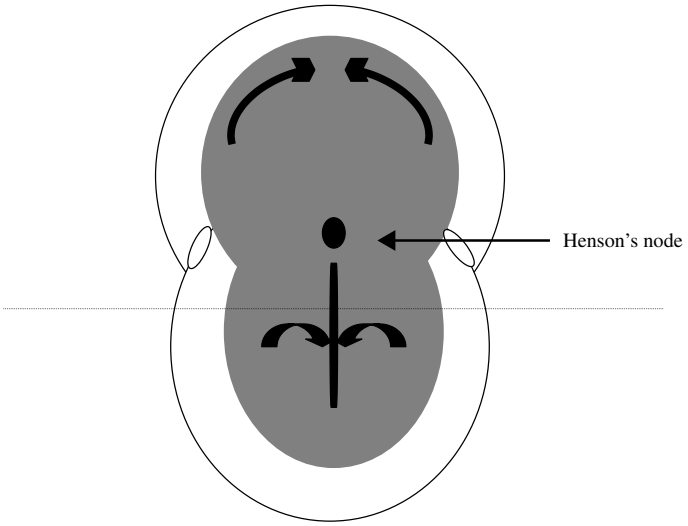
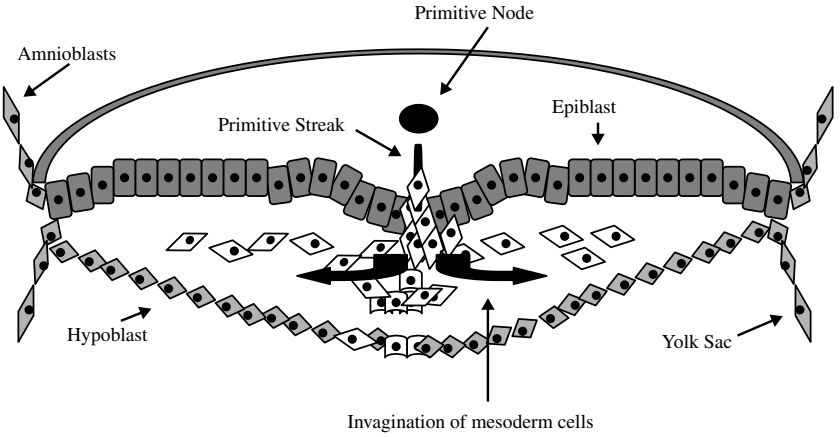


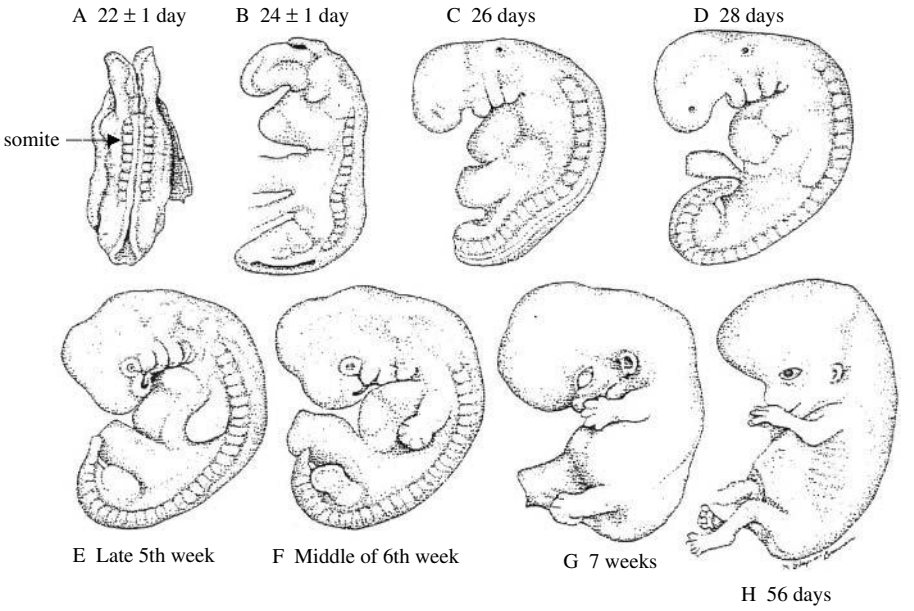
Figure 13.4 Dorsal view of the early embryo with the primitive evident. The horizontal dotted line represents sectioning that gives rise to the transverse view of the embryo in Figure 13.5. Curved arrows indicate migration of cells to the midline.



Invagination of these cells results in formation of the mesoderm and replacement of some of the hypoblast cells to produce the definitive endoderm.

Figure 13.5 View of the transverse section of embryo in Figure 13.6. Note the migration of cells to form the mesoderm (involution).

masses of mesoderm alongside the neural tube. They will form the vertebral column and segmental musculature. They will also develop into the excretory system, gonads, and the outer covering of internal organs. Also formed from mesoderm are mesenchymal cells. These are loose migratory cells forming the dermis (inner skin layer), bones and cartilage, and circulatory system.



Somite: block-like mass of mesoderm alongside neural tube: forms vertebral column and segmental musculature

Figure 13.6 Early stage human embryo with visible somites (represents a 22-day-old human embryo or an 8-day-old mouse embryo).

13.4 CRITICAL PERIODS

Major fetal outcomes depend on the stage of pregnancy affected, as there are critical periods for the development of fetal processes and organs. Although embryogenesis is complex involving cell migrations, proliferation, differentiation, and organogenesis, one may divide the developmental stages in to three large categories: pre-implantation, implantation to organogenesis, and the fetal to neonatal stage. The outcomes associated with exposure during these periods vary. This is not to say there are exceptions based on the type of exposure. However, the primary outcomes are as follows:

Stage of Exposure	Outcome(s)
Pre-implantation	Embryonic lethality
Implantation to time of organogenesis	Morphological defects
Fetal → neonatal stage	Functional disorders, growth retardation, carcinogenesis

The sensitivity of the embryo to the induction of morphological defects is increased during the period of organogenesis. This period is essentially the time of the origination and development of the organs. The critical period graph (Figure 13.7) demonstrates this point and defines the embryonic and fetal periods.

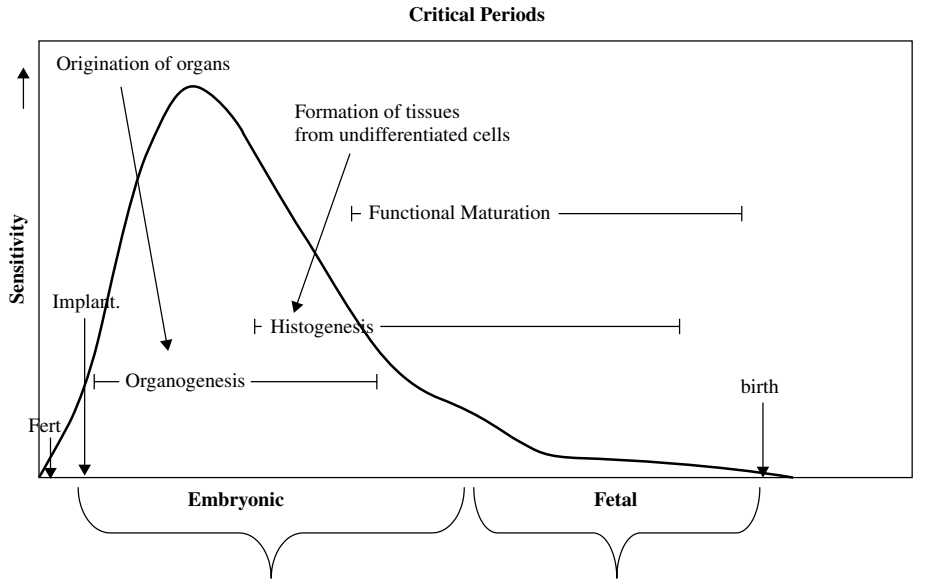


Figure 13.7 Critical periods graph including embryonic and fetal periods. Note the increased sensitivity to teratogenic events during organogenesis.

13.5 HISTORICAL TERATOGENS

13.5.1 Thalidomide

Thalidomide is a sedative-hypnotic drug used in Europe from 1957 to 1961. It was marketed for morning sickness, nausea, and insomnia. It went into general use and was widely prescribed in Europe, Australia, Asia, Africa, and the Americas. Women who had taken the drug from gestation days (GD) 35 to 50 gave birth to offspring suffering from a spectrum of different malformations, mainly amelia (absence of limbs) or phocomelia (severe shortening of limbs). Other malformations included: absence of the auricles with deafness, defects of the muscles of the eye and face, and malformations of the heart, bowel, uterus, and the gallbladder. The compound was withdrawn from the market in 1961 after about 10,000 cases had occurred.

13.5.2 Accutane (Isotetrinoin)

Accutane is a member of a family of drugs called retinoids, which are related to vitamin A. It is approved to treat serious forms of acne. These painful and disfiguring forms of acne do not respond to other acne treatments. Accutane is very effective, but its use is associated with a number of risks including birth defects. Exposure of pregnant women can lead to birth defects such as facial malformations, heart defects, and mental retardation.

13.5.3 Diethylstilbestrol (DES)

DES is a synthetic estrogen that inhibits ovulation by affecting release of pituitary gonadotropins. Some of its uses include treatment for hypogonadism, primary ovarian

failure, and in some cases of prostate cancer. From 1940 to 1970, DES was used to help maintain pregnancy. In utero exposure to DES has been associated with abnormal development of the uterus. It has also been associated with certain types of tumors. Women who were exposed in utero often developed vaginal neoplasia, vaginal adenosis, and cervical erosion. Effects were not seen in offspring until they reached puberty. Clear cell carcinoma of the vagina is a type of adenocarcinoma found in young women who are exposed to diethylstilbestrol in utero. The reproductive organ of males can also be affected subsequent to in utero exposure. The outcomes include hypotrophic testes, poor semen volume and quality.

13.5.4 Alcohol

Fetal Alcohol Syndrome. Fetal alcohol syndrome (FAS) is a pattern of mental and physical defects that develops in some offspring when exposed to alcohol in utero. The first trimester is the most susceptible period. Some babies with alcohol-related birth defects, such as lower birth weight and body size and neurological impairments, do not have all of the classic FAS symptoms. These outcomes are often referred to as fetal alcohol effects (FAE). Currently there is not total agreement among medical scientists concerning the precise differences between FAE and FAS. In addition to growth retardation, the most common outcomes of fetal alcohol syndrome include psychomotor dysfunction and craniofacial anomalies.

The observed growth deficiencies are associated with an inability of the baby to catch up due to a slower than normal rate of development. Other infrequent outcomes include skeletal malformations such as deformed ribs and sternum, scoliosis, malformed digits, and microcephaly. Distinctive facial anomalies have been associated with a diagnosis of fetal alcohol syndrome: small eye openings, epicanthal folds, failure of eyes to move in the same direction, short upturned nose, flat or absent groove between nose and upper lip, and thin upper lip. Visceral deformities may also be present: heart defects, genital malformations, kidney, and urinary defects.

A common concurrent manifestation of FAS include central nervous system defects. These include irregular arrangement of neurons and connective tissue. Mental retardation may also be present and associated with learning disabilities as well as difficulties in controlling body coordination.

13.5.5 "Non Chemical" Teratogens

Teratogens are not only xenobiotics. There may be other factors having the ability to cause malformations in the developing conceptus. Restraint stress in mice (12-hour restraint during early period of organogenesis) elicits axial skeletal defects (primarily supernumerary ribs). The Rubella virus (first reported in 1941, Austria) is associated with a number of fetal outcomes depending on the stage of development that the exposure occurs. Exposure during the first and second month of pregnancy was associated with heart and eye defects. Exposure during the third month was associated with hearing defects (and mental retardation in some cases).

13.6 TESTING PROTOCOLS

Formal testing guidelines were established after thalidomide disaster. In 1966 guidelines were established by the FDA: *Guidelines for Reproduction Studies for Safety Evaluation*

of Drugs for Human Use. Since then (1994) new streamlined testing protocols have been developed with international acceptance. This newer approach, ICH (13.6.2), relies on the investigator to determine the model to access reproductive/developmental toxicity. However, many scientist currently conduct and publish the FDA version of testing (i.e., segment studies). Therefore both (FDA & ICH) approaches will be discussed. Further practical details of testing protocols may be found in Chapter 21 as well as some discussion of the regulatory implications for different agencies.

13.6.1 FDA Guidelines for Reproduction Studies for Safety Evaluation of Drugs for Human Use

Multigenerational Study. This approach involves the continuous exposure of a rodent species (usually mice). The parental animals are exposed shortly after weaning (30–40 days of age). At reproductive maturity, the animals are mated. The first generation is produced (F_1). From these an F_2 is produced and then subsequently an F_3 generation. The effects of the test is monitored through each generation. The measured parameters include fertility, litter size, and neonatal viability. This is a time-consuming effort that usually takes about two years to complete.

Single-Generation Studies. Single-generation studies are short-term studies conducted in three segments:

Segment I: Evaluation of Fertility and Reproductive Performance. Male rodents are treated for 70 days (to expose for one spermatogenic cycle), and nonpregnant females for 14 days (to exposure for several estrous cycles). Treatment is continued in the females during mating, pregnancy, and lactation. Fifty percent of the females are killed and the fetuses are examined for presence of malformations. The other 50% are allowed to give birth. After weaning, these offspring are killed and necropsied.

Segment II: Assessment of Developmental Toxicity. This involves the treatment of pregnant females only during the period covering implantation through organogenesis (typically from gestational days 6 to 15 in mice with 18-day gestational periods). One day prior to birth, the animals are killed and fetuses examined for viability, body weight, and presence of malformation.

Segment II: Postnatal Evaluation. Pregnant animals are treated from the last trimester of pregnancy until weaning. Evaluated are parturition process, late fetal development, neonatal survival, and growth as well as presence of any malformations.

13.6.2 International Conference of Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)—US FDA, 1994

As previously mentioned, new streamlined testing protocols with international acceptance have been developed. Below is a description of these guidelines as it relates to similarity to a particular segment-type study.

ICH 4.1.1: Fertility Assessment. This study duration is typically shorter than segment I studies. Males are exposed for four weeks before mating and females two weeks

before mating. Male reproductive organs are carefully evaluated: organ weights, histological analysis and sperm count, and mobility evaluation. For the females, fertility, litter size, and viability of conceptus are evaluated.

ICH 4.1.2: Postnatal Evaluation and Pregnancy State Susceptibility. This study protocol is similar to the segment III study. Maternal toxicity is evaluated by comparing the degree of toxicity of the nonpregnant female to that of the pregnant female. Postnatal viability and growth are also evaluated. Offspring are also evaluated to assess functional development (i.e., presence of behavioral and reproductive deficits).

ICH 4.1.3: Assessment of Developmental Toxicity. This is almost identical to the segment II study protocol. Pregnant animals are exposed from implantation through organogenesis. The parameters measured in the segment II study are similar. However, the study is usually conducted using at least two species. More specifically, at least one rodent and one nonrodent species.

13.6.3 Alternative Test Methods

A number of alternative test methods have been developed to reduce the number of whole animals used in studies and/or to obtain more rapid information concerning the potential of a compound to be a reproductive/developmental toxicant. Validation of many of the methods has been problematic, since they do not address the contribution of maternal factors or multiorgan contributions to outcomes. Some of these alternative methods include the use of cell or embryo culture. For example, the micromass culture involves the use of limb bud cells from rat embryos grown in micromass culture for five days. The processes of differentiation and cell proliferation are assessed. In the Chernoff/Kavlock Assay, pregnant rodents are exposed during organogenesis and allowed to deliver. Postnatal growth, viability, and gross morphology of litters are recorded (detailed skeletal evaluations are not performed). Other alternative tests involve the use of nontraditional test species such as *Xenopus* embryos (FETAX) and *Hydra. Xenopus* embryos are exposed for 96 hours and then evaluated for morphological defects, viability, and growth. The cells of *Hydra* aggregate to form artificial embryos. The dose response in these "embryos" is compared to that of the adult *Hydra*.

13.7 CONCLUSIONS

Understanding the mechanisms of the induction of birth defects is key to determine how to prevent these effects. Further, increasing the accuracy of experimental animal extrapolation will aid in the interpretation of experimental data in order to more accurately determine the risk of a given compound to elicit birth defects in humans.

SUGGESTED READING

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PART V

ORGAN TOXICITY

CHAPTER 14

Hepatotoxicity

ERNEST HODGSON and PATRICIA E. LEVI

14.1 INTRODUCTION

14.1.1 Liver Structure

The basic structure of the liver consists of rows of hepatic cells (hepatocytes or parenchymal cells) perforated by specialized blood capillaries called sinusoids (see Chapter 10, Figures 10.2 and 10.3). The sinusoid walls contain phagocytic cells called Kupffer cells whose role is to engulf and destroy materials such as solid particles, bacteria, dead blood cells, and so on. The main blood supply comes to the liver from the intestinal vasculature. These vessels, along with those from the spleen and the stomach, merge with each other to form the portal vein (see Chapter 10, Figure 10.4). On entering the liver, the portal vein subdivides and drains into the sinusoids. The blood then perfuses the liver and exits by the hepatic veins, which merge into the inferior vena cava and return blood to the heart. The hepatic artery supplies the liver with oxygenated arterial blood.

Other materials, such as bile acids and many xenobiotics, move from the hepatocytes into the bile-carrying canaliculi, which merge into larger ducts that follow the portal vein branches. The ducts merge into the hepatic duct from which bile drains into the upper part of the small intestine, the duodenum. The gall bladder serves to hold bile until it is emptied into the intestine.

14.1.2 Liver Function

In the liver three main functions occur: storage, metabolism, and biosynthesis. Glucose is converted to glycogen and stored; when needed for energy, it is converted back to glucose. Fat, fat-soluble vitamins, and other nutrients are also stored in the liver. Fatty acids are metabolized and converted to lipids, which are then conjugated with proteins synthesized in the liver and released into the bloodstream as lipoproteins. The liver also synthesizes numerous functional proteins, such as enzymes and blood-coagulating factors. In addition the liver, which contains numerous xenobiotic metabolizing enzymes, is the main site of xenobiotic metabolism.

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14.2 SUSCEPTIBILITY OF THE LIVER

The liver, the largest organ in the body, is often the target organ for chemically induced injuries. Several important factors are known to contribute to the liver's susceptibility. First, most xenobiotics enter the body through the gastrointestinal (GI) tract and, after absorption, are transported by the hepatic portal vein to the liver: thus the liver is the first organ perfused by chemicals that are absorbed in the gut. A second factor is the high concentration in the liver of xenobiotic metabolizing enzymes, primarily the cytochrome P450-dependent monooxygenase system. Although most biotransformations are detoxication reactions, many oxidative reactions produce reactive metabolites (Chapters 7 and 8) that can induce lesions within the liver. Often areas of damage are in the centrilobular region, and this localization has been attributed, in part, to the higher concentration of cytochrome P450 in that area of the liver.

14.3 TYPES OF LIVER INJURY

The types of injury to the liver depend on the type of toxic agent, the severity of intoxication, and the type of exposure, whether acute or chronic. The main types of liver damage are discussed briefly in this section. Whereas some types of damage—for example, cholestasis—are liver specific, others such as necrosis and carcinogenesis are more general phenomena.

14.3.1 Fatty Liver

Fatty liver refers to the abnormal accumulation of fat in hepatocytes. At the same time there is a decrease in plasma lipids and lipoproteins. Although many toxicants may cause lipid accumulation in the liver (Table 14.1), the mechanisms may be different. Basically lipid accumulation is related to disturbances in either the synthesis or the secretion of lipoproteins. Excess lipid can result from an oversupply of free fatty acids from adipose tissues or, more commonly, from impaired release of triglycerides from the liver into the plasma. Triglycerides are secreted from the liver as lipoproteins (very low density lipoprotein, VLDL). As might be expected, there are a number of points at which this process can be disrupted. Some of the more important ones are as follows (Figure 14.1):

- Interference with synthesis of the protein moiety
- Impaired conjugation of triglyceride with lipoprotein
- Interference with transfer of VLDL across cell membranes
- Decreased synthesis of phospholipids
- Impaired oxidation of lipids by mitochondria
- Inadequate energy (adenosine triphosphate [ATP] for lipid and protein synthesis

The role that fatty liver plays in liver injury is not clearly understood, and fatty liver in itself does not necessarily mean liver dysfunction. The onset of lipid accumulation in the liver is accompanied by changes in blood biochemistry, and for this reason blood chemistry analysis can be a useful diagnostic tool.

Table 14.1 Examples of Hepatotoxic Agents and Associated Liver Injury

	Necrosis and fatty liver	
Carbon tetrachloride	Dimethylnitrosamine	Phosphorous
Chloroform	Cyclohexamide	Beryllium
Trichloroethylene	Tetracycline	Allyl alcohol
Tetrachloroethylene	Acetaminophen	Galactosamine
Bromobenzene	Mitomycin	Azaserine
Thioacetamide	Puromycin	Aflatoxin
Ethionine	Tannic acid	Pyrrolizidine alkaloids
	Cholestasis (drug-induced)	
Chlorpromazine	Imipramine	Carbarsone
Promazine	Diazepam	Chlorthiazide
Thioridazine	Methandrolone	Methimazole
Mepazine	Mestranol	Sulfanilamide
Amitriptline	Estradiol	Phenindione
	Hepatitis (drug-induced)	
Iproniazid	Methoxyflurane	Halothane
Isoniazid	Papaverine	Zoxazolamine
Imipramine	Phenyl butazone	Indomethacin
6-Mercaptopurine	Cholchicine	Methyldopa
	Carcinogenesis (experimental animals)	
Aflatoxin B1	Dimethylbenzanthracene	Acetylaminofluorene
Pyrrolizidine alkaloids	Dialkyl nitrosamines	Urethane
Cycasin	Polychlorinated biphenyls	
Safrole	Vinyl chloride	

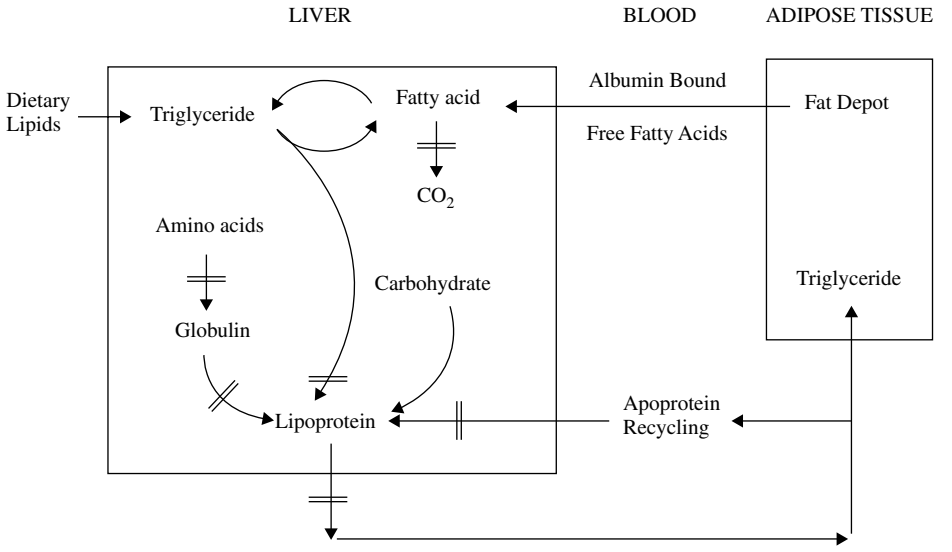

Figure 14.1 Triglyceride cycle in the pathogenesis of fatty liver. "=" are metabolic blocks. (From S. A. Meyer, *Introduction to Biochemical Toxicology*, 3rd ed., Wiley, 2001.)

14.3.2 Necrosis

Cell necrosis is a degenerative process leading to cell death. Necrosis, usually an acute injury, may be localized and affect only a few hepatocytes (focal necrosis), or it may involve an entire lobe (massive necrosis). Cell death occurs along with rupture of the plasma membrane, and is preceded by a number of morphologic changes such as cytoplasmic edema, dilation of the endoplasmic reticulum, disaggregation of polysomes, accumulation of triglycerides, swelling of mitochondria with disruption of cristae, and dissolution of organelles and nucleus. Biochemical events that may lead to these changes include binding of reactive metabolites to proteins and unsaturated lipids (inducing lipid peroxidation and subsequent membrane destruction) disturbance of cellular $Ca+^{2}$ homeostasis, inference with metabolic pathways, shifts in Na+ and K+ balance, and inhibition of protein synthesis. Changes in blood chemistry resemble those seen with fatty liver, except they are quantitatively larger. Because of the regenerating capability of the liver, necrotic lesions are not necessarily critical. Massive areas of necrosis, however, can lead to severe liver damage and failure.

14.3.3 Apoptosis

Apoptosis is a controlled form of cell death that serves as a regulation point for biologic processes and can be thought of as the counterpoint of cell division by mitosis. This selective mechanism is particularly active during development and senescence. Although apoptosis is a normal physiological process, it can also be induced by a number of exogenous factors, such as xenobiotic chemicals, oxidative stress, anoxia, and radiation. (A stimulus that induces a cell to undergo apoptosis is known as an *apogen*.) If, however, apoptosis is suppressed in some cell types, it can lead to accumulation of these cells. For example, in some instances, clonal expansion of malignant cells and subsequent tumor growth results primarily from inhibition of apoptosis.

Apoptosis can be distinguished from necrosis by morphologic criteria, using either light or electron microscopy. Toxicants, however, do not always act in a clear-cut fashion, and some toxicants can induce both apoptosis and necrosis either concurrently or sequentially.

14.3.4 Cholestasis

Cholestasis is the suppression or stoppage of bile flow, and may have either intrahepatic or extrahepatic causes. Inflammation or blockage of the bile ducts results in retention of bile salts as well as bilirubin accumulation, an event that leads to jaundice. Other mechanisms causing cholestasis include changes in membranes permeability of either hepatocytes or biliary canaliculi. Cholestasis is usually drug induced (Table 14.1) and is difficult to produce in experimental animals. Again, changes in blood chemistry can be a useful diagnostic tool.

14.3.5 Cirrhosis

Cirrhosis is a progressive disease that is characterized by the deposition of collagen throughout the liver. In most cases cirrhosis results from chronic chemical injury. The

accumulation of fibrous material causes severe restriction in blood flow and in the liver's normal metabolic and detoxication processes. This situation can in turn cause further damage and eventually lead to liver failure. In humans, chronic use of ethanol is the single most important cause of cirrhosis, although there is some dispute as to whether the effect is due to ethanol alone or is also related to the nutritional deficiencies that usually accompany alcoholism.

14.3.6 Hepatitis

Hepatitis is an inflammation of the liver and is usually viral in origin; however, certain chemicals, usually drugs, can induce a hepatitis that closely resembles that produced by viral infections (Table 14.1). This type of liver injury is not usually demonstrable in laboratory animals and is often manifest only in susceptible individuals. Fortunately, the incidence of this type of disease is very low.

14.3.7 Oxidative Stress

Oxidative stress has been defined as an imbalance between the prooxidant/antioxidant steady state in the cell, with the excess of prooxidants being available to interact with cellular macromolecules to cause damage to the cell, often resulting in cell death. Although the occurrence of reactive oxygen species in normal metabolism and the concept of oxidative stress was derived from these studies, it is apparent that oxidative stress can occur in almost any tissue, producing a variety of deleterious effects. To date, a number of liver diseases, including alcoholic liver disease, metal storage diseases, and cholestatic liver disease, have been shown to have an oxidative stress component.

Reactive oxygen and reactive nitrogen radicals can be formed in a number of ways (Figure 14.2), the former primarily as a by-product of mitochondrial electron transport. Superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl can all arise from this source. Other sources include monooxygenases and peroxisomes. If not detoxified, reactive oxygen species can interact with biological macromolecules such as DNA and protein or with lipids. Once lipid peroxidation of unsaturated fatty acids in phospholipids is initiated, it is propagated in such a way as to have a major damaging effect on cellular membranes. The formation, detoxication by superoxide dismutase and by glutathione-dependent mechanisms, and interaction at sites of toxic action are illustrated in Figure 14.2.

14.3.8 Carcinogenesis

The most common type of primary liver tumor is hepatocellular carcinoma; other types include cholangiocarcinoma, angiosarcoma, glandular carcinoma, and undifferentiated liver cell carcinoma. Although a wide variety of chemicals are known to induce liver cancer in laboratory animals (Table 14.1), the incidence of primary liver cancer in humans in the United States is very low.

Some naturally occurring liver carcinogens are aflatoxin, cycasin, and safrole. A number of synthetic chemicals have been shown to cause liver cancer in animals, including the dialkylnitrosamines, dimethylbenzanthracene, aromatic amines such as

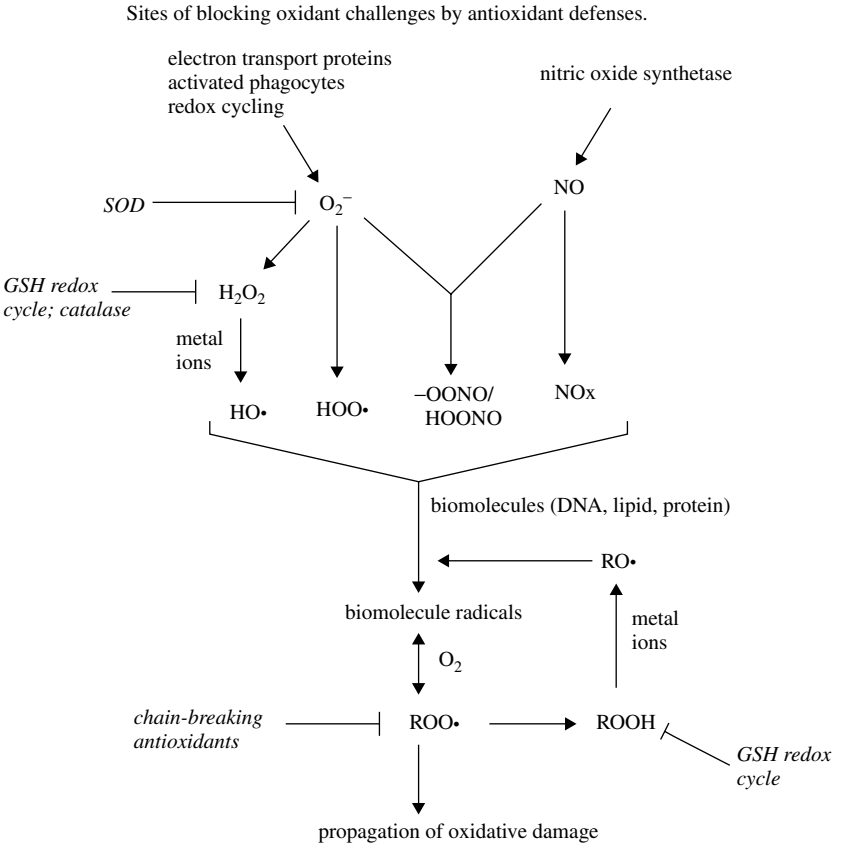

Figure 14.2 Molecular targets of oxidative injury. (From D. J., Reed, *Introduction to Biochemical Toxicology*, 3rd ed., Wiley, 2001.)

2-naphthylamine and acetylaminofluorene, and vinyl chloride. The structure and activation of these compounds can be found in Chapters 7 and 8. In humans, the most noted case of occupation-related liver cancer is the development of angiosarcoma, a rare malignancy of blood vessels, among workers exposed to high levels of vinyl chloride in manufacturing plants. For a discussion of chemical carcinogenesis, see Chapter 12.

14.4 MECHANISMS OF HEPATOTOXICITY

Chemically induced cell injury can be thought of as involving a series of events occurring in the affected animal and often in the target organ itself:

- The chemical agent is activated to form the initiating toxic agent.
- The initiating toxic agent is either detoxified or causes molecular changes in the cell.
- The cell recovers or there are irreversible changes.
- Irreversible changes may culminate in cell death.

Cell injury can be initiated by a number of mechanisms, such as inhibition of enzymes, depletion of cofactors or metabolites, depletion of energy (ATP) stores, interaction with receptors, and alteration of cell membranes. In recent years attention has focused on the role of biotransformation of chemicals to highly reactive metabolites that initiate cellular toxicity. Many compounds, including clinically useful drugs, can cause cellular damage through metabolic activation of the chemical to highly reactive compounds, such as free radicals, carbenes, and nitrenes (Chapters 7 and 8).

These reactive metabolites can bind covalently to cellular macromolecules such as nucleic acids, proteins, cofactors, lipids, and polysaccharides, thereby changing their biologic properties. The liver is particularly vulnerable to toxicity produced by reactive metabolites because it is the major site of xenobiotic metabolism. Most activation reactions are catalyzed by the cytochrome P450 enzymes, and agents that induce these enzymes, such as phenobarbital and 3-methylcholanthrene, often increase toxicity. Conversely, inhibitors of cytochrome P450, such as SKF-525A and piperonyl butoxide, frequently decrease toxicity.

Mechanisms such as conjugation of the reactive chemical with glutathione are protective mechanisms that exist within the cell for the rapid removal and inactivation of many potentially toxic compounds. Because of these interactions, cellular toxicity is a function of the balance between the rate of formation of reactive metabolites and the rate of their removal. Examples of these interactions are presented in the following discussions of specific hepatotoxicants.

14.5 EXAMPLES OF HEPATOTOXICANTS

14.5.1 Carbon Tetrachloride

Carbon tetrachloride has probably been studied more extensively, both biochemically and pathologically, than any other hepatotoxicant. It is a classic example of a chemical activated by cytochrome P450 to form a highly reactive free radical (Figure14.3). First, CCl_4 is converted to the trichloromethyl radical ($CCl_3\bullet$) and then to the trichloromethylperoxy radical ($CCl_3O_2\bullet$). Such radicals are highly reactive and generally have a small radius of action. For this reason the necrosis induced by CCl_4 is most severe in the centrilobular liver cells that contain the highest concentration of the P450 isozyme responsible for CCl_4 activation.

Typically free radicals may participate in a number of events (Figure 14.4), such as covalent binding to lipids, proteins, or nucleotides as well as lipid peroxidation. It

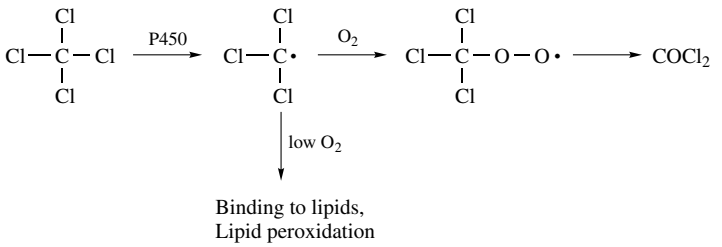

Figure 14.3 Metabolism of carbon tetrachloride and formation of reactive metabolites. (From P. E. Levi, *A Textbook of Modern Toxicology*, 2nd ed., Appleton and Lange, 1997.)

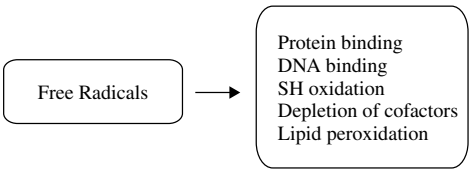

Figure 14.4 Summary of some toxic effects of free radicals. (From P. E. Levi, *A Textbook of Modern Toxicology*, 2nd ed., Appleton and Lange, 1997.)

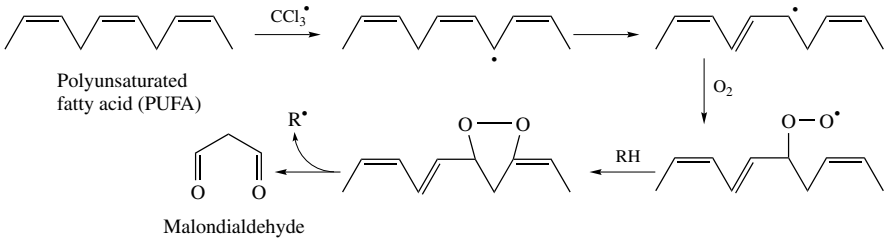

Figure 14.5 Schematic illustrating lipid peroxidation and destruction of membranes. (From P. E. Levi, *A Textbook of Modern Toxicology*, 2nd ed., Appleton and Lange, 1997.)

is now thought that CCl_3•, which forms relatively stable adducts, is responsible for covalent binding to macromolecules, and the more reactive CCl_3O_2•, which is formed when CCl_3• reacts with oxygen, is the prime initiator of lipid peroxidation.

Lipid peroxidation (Figure 14.5) is the initiating reaction in a cascade of events, starting with the oxidation of unsaturated fatty acids to form lipid hydroperoxides, which then break down to yield a variety of end products, mainly aldehydes, which can go on to produce toxicity in distal tissues. For this reason cellular damage results not only from the breakdown of membranes such as those of the endoplasmic reticulum, mitochondria, and lysosomes but also from the production of reactive aldehydes that can travel to other tissues. It is now thought that many types of tissue injury, including inflammation, may involve lipid peroxidation.

14.5.2 Ethanol

Alcohol-related liver diseases are complex, and ethanol has been shown to interact with a large number of molecular targets. Ethanol can interfere with hepatic lipid metabolism in a number of ways and is known to induce both inflammation and necrosis in the liver. Ethanol increases the formation of superoxide by Kupffer cells thus implicating oxidative stress in ethanol-induced liver disease. Similarly prooxidants (reactive oxygen species) are produced in the hepatocytes by partial reactions in the action of CYP2E1, an ethanol-induced CYP isoform. The formation of protein adducts in the microtubules by acetaldehyde, the metabolic product formed from ethanol by alcohol dehydrogenase, plays a role in the impairment of VLDL secretion associated with ethanol.

14.5.3 Bromobenzene

Bromobenzene is a toxic industrial solvent that is known to produce centrilobular hepatic necrosis through the formation of reactive epoxides. Figure 14.6 summarizes

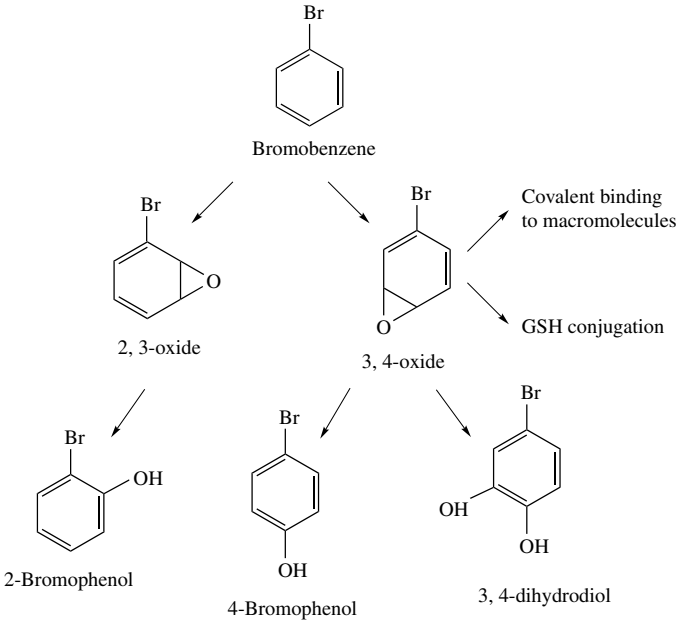

Figure 14.6 Metabolism of bromobenzene. (From P. E. Levi, *A Textbook of Modern Toxicology*, 2nd ed., Appleton and Lange, 1997.)

the major pathways of bromobenzene metabolism. Both bromobenzene 2,3-epoxide and bromobenzene 3,4-epoxide are produced by P450 oxidations. The 2,3-epoxide, however, is the less toxic of the two species, reacting readily with cellular water to form the nontoxic 2-bromophenol. The more stable 3,4-epoxide is the form most responsible for covalent binding to cellular proteins. A number of pathways exist for detoxication of the 3,4-epoxide: rearrangement to the 4-bromophenol, hydration to the 3,4-dihydrodiol catalyzed by epoxide hydrolase, or conjugation with glutathione. When more 3,4-epoxide is produced than can readily be detoxified, cell injury increases.

Pretreatment of animals with inhibitors of cytochrome P450 is known to decrease tissue necrosis by slowing down the rate of formation of the reactive metabolite, whereas pretreatment of animals with certain P450 inducers can increase the toxicity of bromobenzene, (e.g., the P450 inducer phenobarbital increases hepatotoxicity by inducting a P450 isozyme that preferentially forms the 3,4-epoxide). However, pretreatment with another P450 inducer, 3-methylcholanthrene, decreases bromobenzene hepatotoxicity by inducing a form of P450 that produces primarily the less toxic 2,3-epoxide.

14.5.4 Acetaminophen

Acetaminophen is widely used analgesic that is normally safe when taken at therapeutic doses. Overdoses, however, may cause an acute centrilobular hepatic necrosis that can be fatal. Although acetaminophen is eliminated primarily by formation of glucuronide and sulfate conjugates, a small proportion is metabolized by cytochrome P450 to a reactive electrophilic intermediate believed to be a quinoneimine (see Chapter 8). This reactive intermediate is usually inactivated by conjugation with reduced glutathione and excreted. Higher doses of acetaminophen will progressively deplete hepatic glutathione

levels, however, resulting in extensive covalent binding of the reactive metabolite to liver macromolecules with subsequent hepatic necrosis. The early administration of sulfhydryl compounds such as cysteamine, methionine, and *N*-acetylcysteine is very effective in preventing liver damage, renal failure, and death that would otherwise follow an acetaminophen overdose. These agents are thought to act primarily by stimulating glutathione synthesis.

In laboratory animals the formation of the acetaminophen-reactive metabolite, the extent of covalent binding, and the severity of hepatotoxicity can be influenced by altering the activity of various P450 isozymes. Induction of P450 isozymes with phenobarbital, 3-methylcholanthrene, or ethanol increases toxicity, whereas inhibition of P450 with piperonyl butoxide, cobalt chloride, or metyrapone decreases toxicity. Consistent with these effects in animals, it appears that the severity of liver damage after acetaminophen overdose is greater in chronic alcoholics and patients taking drugs that induce the levels of the P450 isozymes responsible for the activation of acetaminophen.

14.6 METABOLIC ACTIVATION OF HEPATOTOXICANTS

Studies of liver toxicity caused by bromobenzene, acetaminophen, and other compounds have led to some important observations concerning tissue damage:

- Toxicity may be correlated with the formation of a minor but highly reactive intermediate.
- A threshold tissue concentration of the reactive metabolite must be attained before tissue injury occurs.
- Endogenous substances, such as glutathione, play an essential role in protecting the cell from injury by removing chemically reactive intermediates and by keeping the sulfhydryl groups of proteins in the reduced state.
- Pathways such as those catalyzed by glutathione transferase and epoxide hydrolases play an important role in protecting the cell.
- Agents that selectively induce or inhibit the xenobiotic metabolizing enzymes may alter the toxicity of xenobiotic chemicals.

These same principles are applicable to the toxicity caused by reactive metabolites in other organs, such as kidney and lung as will be illustrated in the following sections.

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CHAPTER 15

Nephrotoxicity

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15.1 INTRODUCTION

15.1.1 Structure of the Renal System

The renal system consists of the kidneys and their vasculature and innervation, the kidneys each draining through a ureter into a single median urinary bladder, and the latter draining to the exterior via a single duct, the urethra. The kidney has three major anatomical areas: the cortex, the medulla, and the papilla.

The renal cortex is the outermost region of the kidney and contains glomeruli, proximal and distal tubules, and peritubular capillaries. Cortical blood flow is high, the cortex receiving approximately 90% of the renal blood flow. Since blood-borne toxicants will be delivered preferentially to the cortex, they are more likely to affect cortical functions rather than those of medulla or papilla. The renal medulla is the middle portion and contains primarily loops of Henle, vasa recta, and collecting ducts. Although the medulla receives only about 6% of the renal blood flow, it may be exposed to high concentrations of toxicants within tubular structures. The papilla is the smallest anatomical portion of the kidney and receives only about 1% of the renal blood flow. Nevertheless, because the tubular fluid is maximally concentrated and luminal fluid is maximally reduced, the concentrations of potential toxicants in the papilla my be extremely high, leading to cellular injury in the papillary tubular and/or interstitial cells.

The nephron is the functional unit of the kidney. It is described in detail in Chapter 10 and illustrated in Figure 10.1.

15.1.2 Function of the Renal System

The primary function of the renal system is the elimination of waste products, derived either from endogenous metabolism or from the metabolism of xenobiotics. The latter function is discussed in detail in Chapter 10. The kidney also plays an important role in regulation of body homeostasis, regulating extracellular fluid volume, and electrolyte balance.

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Other functions of the kidney include the synthesis of hormones that affect metabolism. For example, 25-hydroxy-vitamin D_3 is metabolized to the active form, 1,25-dihydroxy-vitamin D_3. Renin, a hormone involved in the formation of angiotensin and aldosterone, is formed in the kidney as are several prostaglandins. While kidney toxicity could affect any of these functions, the effects used clinically to diagnose kidney damage are related to excretory function damage, such as increases in urinary glucose, amino acids, or protein, changes in urine volume, osmolarity, or pH. Similarly changes in blood urea nitrogen (BUN), plasma creatinine, and serum enzymes can be indicative of kidney damage.

In animal studies of nephrotoxicity not only can histopathology be carried out but various biochemical parameters can be compared with those from untreated animals. They include lipid peroxidation and covalent binding to tissue macromolecules.

15.2 SUSCEPTIBILITY OF THE RENAL SYSTEM

Several factors are involved in the sensitivity of the kidney to a number of toxicants (Table 15.1), although the high renal blood flow and the increased concentration of excretory products following reabsorption of water from the tubular fluid are clearly of major importance. Although the kidneys comprise less than 1% of the body mass, they receive around 25% of the cardiac output. Thus significant amounts of exogenous chemicals and/or their metabolites are delivered to the kidney.

A second important factor affecting the kidneys sensitivity to chemicals is its ability to concentrate the tubular fluid and, as a consequence, as water and salts are removed, to concentrate any chemicals it contains. Thus a nontoxic concentration in the plasma may be converted to one that is toxic in the tubular fluid. The transport characteristics of the renal tubules also contribute to the delivery of potentially toxic concentrations of chemicals to the cells. If a chemical is actively secreted from the blood into the tubular fluid, it will accumulate initially within the cells of the proximal tubule or, if it is reabsorbed from the tubular fluid, it will pass into the cells in relatively high concentration.

The biotransformation of chemicals to reactive, and thus potentially toxic, metabolites is a key feature of nephrotoxicity. Many of the same activation reactions found in the liver are also found in the kidney and many toxicants can be activated in either organ, including acetaminophen, bromobenzene, chloroform, and carbon tetrachloride, thus having potential for either hepatotoxicity or nephrotoxicity. Some regions of the kidney have considerable levels of xenobiotic metabolizing enzymes, particularly cytochrome P450 in the pars recta of the proximal tubule, a region particularly susceptible to chemical damage. Since reactive metabolites are generally unstable, and therefore more or less transient, they are likely to interact with cellular macromolecular close to the site of generation. Thus, although the activity of activation enzymes such as

Table 15.1 Factors Affecting the Susceptibility of the Kidney to Toxicants

High renal blood flow
Concentration of chemicals in tubular fluid
Reabsorption and/or secretion of chemicals through tubular cells
Activation of protoxicants to reactive, and potentially toxic, metabolites

cytochrome P450 is lower in the kidney that in the liver, they are of greater importance in nephrotoxicity than those of the liver due to their proximity to site of action.

As with toxicity in other organs the ultimate expression of a toxic end point is the result of a balance between the generation of reactive metabolites and their detoxication. The high levels of glutathione found in the kidney doubtless play an important role in the detoxication process.

15.3 EXAMPLES OF NEPHROTOXICANTS

15.3.1 Metals

Many heavy metals are potent nephrotoxicants, and relatively low doses can produce toxicity characterized by glucosuria, aminoaciduria, and polyuria. As the dose increases, renal necrosis, anuria, increased BUN, and death will occur. Several mechanisms operate to protect the kidney from heavy metal toxicity. After low dose exposure and often before detectable signs of developing nephrotoxicity, significant concentrations of metal are found bound to renal lysosomes. This incorporation of metals into lysosomes may result from one or more of several mechanisms, including lysosomal endocytosis of metal-protein complexes, autophagy of metal-damaged organelles such as mitochondria, or binding of metals to lipoproteins within the lysosome. Exposure to high concentrations, however, may overwhelm these mechanisms, resulting in tissue damage.

Cadmium. In humans, exposure to cadmium is primarily through food or industrial exposure to cadmium dust. In Japan, a disease called Itai-itai Byo is known to occur among women who eat rice grown in soils with a very high cadmium content. The disease is characterized by anemia, damage to proximal tubules, and severe bone and mineral loss. Cadmium is excreted in the urine mainly as a complex (CdMT) with the protein metallothionein (MT). MT is a low molecular weight protein synthesized in the liver. It contains a large number of sulfhydryl groups that bind certain metals, including cadmium. The binding of cadmium by MT appears to protect some organs such as the testes from cadmium toxicity. At the same time, however, the complex may enhance kidney toxicity because the complex is taken up more readily by the kidney than is the free metal ion. Once inside the cell, it is thought that the cadmium is released, presumably by decomposition of the complex within the lysosomes.

Cadmium has a long biological half-life, 10 to 12 years in humans; thus low-level chronic exposure will eventually result in accumulation to toxic concentrations.

Lead. Lead, as Pb^{2+}, is taken up readily by proximal tubule cells, where it damages mitochondria and inhibits mitochondrial function, altering the normal absorptive functions of the cell. Complexes of lead with acidic proteins appear as inclusion bodies in the nuclei of tubular epithelium cells. These bodies, formed before signs of lead toxicity occur, appear to serve as a protective mechanism.

Mercury. Mercury exerts its principle nephrotoxic effect on the membrane of the proximal tubule cell. In low concentrations, mercury binds to the sulfhydryl groups of membrane proteins and acts as a diuretic by inhibiting sodium reabsorption. Organomercurial diuretics were introduced into clinical practice in the 1920s and were used

clinically into the 1960s. Despite their widespread acceptance as effective therapeutic diuretics, it was well known that problems related to severe kidney toxicity were possible. However, in the absence of other effective drugs, the organomercurials proved to be effective, sometimes life-saving, therapeutic agents. More recently organomercurial chemicals have been implicated as environmental pollutants, responsible for renal damage in humans and animals.

Uranium. About 50% of plasma uranium is bound, as the uranyl ion, to bicarbonate, which is filtered by the glomerulus. As a result of acidification in the proximal tubule, the bicarbonate complex dissociates, followed by reabsorption of the bicarbonate ion; the released UO_2^{2+} then becomes attached to the membrane of the proximal tubule cells. The resultant loss of cell function is evidenced by increased concentrations of glucose, amino acids, and proteins in the urine.

15.3.2 Aminoglycosides

Certain antibiotics, most notably the aminoglycosides, are known to be nephrotoxic in humans, especially in high doses or after prolonged therapy. The group of antibiotics includes streptomycin, neomycin, kanamycin, and gentamycin. Aminoglycosides are polar cations that are filtered by the glomerulus and excreted unchanged into the urine. In the proximal tubule, the aminoglycosides are reabsorbed by binding to anionic membrane phospholipids, followed by endocytosis and sequestration in lysosomes (Figure 15.1). It is thought that when a threshold concentration is reached, the lysosomes rupture, releasing hydrolytic enzymes that cause tissue necrosis.

15.3.3 Amphotericin B

With some drugs, renal damage may be related to the drugs' biochemical mechanism of action. For example, the polymycins, such as amphotericin B, are surface-active agents that bind to membrane phospholipids, disrupting the integrity of the membrane and resulting in leaky cells.

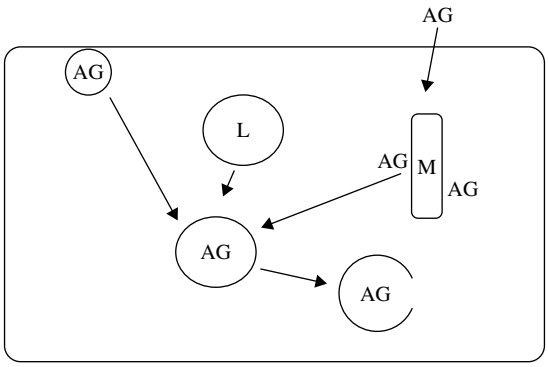

Figure 15.1 Possible cellular interactions of aminoglycosides. AG = aminoglycoside; M = mitochondrion; L = lysosome. (From E. Hodgson and P. E. Levi, eds., *A Textbook of Modern Toxicology*. 2nd ed., Appleton and Lange, Stamford, CT, 1997.)

15.3.4 Chloroform

Chloroform is a common industrial organic solvent that can be a hepatotoxicant or a nephrotoxicant in both humans and animals. As a nephrotoxicant it is both species and gender dependent. For example, following chloroform administration male mice develop primarily kidney necrosis whereas female develop liver necrosis.

As a nephrotoxicant, chloroform most probably undergoes metabolic activation in the kidney itself. Chloroform is metabolized to phosgene (Figure 15.2) by a cytochrome P450-dependent reaction, probably proceeding via an unstable hydroxylated product, trichloromethanol. Phosgene is capable of binding to cellular proteins to produce the cellular necrosis associated with chloroform toxicity to the kidney. Phosgene can also be further metabolized by a number of reactions (Figure 15.2), and as with most chemical-induced toxicity, the final expression of toxicity depends on a balance between activation and detoxication.

15.3.5 Hexachlorobutadiene

Hexachlorobutadiene is an industrial solvent and heat-transfer agent. It is a widespread environmental contaminant that is a potent and relatively specific nephrotoxicant. Hexachlorobutadiene first forms a glutathione conjugate, which is further metabolized by the mercapturic acid pathway to a cysteine conjugate (see Chapter 7 for details of glutathione conjugation and the mercapturic acid pathway). In the kidney, the cysteine conjugate is cleaved to a reactive intermediate by the enzyme, cysteine conjugate β-lyase.

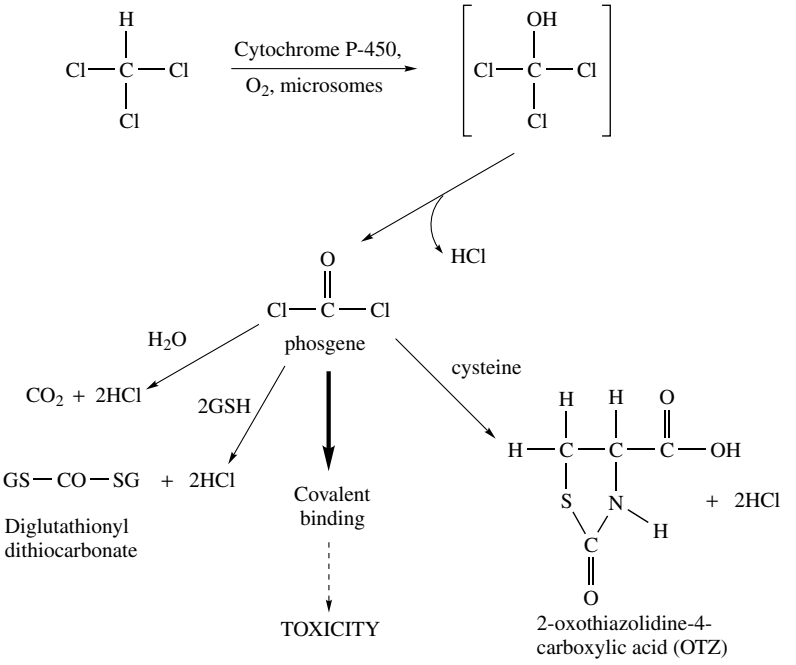

Figure 15.2 Proposed mechanism of chloroform biotransformation. (From J. B. Tarloff in *An Introduction to Biochemical Toxicology*, 3rd ed., E. Hodgson and R. C. Smart eds., Wiley, 2001.)

15.3.6 Tetrafluoroethylene

The nephrotoxic mode of action of tetrafluoroethylene is similar to that of hexachlorobutadiene. It is first metabolized to a cysteine conjugate, which is metabolized by cysteine conjugate β-lyase to a reactive product that can bind to cellular macromolecules.

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